Fungal Pathogenesis in Plants and Crops

Molecular Biology and Host Defense Mechanisms

Second Edition

P. Vidhyasekaran

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Second Edition

P. Vidhyasekaran
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Preface

Ever since the publication of the first edition of *Fungal Pathogenesis in Plants and Crops: Molecular Biology and Host Defense Mechanisms* in 1997, several pathbreaking studies have been carried out in the field of molecular biology of fungal pathogenesis. Several important fungal pathogens have been now reclassified as Oomycetes belonging to kingdom Chromista. The oomycetes are considered as false fungi, and this book includes both fungal and oomycete pathogenesis under the same title, "Fungal Pathogenesis." Molecular communications between plants and fungal pathogens are now being deciphered and several signaling systems have been identified. The major breakthrough in deciphering the signals is the cloning of numerous disease-resistance genes and identifying their role in signal transduction systems in plants. Several defense-related genes have been cloned and characterized. Signals activating these defense genes have been identified. Transgenic plants overexpressing these signals have been developed to suppress the fungal pathogenesis in plants. Pathogens are able to suppress these signaling systems and evade the induced host-defense mechanisms. The book describes the weapons used by fungal pathogens to evade or suppress the host-defense mechanisms. Several new defense genes have been identified. The number of PR protein groups identified has increased from 5 groups in the first edition to 17 in this book. Knowledge of the molecular mechanism of evasion of these defense genes by the fungal pathogens has helped to evolve novel genetically engineered disease-resistant plants. Developing plants expressing elicitor genes to elicit early induction of defense genes, plants overexpressing the signal molecules or activating signal transduction systems, plants expressing "foreign" phytoalexins and PR proteins that cannot be degraded by the fungal pathogens, plants expressing detoxification genes to detoxify the toxins produced by pathogens, and plants expressing polygalacturonase inhibitor proteins are the classical examples demonstrating the practical use of our knowledge of molecular biology of fungal pathogenesis. This book describes each of the fungal infection processes from initial contact and penetration to subsequent invasion and symptom development. This book will be a valuable resource for researchers and students studying plant pathology, molecular plant pathology, plant molecular biology, plant biochemistry, plant biotechnology, plant physiology, applied botany, and other branches of biological sciences.

Author

Dr. P. Vidhyasekaran, PhD, is a former director of the Center for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, India. He is the author of 12 books, published by leading publishers in the United States and India. He has published more than 400 articles in 32 international journals as well as several book chapters. Dr. Vidhyasekaran has served on the editorial boards of several journals and has been elected president of the Indian Society of Plant Pathologists. He is a fellow of the National Academy of Agricultural Sciences and several other scientific societies, and a member of the New York Academy of Sciences. Dr. Vidhyasekaran has won several national awards as well as the Tamil Nadu Best Scientist Award. He has served as a visiting scientist in several countries including the United States, Philippines, and Denmark. He is the author of *Physiology of Disease Resistance in Plants* (CRC Press) and *Handbook of Molecular Technologies in Crop Disease Management* (Haworth Press).

1 Perception and Transduction of Plant Signals in Pathogens

1.1 INTRODUCTION

Interplay of signaling systems in plant–pathogen interactions is the key component in the development of diseases in plants (Vidhyasekaran, 1997, 2002, 2004, 2007; Yang et al., 2005). Signaling between fungal pathogens and plants begins when they meet on the plant surface (Fujita et al., 2004a). The evidence is strong that plant–fungal interactions begin, and perhaps their outcomes (susceptibility or resistance) are determined, during the very first few minutes of association and perhaps even earlier (Heath, 2000). If the signals at the plant surface are perceived as favorable by the fungi, the fungal spores germinate and either directly penetrate the cuticle or penetrate the cuticle by producing appressorium and infection peg (Nielsen et al., 2000; Wright et al., 2000; Apoga et al., 2001; Lev and Horwitz, 2003; Fujita et al., 2004b; Li et al., 2004). Both the physical and chemical plant signals may be involved in the penetration process. The plant signals may be used by the fungus to trigger the expression of the genes necessary to penetrate into the host (Dickman et al., 2003; Tsuji et al., 2003; Yang et al., 2005).

Plants have evolved sophisticated detection and response systems that decipher pathogen signals and induce synthesis of various antifungal compounds and fortify their cell wall components to defend themselves. For successful pathogenesis, the pathogens have to overcome these defense mechanisms by producing appropriate signals. On perception of pathogen signals, several signaling pathways are activated in plants mounting resistance response to invading pathogens (Glazebrook et al., 2003; Nandi et al., 2003; Heath, 2004; Thaler et al., 2004; Uppalapati et al., 2004). The interplay of the plant and pathogen signals allows the plant to fine-tune defense responses (Mould et al., 2003) and the pathogen to succumb to the toxic environment in the plants or to overcome these defense mechanisms, resulting in disease susceptibility or resistance (Yang et al., 2004). Susceptibility and resistance are opposite sides of the same coin (Neu et al., 2003; Christensen et al., 2004) and indicate the winning candidate in the battle between plant and pathogen. Resistance and susceptibility are often determined at the single-cell level (Gjetting et al., 2004). These complex signaling systems in pathogens and plants during pathogenesis in susceptible and resistant conditions are described in this book. In this chapter, the molecular events occurring during the fungal development in host plants on perception of plant signals are described.

1.2 SIGNALING AND TRANSDUCTION SYSTEMS IN "FIRST TOUCH" AND ADHESION OF FUNGAL SPORES

1.2.1 First Touch or Initial Contact Triggers the Infection Process

A physical contact of the pathogen, what is called "first touch," itself has been shown to initiate various signaling and transduction systems in the fungal infection process. First touch

or initial surface contact may induce the fungal spores to germinate and differentiate to form infection structures, such as appressoria. Any solid-surface contact is sufficient for inducing spore germination in *Colletotrichum* spp. (Kim et al., 1998). Conidia of *Colletotrichum gloeosporioides* are induced to germinate and differentiate to form appressoria by different plant signals (Podila et al., 1993; Flaishman and Kolattukudy, 1994). However, contact with a hard surface is necessary for the plant signals to induce appressorium formation (Liu and Kolattukudy, 1998). *C. gloeosporioides* conidia formed appressoria on both a hydrophilic cover glass and a hydrophobic polystyrene petri dish when exposed to plant-surface wax or ethylene signals. On the other hand, on soft hydrophilic or hydrophobic substrates, such as 2% agar or petrolatum, respectively, only germination occurred (Liu and Kolattukudy, 1998). All hard surfaces tested induced appressorium development, including both hydrophobic and hydrophilic surfaces of GelBond, whereas soft surfaces such as water agar (2%) failed to induce appressorium formation in *C. gloeosporioides* (Figure 1.1; Uhm et al., 2003). It appears that a chain of molecular events that ultimately lead to differentiation of the germ tubes into appressoria is initiated upon contact with a hard surface.

The fungal spores, which land on the host surface, first recognize certain substratum characteristics within seconds of making contact (Carver et al., 1999). On the first touch, the spore releases copious extracellular matrix (ECM) in advance of germination (Kunoh et al., 1988; Nicholson et al., 1988, 1993; Pascholati et al., 1992; Carver et al., 1999; Wright et al., 2002b; Fujita et al., 2004b). The ECM production in the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* was associated with changes in spore surface features and the ECM was shown to be released biphasically; an initial release was evident after 2 min, and a second release after 15 min (Nicholson et al., 1988). In fact, the release of ECM from the conidia of *B. graminis* f. sp. *hordei* could be observed within 20 s of first touch (Carver et al., 1999). Nielsen et al. (2000) demonstrated hydrolytic enzyme activity in the region corresponding to conidial ECM, within 3 min of spore deposition. The ECM continued to accumulate at the conidium–substratum interface for up to ~60 min after contact (Carver et al., 1999).

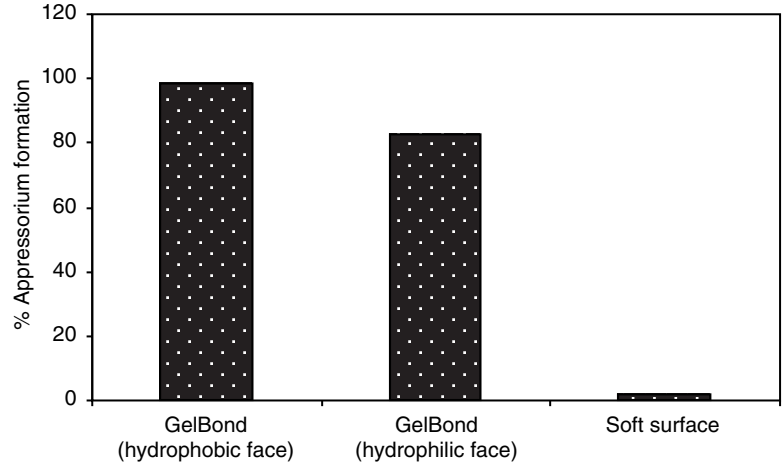

FIGURE 1.1 Relative efficacy of hard (hydrophobic or hydrophilic) and soft surfaces on appressorium formation in *Colletotrichum gloeosporioides*. (Adapted from Uhm, K.-H., Ahn, I.-P., Kim, S., and Lee, Y.-H., *Phytopathology*, 93, 82, 2003.)

1.2.2 Adhesion or Close Contact Triggers Fungal Infection Process

The spores of several pathogens adhere to the host plant surface immediately after landing on the plant surface (Pringle, 1981; Evans et al., 1982; Lippincott and Lippincott, 1984; Young and Kauss, 1984; Epstein et al., 1985; Hamer et al., 1988; Jones and Epstein, 1989; Beckett et al., 1990; Nicholson and Epstein, 1991; Sela-Buurlage et al., 1991; Braun and Howard, 1994b; Ding et al., 1994; Mercure et al., 1994a; Knogge, 1996; Nicholson, 1996; Apoga et al., 2001; Jansson and Åkesson, 2003). Adhesion of conidia has been reported for *B. graminis* (Yamaoka and Takeuchi, 1999), *Uromyces appendiculatus* (Epstein et al., 1987; Terhune and Hoch, 1993), *U. viciae-fabae* (Beckett et al., 1990; Deising et al., 1992), *Botrytis cinerea* (Doss et al., 1993), *Colletotrichum musae* (Sela-Buurlage et al., 1991), *C. lindemuthianum* (Young and Kauss, 1984), *C. graminicola* (Mercure et al., 1994a), *Magnaporthe grisea* (Hamer et al., 1988), *Haematonectria haematococca* (anamorph: *Fusarium solani*) (Jones and Epstein, 1989, 1990; Kwon and Epstein, 1993), *Cochliobolus heterostrophus* (Braun and Howard, 1994b; Zhu et al., 1998; Apoga and Jansson, 2000), and *Bipolaris maydis*, *B. zeicola*, and *B. turcicum* (Pringle, 1981; Evans et al., 1982; Braun and Howard, 1994b; Jansson and Åkesson, 2003). Adhesion of spores on leaf surfaces by foliar pathogens (Hamer et al., 1988; Mercure et al., 1994a; Jansson and Åkesson, 2003) and root surfaces by soil-borne pathogens (Hinch and Clarke, 1980; Longman and Callow, 1987; Carlson et al., 1991b) has been reported. Adhesion appears to be essential for spore germination, germ tube elongation, appressorium formation, and penetration hyphal development of several pathogens (Ding et al., 1994; Mercure et al., 1994a,b; Yamaoka and Takeuchi, 1999; Jansson and Åkesson, 2003). Adhesion to the host surface has been shown to be an important step in fungal infection of plants (Epstein and Nicholson, 1997). Jones and Epstein (1990) showed that an adhesion-deficient mutant of *H. haematococca* (anamorph: *F. solani*) had lower virulence than the wild type. Adhesion of conidia is reported to be an absolute requirement for germination and subsequent infection in the grape pathogen *Phyllosticta ampelicida* (Kuo and Hoch, 1996). Adhesion of spores to host surfaces is the first vital step toward successful infection (Wright et al., 2002a).

1.2.3 Adhesion of Spores due to Hydrophobic Interaction

Surface hydrophobicity appears to be important for adhesion of the conidia of several plant pathogens (Jones and Epstein, 1989; Beckett et al., 1990). Conidia of *Colletotrichum graminicola* adhered to a greater extent to the hydrophobic surface of polystyrene and they did not adhere to the glass, which is a hydrophilic surface (Mercure et al., 1994b). The conidial adhesion of *Bipolaris sorokiniana* appeared to be selective to hydrophobic surfaces, since the conidia did not adhere to hydrophilic glass surfaces but adhered to the hydrophobic surface of polystyrene. The detergent Triton X-100, which interferes with hydrophobic binding, disrupted the adhesion of the conidia to the polystyrene (Apoga et al., 2001). The conidial surface is hydrophobic, and several studies have demonstrated a correlation between cell-surface hydrophobicity and adhesion to polystyrene (Doss et al., 1993; Kuo and Hoch, 1996). Preference for adhesion to hydrophobic compared to hydrophilic surfaces has been observed for spores of *Colletotrichum musae* (Sela-Buurlage et al., 1991), *C. lindemuthianum* (Young and Kauss, 1984; Mercure et al., 1994b), *Botrytis cinerea* (Doss et al., 1993), and *Uromyces viciae-fabae* (Clement et al., 1994). Requirement of a hydrophobic surface for adhesion of spores has been observed in *U. appendiculatus* (Terhune and Hoch, 1993), and *P. ampelicida* (Kuo and Hoch, 1996). The importance of hydrophobicity in adhesion was demonstrated by Doss et al. (1993) who demonstrated that the oxidation of a polyethylene hydrophobic surface lowered the water contact angle and decreased the adhesion of conidia of *B. cinerea*.

Hydrophobins are components of fungal cell walls that contribute to cell-surface hydrophobicity. They are required for the formation of the hydrophobic rodlet layer of

spores (Stringer et al., 1991; Bell-Pedersen et al., 1992; Lauter et al., 1992). The hydrophobins are also secreted (Wessels, 1993). Hydrophobins are relatively small cysteine-rich proteins. The hydrophobins are between 96 and 157 amino acids in length, contain 8 cysteine residues, and are strongly hydrophobic (Wessels et al., 1991; Stringer and Timberlake, 1993). Another common feature is that the second and third cysteines form a doublet and are usually followed by an asparagine residue (Templeton et al., 1994). The hydrophobic nature of the surfaces of fungal pathogens is important for adhesion of pathogens to host structures (Beever and Dempsey, 1978; Stringer et al., 1991). The external location of the rodlets means that they could mediate the initial contact in fungal interactions (Templeton et al., 1994).

1.2.4 Adhesion of Spores Is Accompanied by Release of Extracellular Material

Adhesion of fungal spores may be due to the presence of some preformed as well as newly secreted adhesive materials (mucilaginous extracellular matrices) (Epstein et al., 1987; Deising et al., 1992; Braun and Howard, 1994b). Wright et al. (2002b) demonstrated the adhesive function of ECM of *Blumeria graminis* f. sp. *hordei*, the barley powdery mildew pathogen. Strength of adhesion by the conidia correlated to the speed of ECM release and to the quantitative of ECM released on the substrata (Wright et al., 2002b). Before germinating, conidia of *B. graminis* f. sp. *hordei*, *B. graminis* f. sp. *tritici*, and *Erysiphe pisi* rapidly released ECM onto barley coleoptile cells. More was released if full rather than partial cell contact was made (Fujita et al., 2004a).

M. grisea contains presynthesized material in the conidial apex that is released upon hydration, at the time when the tip of the conidium becomes anchored to the substratum (Hamer et al., 1988). *H. haematococca* (anamorph: *F. solani*) synthesizes a spore tip material that is associated with the attachment of the spore to the host plant (Jones and Epstein, 1989, 1990). Many other fungi produce conidial mucilages when contacting a substratum (Nicholson and Moraes, 1980; Nicholson et al., 1986; Nicholson and Epstein, 1991; Sela-Buurlage et al., 1991; Clement et al., 1993a,b; Braun and Howard, 1994a; Kuo and Hoch, 1995; Nicholson and Kunoh, 1995; Apoga and Jansson, 2000; Wright et al., 2002a; Fujita et al., 2004a). Scanning electron microscopy revealed the presence of a film of material in the contact surface of *C. graminicola* conidia that adhered to corn (*Zea mays*) leaves (Mercure et al., 1994a). It indicates that a material is released from conidia as a result of contact of the conidium with a substrate. Treatment of *C. graminicola* conidia with either cycloheximide (a protein synthesis inhibitor) or brefeldin A (a glycoprotein synthesis and transport inhibitor) significantly reduced adhesion (Mercure et al., 1994b). It suggests that blocking of synthesis of a new material (protein or glycoprotein) may lead to loss of adhesion of conidia. The capacity of conidia of *C. lindemuthianum* (Young and Kauss, 1984), *C. musae* (Sela-Buurlage et al., 1991), and *H. haematococca* (Jones and Epstein, 1989) to adhere was inhibited by the exposure to respiration inhibitors. It suggests that adhesion requires active metabolism of spores to synthesize new adhesive materials.

Release of adhesive materials from spores of different pathogens has been demonstrated using electron microscopy (Sing and Bartnicki-Garcia, 1975; Hamer et al., 1988) or by fluorescein-labeled lectins or antibodies (Hardham, 1985; Hardham and Suzaki, 1986; Gubler and Hardham, 1988; Freytag and Hardham, 1991; O'Connell, 1991; Kwon and Epstein, 1993). The glycoprotein adhesive material was released from *C. graminicola* conidia at an extremely early time in the infection process (Mercure et al., 1995). A mucilage is released from the apex of *M. grisea* conidia during adhesion (Hamer et al., 1988). Macroconidia of *H. haematococca* release an extracellular material from the tip of the conidium and this material was involved in adhesion (Schuerger and Mitchell, 1993). Zoospores of *Phytophthora palmivora* (Sing and Bartnicki-Garcia, 1975) and *P. cinnamomi* (Hardham and Suzaki, 1986;

Gubler and Hardham, 1988; Gubler et al., 1989) secrete adhesive materials. Similar adhesive material has been detected in urediniospores of *Uromyces viciae-fabae* (Clement et al., 1993a,b).

The materials associated with adhesion have been identified as proteins (Gubler and Hardham, 1988; Mercure et al., 1995) or glycoproteins (Sing and Bartnicki-Garcia, 1975; Hardham and Suzaki, 1986; Clement et al., 1993a,b; Kwon and Epstein, 1993; Mercure et al., 1995) or carbohydrates (Kwon and Epstein, 1993, 1997; Sugui et al., 1998) in spores of many fungal pathogens. A protein has been shown to be involved in attaching *P. cinnamomi* zoospores to the host surface and its molecular weight was more than 200 kDa (Gubler and Hardham, 1988). A mannoprotein was detected in the ECM released by adherent macroconidia of *H. haematococca* (Kwon and Epstein, 1993, 1997). The ECM released onto hydrophobic surfaces by conidia and conidial germlings of *C. graminicola* contained both carbohydrate and protein. The carbohydrate analysis revealed that mannose was the predominant sugar (Sugui et al., 1998). Two kinds of adhesive materials have been detected in *Colletotrichum graminicola* conidia. The preformed adhesive material was a protein, whereas the newly secreted material was a glycoprotein (Mercure et al., 1994b).

Some of the adhesive materials secreted by fungal spores have properties of lectins in binding specific sugar moieties. The adhesive materials secreted by zoospores of *Pythium aphanidermatum*, which are involved in adhesion to root surfaces of cress (*Lepidium sativum*) (Longman and Callow, 1987) and *Phytophthora* spp., which are involved in adhesion to cells of soybean (*Glycine max*) and other plants (Hohl and Balsiger, 1986; Guggenbuhl and Hohl, 1992) have been identified as lectins.

1.2.5 Involvement of Cutinases in Spore Adhesion

Cutinases and other esterases have been shown to be involved in the adhesion of some fungi. Conidia of *Blumeria graminis* f. sp. *hordei*, the barley powdery mildew pathogen, rapidly released a liquid upon contact with a barley leaf. The liquid contained esterase activity resembling fungal cutinase (Nicholson et al., 1988). The liquid film flowed off the conidium and onto the contact surface again forming a film on the contact surface (Kunoh et al., 1988, 1990; Nicholson et al., 1988). The release of exudate onto the surface of barley leaves resulted in an apparent loss of structural integrity of the underlying cuticular surface (Kunoh et al., 1988, 1990). The exudate containing cutinase activity was involved in the erosion of the leaf cuticle (Pascholati et al., 1992). Cuticular erosion presents a surface that triggers recognition, which initiates the infection process (Nicholson and Epstein, 1991). Such erosion may be necessary either for recognition of the host surface by the spores or for adhesion (Pascholati et al., 1992).

Possible role of cutinases in adhesion has been reported for many fungi (Purdy and Kolattukudy, 1975; Nicholson and Epstein, 1991). Cutinase and nonspecific esterases have been shown to be present on the surface of the wall of urediniospores of the rust fungus *Uromyces viciae-fabae* (Deising et al., 1992). Upon hydration, the enzymes were released from the spore wall and were located at the interface of the spore and the underlying substratum. These enzymes have been shown to function in the adhesion of the spore to the leaf surface (Deising et al., 1992). It has been suggested that fungal spore senses contact with the plant surface through the unique monomers of plant cuticle generated by the small amount of cutinase carried by the spore (Kolattukudy, 1980; Woloshuk and Kolattukudy, 1986).

1.2.6 Some Plant Signals May Be Needed for Adhesion of Spores

Some plant signals may be needed for adhesion. Macroconidia of the cucurbit pathogen *H. haematococca* did not attach to a polystyrene substratum. However, within minutes after

exposure to its plant host (zucchini) extract, the macroconidia became adhesion competent (Jones and Epstein, 1989). They produced fluorescein isothiocyanate-conjugated ConA-labeled mucilage at the spore apex and adhered at the macroconidial apex. The macroconidia produced a 90 kDa glycopeptide when incubated in zucchini plant extract. Similar glycopeptide was not produced when the macroconidia was incubated in Czapek-Dox medium or water and the macroconidia did not adhere to the substratum (Kwon and Epstein, 1993). Thus production of the 90 kDa glycopeptide was associated with the induction of adhesion competence.

In mammalian systems, substrate adhesion molecules such as fibronectin, vitronectin, laminin and collagen, and their receptors have been shown to be important in cell-to-substratum adhesion processes. These molecules have been detected in plant cells also (Calderone and Braun, 1991; Sanders et al., 1991; Tronchin et al., 1991; Axelos et al., 1993; Zhu et al., 1993, 1994). Vitronectin is a glycoprotein. Vitronectin connects the extracellular matrix with the intracellular network through a subset of plasma membrane receptors known as integrins (Vogel et al., 1993). Integrins are a large family of heterodimeric glycoproteins. They are cell-surface transmembrane receptors that mediate and coordinate cellular responses to the extracellular matrix. Cellular signaling pathways can regulate cell adhesion by altering affinity and avidity of integrins for the ECM. Integrins interact with ECM components through their extracellular domains, usually through recognition of an RGD (arginine–glycine–aspartic acid) tripeptide, whereas their cytoplasmic domains play a pivotal role in mediating integrin-dependent cellular functions. The integrin cytoplasmic tails interact with the signaling molecules and other cellular proteins, resulting in regulation of many biological functions (French-Constant and Colognato, 2004).

One of the vitronectin-like proteins, PVN1, has been isolated from tobacco (*Nicotiana tabacum*) cells and it binds tobacco cells to glass surfaces. This plant adhesion protein has been found localized in the cell wall of tobacco cells (Zhu et al., 1994). The vitronectin-like proteins have been implicated in attachment of bacterial pathogens to plant cells (Wagner and Matthysse, 1992). However, role of similar adhesion molecules in fungal adhesion has not yet been demonstrated.

1.3 SIGNALING IN FUNGAL SPORE GERMINATION

1.3.1 Plant Signals Trigger Structural Changes in Spores before Germination

When spores land on the plant surface, the spores sense the plant signals and prepare for germination. Conidia of *Blumeria graminis* and *Erysiphe pisi* release ECM within seconds, or at most a few minutes, after the deposition on plant leaves (Carver et al., 1999; Wright et al., 2002b; Fujita et al., 2004b). The ECM release promotes spore germination (Meguro et al., 2001). Nielsen et al. (2000) showed that the contact of the conidium of *B. graminis* f. sp. *hordei* with an appropriate, inductive substratum initiated the uptake of anionic low molecular weight compounds. This uptake was preceded by the release of a proteinaceous ECM from the body of the conidium. Following the release of the matrix and its associated hydrolytic enzyme activities, low molecular weight molecules moved into the conidium from the host. The anionic low molecular weight materials showed physical properties similar to anionic cutin monomers (Nielsen et al., 2000). The cutin monomers should be from host cuticle and would have been released from the host by the ECM containing necessary enzymes (Nicholson et al., 1988; Kunoh et al., 1990; Pascholati et al., 1992). The host signals entering the conidium may trigger spore germination and other infection structures (Nielsen et al., 2000).

The fungal spores undergo major structural changes after adhesion to the substrate (Kunoh et al., 1988; Caesar-TonThat and Epstein, 1991). Within 5 min of contact, the

reticulate surface of *B. graminis* f. sp. *hordei* conidium began to disappear. By 10 min, only the spine-like surface protrusions of the conidium were visible, and by 30 min globose bodies appeared on the conidium surface. These events took place before germination of the conidium (Kunoh et al., 1988). The release of liquid containing esterase activity from the conidium within 5 min of contact with the barley leaf has been suggested to be responsible for these structural changes (Nicholson et al., 1988; Kunoh et al., 1990). The surface of macroconidia of *H. haematococca* undergoes major structural changes hours before germ tube emergence (Caesar TonThat and Epstein, 1991).

Ascospores of *Hypoxylon fuscum*, a pathogen of beech (*Fagus sylvatica*) were irreversibly activated, before germination, within minutes of contact with a potential host (Chapela et al., 1991). Some molecules found in the host cell wall were found to induce structural changes in spores and these molecules have been identified as compounds closely related to monolignol glucosides z-isoconiferin (glucosylated form of coniferyl alcohol) and z-syringin (Chapela et al., 1991).

1.3.2 Plant-Surface Signals Trigger Spore Germination

For several plant pathogens, it has been shown that the germination of conidia is stimulated by contact with or after adhesion to a solid surface. Conidial adhesion is required to stimulate germination in *M. grisea* (Liu and Kolattukudy, 1999) and *Phyllosticta ampelicida* (Kuo and Hoch, 1996), whereas solid-surface contact is sufficient for inducing germination in *Colletotrichum* spp. (Kim et al., 1998). In *Bipolaris sorokiniana* adhesion was not needed for conidial germination since germination occurred in the bulk media. However, germination was stimulated on a solid surface when the fungus was incubated in water, indicating that surface contact or adhesion can stimulate germination (Apoga et al., 2001).

Many published micrographs of germinating conidia of *Blumeria graminis* show that most germ tubes emerge from the spore body close to its site of contact with the host leaf so that the growing germ tubes are highly likely to make contact with the substratum (Carver et al., 1995a,b, 1999). The development of appressoria by germinating *B. graminis* conidia depended on its germ tubes making contact with the host surface (Wright et al., 2000). First formed germ tubes of *B. graminis* f. spp. *hordei*, *tritici*, and *avenae* conidia emerged from close to the host leaf surface, and so made contact with it allowing them to become functional primary germ tubes (Wright et al., 2000). The germ tube emergence requires contact with the plant surface (or a substratum). However, only a small percentage of a conidium's surface needs to be in contact with a ''substratum'' to stimulate germ tube emergence close to the contact site (Wright et al., 2000). The germ tubes of *B. graminis* f. sp. *hordei* conidia suspended on spider's thread often emerged close to the site of contact with the tip of a microneedle. The fact that the needle tip was allowed contact for only 2 min shows the rapidity of the response (Wright et al., 2000). Contact with a spider's suspension thread itself was also sufficient for many conidia to produce germ tube. The results suggest that the area of contact required for *B. graminis* conidia to stimulate directional germ tube emergence is very small. On leaves, the contact between the tips of a limited number of conidial wall projections and the edges of epicuticular leaf wax plates was sufficient to initiate the infection structures (Wright et al., 2000). It was shown that the site of germ tube emergence was determined within 1 min of deposition on plant surfaces (Wright et al., 2000).

Several molecular events may precede germ tube emergence from spores. Most germ tubes emerge from the spore body close to its site of contact with the host leaf so that the growing germ tubes are highly likely to make contact with the substratum. These would include the engagement of signaling and transduction systems controlling the site of germ tube emergence, degradation of the conidial wall at the site of germ tube emergence, and relocation of existing resources, or the de novo synthesis of materials, necessary for germ tube construction (Wright et al., 2000). The rapid ECM release from the spores on the

contact surface (Carver et al., 1999; Nielsen et al., 2000) may play a role in perception of the plant-surface contact site and subsequent intracellular signaling systems controlling the site of germ tube emergence. The activation of intracellular signaling has been demonstrated by showing the fluctuations in cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) levels within developing *B. graminis* f. sp. *hordei* germlings (Hall and Gurr, 2000). The increased endogenous cAMP levels could be detected within 15 min in the conidia of the powdery mildew fungus after contact with a cellulose membrane (Kinane et al., 2000). The activation of the cAMP pathway preceded germ tube emergence.

1.3.3 Flavonoids Signaling Spore Germination

Spores of soil-borne fungal pathogens remain dispersed in soil for months or years in a quiescent state until the appearance of a potential host stimulates their germination (Bagga and Straney, 2000). Flavonoids exuded by roots and seeds (Graham, 1991; Dakora et al., 1993) act as stimulatory signal in inducing spore germination (Morris and Ward, 1992; Ruan et al., 1995; Bagga and Straney, 2000). Flavonoids signaled spore germination in *H. haematococca* (anamorph: *Fusarium solani*). Treatment of spores of *H. haematococca* with specific inhibitors of cAMP-dependent PKA inhibited the ability of flavonoids to stimulate spore germination (Ruan et al., 1995). The results suggest that the cAMP pathway may be involved in the flavonoid signal-induced spore germination. The cAMP levels in the macroconidia of *H. haematococca* were transiently induced by flavonoid treatment (Bagga and Straney, 2000). The cAMP levels can be modulated by changes in cAMP degradation, catalyzed by cAMP phosphodiesterase (Riley and Barclay, 1990). Bagga and Straney (2000) showed a correlation between strength of induction of cAMP levels by the flavonoid and inhibition of phosphodiesterase enzyme activity. The competitive phosphodiesterase inhibitors, IBMX and theophyllin, stimulated *H. haematococca* germination (Ruan et al., 1995). These observations suggest that the flavonoids would have modulated cAMP levels through direct inhibition of cAMP phosphodiesterase and the increased cAMP levels would have signaled the spore germination in *H. haematococca* (Bagga and Straney, 2000).

1.4 SIGNALING IN DIFFERENTIATION OF GERM TUBES INTO INFECTION STRUCTURES

1.4.1 Adhesion of Germlings and Infection Structures

The infection process of many fungal pathogens involves an orderly sequence of morphological differentiation as a prerequisite for successful infection. These include first the formation of a germ tube, which is followed by differentiation into appressorium. Penetration hypha arises from appressorium and it penetrates the host epidermal cell wall (Carver et al., 1995a; Nicholson and Kunoh, 1995). The orderly sequence of differentiation of fungal structures is driven by the perception of host plant signals (Nielsen et al., 2000). There is evidence that germ tubes recognize substratum hydrophobicity (Kamakura et al., 2002), cutin monomers (Francis et al., 1996), that could be released from the plant cuticle by the action of fungal cutinase (Pascholati et al., 1992), and possibly cellulose breakdown products (Carver et al., 1996) released by the action of fungal cellulases (Suzuki et al., 1998; Pryce-Jones et al., 1999). When favorable plant signals are perceived by the fungal germ tubes, the fungal germlings grow, form appressoria, and penetrate into host tissue producing penetration peg or in some cases, directly penetrate the cuticle (Kolattukudy et al., 1995). Close contact of germ tubes, appressoria, and infection hyphae with the plant surface and the signal perception and transduction system have been shown to be necessary for the development of infection structures and pathogenesis (Jansson and Åkesson, 2003).

Germ tube may adhere to plant surface during its differentiation into appressorium (Epstein et al., 1985, 1987; Chaubal et al., 1991; Guggenbuhl and Hohl, 1992; Clement et al., 1993a; Xiao et al., 1994a; Pain et al., 1996; Wright et al., 2002a). The germling growth in many leaf pathogens was dependent on the ability to grow in close contact with the plant surface (Epstein and Nicholson, 1997; Staples and Hoch, 1997). Treatments that interfere with adhesion of hyphae to substrate block the development of appressoria of the rice blast pathogen *M. grisea* (Xiao et al., 1994a).

The differentiated appressorium is also tightly attached to the host tissue (Howard et al., 1991; Xiao et al., 1994b; Pain et al., 1996; Jones et al., 2000). The appressorium of *M. grisea* is tightly attached to the host surface for the penetration of the host tissue (Howard et al., 1991). After penetration of the cuticle, the fungal pathogen comes into contact with mesophyll cells. Adhesion of the penetration hyphae with mesophyll cell walls has also been reported (Ding et al., 1994). In contrast to cuticular adhesion, the interactions between host cell wall and fungal penetration hyphae involve primarily hydrophilic surfaces (Ding et al., 1994).

Germlings may require hydrophobic surface for adhesion. Germ tubes of *Uromyces appendiculatus*, the bean (*Phaseolus vulgaris*) rust fungus, required a hydrophobic surface for adhesion. By using interference reflection microscopy, Terhune and Hoch (1993) demonstrated that the area of contact of germ tubes of *U. appendiculatus* was greater on a hydrophobic than on a hydrophilic surface. Fungal hydrophobins may be involved in adhesion of the germlings (Wösten et al., 1994). The hydrophobins represent a class of cell wall proteins that influence the hydrophobic character of the cell surface (Wessels et al., 1991). Hydrophobins form the rodlet layer visible on the surface of conidia of many fungal pathogens. The assembly of rodlets is catalyzed at hydrophilic–hydrophobic interfaces and can contribute to the adhesion of hyphae to hydrophobic surfaces (Wösten et al., 1994).

1.4.2 Extracellular Matrix in Germling Adhesion

The adhesion may involve the formation of ECM in germlings (Braun and Howard, 1994a; Apoga et al., 2001). Production of an ECM has commonly been related to germling adhesion (Ben-Naim and Yaacobi, 1974; Evans et al., 1982; Chaubal et al., 1991; Jones, 1994; Braun and Howard, 1994b; Doss et al., 1995; Epstein and Nicholson, 1997; Apoga and Jansson, 2000; Rumbolz et al., 2000; Apoga et al., 2001). Adhesion of germlings of *Bipolaris sorokiniana* (Apoga et al., 2001), *Botrytis cinerea* (Doss et al., 1995; Doss, 1999), *Blumeria graminis* (Carver et al., 1995a,b; Wright et al., 2002a), and *Cochliobolus heterostrophus* (Braun and Howard, 1994a) was accompanied by release of ECM. Germ tubes of *Uromyces viciae-fabae* adhered in association with the release of an extracellular matrix or mucilage (Clement et al., 1993a). ECM produced by fungi during germination has been implicated in adhesion (Nicholson and Epstein, 1991). ECM has been shown to lie beneath germ tubes of the tomato powdery mildew pathogen *Oidium neolycopersici*, and around the margins of the appressorium, but not beneath ungerminated spores (Jones et al., 2000).

Two-layered ECM has been visualized by histological staining in germ tubes of *Bipolaris maydis*, *B. zeicola*, and *Setosphaeria turcica* (= *Bipolaris turcicum*) (Evans et al., 1982; Braun and Howard, 1994b). The germ tube of *B. sorokiniana* is surrounded by a two-layered ECM and this material is important in adhesion of the fungus to the host surface (Pringle, 1981; Evans et al., 1982; Carlson et al., 1991a; Apoga and Jansson, 2000; Apoga et al., 2001). Germlings of *Cochliobolus heterostrophus* is surrounded by a two-layered ECM. By using a mutant of *C. heterostrophus*, defective in the outer germ tube ECM layer, Zhu et al. (1998) showed that the two layers may exist independently and still adhere to the leaf surface; thus the inner layer may be responsible for the adhesion.

The adhesion of germlings to the host surface may be due to the ECM which may contain proteins/glycoproteins. Epstein et al. (1987) showed that pronase E treatment effectively

reduced adhesion of germlings of the bean rust pathogen *Uromyces appendiculatus*. It also reduced the germling directional growth and nuclear division (appressorium formation). Germlings treated with pronase E appeared to grow as vigorously as the controls, but were rounded, lacked visible extracellullar material between the fungus and the substratum and were not tightly adhered to the surface. Pronase E was also effective in removing germlings previously adhered to bean leaf discs. Washing with water or heat-denatured pronase E did not significantly affect germling adhesion. Bean rust germlings adhered to the sides of glass vials when incubated in stirred solutions of heat-denatured pronase E. In contrast, germlings with pronase E remained in suspension. All these observations strongly suggest the involvement of an extracellular protein in adhesion (Epstein et al., 1987). The composition of the bean rust pathogen's extracellular material was partially characterized. Six predominant extracellular peptides were detected (Epstein et al., 1987).

Several reports have indicated that the fungal adhesives consist of high molecular weight glycoproteins (Chaubal et al., 1991; Ding et al., 1994; Jones, 1994; Kuo and Hoch, 1995; Kwon and Epstein, 1997; Sugui et al., 1998; Hughes et al., 1999; Apoga et al., 2001). Adhesion of *Phytophthora palmivora* is mediated by surface glycoproteins (Bircher and Hohl, 1997). The carbohydrate portion of fungal glycoproteins contains α-mannosides and α-glycosides that can bind to the lectin concanavalin A (ConA). Treating germ tubes of several fungi with ConA significantly decreased adhesion (Hamer et al., 1988; Kwon and Epstein, 1993; Clay et al., 1994; Mercure et al., 1994a; Bircher and Hohl, 1997; Shaw and Hoch, 1999; Apoga and Jansson, 2000; Apoga et al., 2001). The importance of glycoproteins in adhesion, which is essential for pathogenesis, has been demonstrated by inactivating the action of glycoproteins by the lectin ConA in *Phytophthora megasperma* f. sp. *glycinea* (Ding et al., 1994). Concanavalin A treatment prevented the adhesion of germ tubes of *P. megasperma* f. sp. *glycinea* to the tissues of soybean leaf disc. It strongly inhibited fungal colonization of the host tissue (Figure 1.2; Ding et al., 1994).

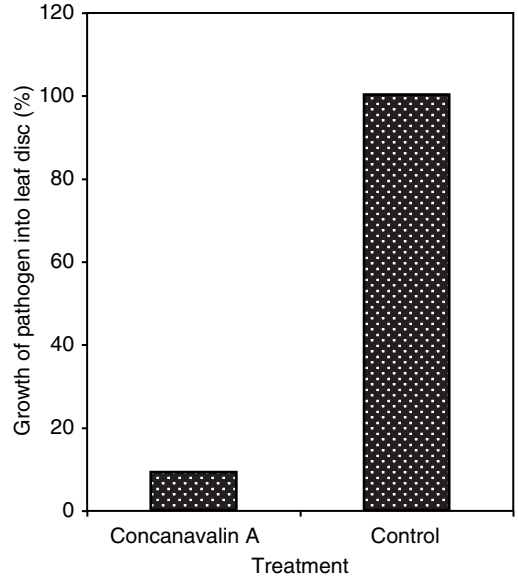

FIGURE 1.2 Effect of concanavalin A on colonization of soybean leaf discs by *Phytophthora megasperma* f. sp. *glycinea*. (Adapted from Ding, H., Balsiger, S., Guggenbuhl, C., and Hohl, H.R., *Physiol. Mol. Plant Pathol.*, 44, 363, 1994.)

When a drop of suspension of conidia of *M. grisea*, the rice blast pathogen, was dispersed on the surface of a polycarbonate film, the conidia settled to the contact surface and some of the settled conidia were observed to adhere to the contact surface within 30 min before germ tube emergence. Germinated conidia adhered firmly to the contact surface and were completely resistant to removal by rotating in water for 2 min or even overnight (Xiao et al., 1994a). Scanning electron microscopy showed abundant mucilaginous substances around germ tubes (Xiao et al., 1994a,b). α-Glucosidase, α-mannosidase, and protease strongly inhibited sporeling adhesion. In the presence of these enzymes, germinated conidia failed to adhere to the contact surface and floated in the water droplet. It suggests that the mucilage substance may be a glycoprotein since the proteolytic and glycolytic enzymes degraded the mucilage. The mucilaginous substances were observed around germ tubes on plant also. The results suggest that the adhesion of germinated conidia on plant surfaces may be due to a glycoprotein molecule (Xiao et al., 1994a).

Ultrastructural studies of barley root infection by *Bipolaris sorokiniana* revealed a fibrillar ECM beneath hyphae in contact with the root surface (Carlson et al., 1991b). The adhesion of germlings of *B. sorokiniana* incubated in the presence of the protein glycosylation inhibitor tunicamycin or the lectins ConA and GNA (from *Galanthus nivalis*) was significantly reduced (Apoga et al., 2001). It indicates the involvement of surface glycoproteins in this adhesion process.

1.4.3 Extracellular Matrix in Appressorial Adhesion

Adhesion of appressoria to the host plant surface appears to be needed for signaling the infectious structure development. ECM seemed pivotal for successful adhesion of appressoria to the host (Jones et al., 2001). ECM has been reported to coat appressoria of several fungi (Tunlid et al., 1992; Nicole et al., 1994; Apoga and Jansson, 2000). Scanning electron microscopy showed abundant mucilaginous substances around appressoria (Xiao et al., 1994a,b). Adhesion and differentiation of appressoria of fungi appear to be mediated by the ECM glycoproteins. Adhesion of appressoria of *Phytophthora palmivora* is mediated by surface glycoproteins found in ECM (Bircher and Hohl, 1997). Glycoprotein molecule has been reported to be responsible for adhesion of *M. grisea* appressoria (Xiao et al., 1994a).

Cutinases present in the ECM also may be involved in adhesion of appressorium. Adhesion of appressorium of the maize pathogen *Colletotrichum graminicola* to the host surface appears to be due to the presence of cutinases in the ECM (Pascholati et al., 1993). When conidia of *C. graminicola* are produced in acervuli on infected plant tissues or in culture, they are surrounded by a mucilage. The mucilage contains four cutinases (Pascholati et al., 1993). Diisopropyl fluorophosphate (DIPF) inhibits the cutinase activity. DIPF-treated conidia developed normal appressoria but failed to cause disease. DIPF prevented firm adhesion of appressoria and firm adhesion was necessary for penetration. The results suggest that cutinases play a role in adhesion of appressorium of the fungus (Pascholati et al., 1993).

1.4.4 Topographic Signals in Appressorium Formation

Appressorial formation is an active process and is a prerequisite for invasion of host plants by several fungi (Emmett and Parbery, 1975; Staples and Macko, 1980; Staples and Hoch, 1987; Ding et al., 1994). Plant signals may trigger germling development and appressorium formation. Chemical and physical signals are known to trigger appressorium formation (Dickinson, 1977; Dickson, 1979; Hoch et al., 1987a,b; Hoch and Staples, 1991; Gilbert et al., 1996).

Thigmotropic (physical contact with a hard substrate) growth of germ tubes of many fungal pathogens has been observed on leaf replicas (Clay et al., 1994; Staples and Hoch, 1997).

Germ tubes of the bean rust fungus, *Uromyces appendiculatus*, are able to sense the height of the stomatal guard cells as a signal to form appressorium (Hoch et al., 1987b). The thigmotropic growth of hyphae of *Bipolaris sorokiniana* in grooves between epidermal cells of barley leaves was observed (Jansson and Åkesson, 2003). However, hyphae often crossed the epidermal ridges and were then attached to the ridges by the ECM. A close contact of germ tubes and hyphae with the cuticular surface was necessary for directed growth.

The extensive growth of hyphae on the leaf surface before appressorial formation and penetration of the host by *B. sorokiniana* suggests that appressoria are mainly formed when the nutrients stored in the conidia are depleted. However, no appressoria were formed on the artificial surfaces used, indicating that appressoria also need thigmotropic signal to be formed (Jansson and Åkesson, 2003). Studies on appressorial formation by *B. sorokiniana* on leaf replicas showed that appressoria were frequent over grooves formed by the juncture of anticlinal walls of epidermal cells, suggesting the importance of thigmotropic signal (Clay et al., 1994). Thigmotropic growth of germ tubes of several other fungal pathogens has been reported (Staples and Hoch, 1997).

Topographic (surface features of plant organ) signals from plant surface may trigger appressorium formation. When ridges were microfabricated on silicon wafers, 0.5 μm ridges were found to be optimal for induction of appressorium formation in the rust fungus *U. appendiculatus*. On bean-leaf surfaces, the stomatal lip constitutes a similar ridge, which induces appressorium formation (Hoch et al., 1987b). Mechanosensitive chemicals that could transduce membrane stress induced by the leaf topography into an influx of ions including Ca^{2+} would have triggered the appressorium formation process (Zhou et al., 1991). The topographic signals induced some specific genes in *U. appendiculatus* during appressorium formation (Bhairi et al., 1989; Xuei et al., 1992, 1993). One of the upregulated genes during appressorium formation, *INF24*, contains a 450 bp open reading frame (Bhairi et al., 1989; Xuei et al., 1992, 1993) and the other gene, *INF56*, contains two open reading frames, one nested in the other (Xuei et al., 1992). Although both these genes were uniquely expressed during the thigmotropic differentiation of *U. appendiculatus*, the functions of these genes are not known.

Surface hardness may be important in substrate recognition by *M. grisea* (Jelitto et al., 1994; Xiao et al., 1994b). If conidia of the fungus are deposited on a noninductive soft substrate, germ tubes fail to develop appressoria (Talbot et al., 1993). Eight genes, designated *chip* (*Colletotrichum* hard-surface induced protein) genes, which are expressed during the early stage of hard-surface treatment, were cloned from *Colletotrichum gloeosporioides*. One of the cloned genes, *chip1*, encoded an ubiquitin-conjugating enzyme. The gene was induced by hard-surface contact and ethylene treatment enhanced this induction (Liu and Kolattukudy, 1998). Other cloned genes include *chip2* and *chip3* and these genes encode 65 and 64 kDa proteins, respectively (Kim et al., 2000). The *chip2* product would contain a putative nuclear localization signal, a leucine zipper motif, and a heptad repeat region, which might dimerize into coiled-coil structure. The *chip3* product would be a nine-transmembrane-domain-containing protein. Both *chip2* and *chip3* genes were induced by a 2 h hard-surface contact. Disruption of these two genes did not affect appressorium-forming ability of *C. gloeosporioides* suggesting that the fungus might have genes functionally redundant to *chip2* and *chip3* (Kim et al., 2000). It appears that hard-surface contact probably signals a chain of molecular events that involve reprogramming of protein synthesis needed for conidial germination and differentiation into appressoria (Liu and Kolattukudy, 1998).

Surface hydrophobicity has been implicated as an important cue for appressoria development in the rice blast fungus, *Magnaporthe grisea* (Lee and Dean, 1994). It has been demonstrated that a gene, *CBP1*, encoding a putative extracellular chitin-binding protein, may play an important role in the hydrophobic surface sensing of *M. grisea* during appressorium differentiation (Kamakura et al., 2002). *CBP1* coded for a putative extracellular

protein (signal peptide) with two similar chitin-binding domains at both ends of a central domain with homology to fungal chitin deacetylases and with a C-terminus domain rich in Ser/Thr related extracellular matrix protein such as agglutinin. Null mutants of *CBP1* failed to differentiate appressoria normally on artificial surface but succeeded in normally differentiating appressoria on the plant leaf surface. It suggests that *CBP1* may play an important role in the recognition of physical factors on solid surfaces (Kamakura et al., 2002).

It is suggested that dimeric transmembrane proteins, termed integrins, may play a key role in cell adhesion, recognition, and intracellular signaling (Hostetter, 2000). In *Candida albicans*, the application of the tripeptide arginine–glycine–aspartic acid (RGD) blocks both adhesion to and perception of the host (Bendel and Hostetter, 1993). In *U. appendiculatus*, exogenous application of the RGD blocked the fungal perception of the topographical features of the host surface (Hoch et al., 1987b; Corrêa et al., 1996). The colocalization of integrins with activated Ca^{2+} channels has been reported (Levina et al., 1994). The importance of integrins in appressorial function in *Oidium neolycopersici* has been reported (Jones et al., 2001). Integrins have been proposed to be involved in specific interactions of pathogenic fungi with their hosts and intracellular signaling cascades (Corrêa et al., 1996; Jones et al., 2001).

Several reports have suggested that the substrate characteristics may be very important for the induction of appressorium development in fungi. In many cases, appressorium formation is induced on hydrophobic, but not on hydrophilic surfaces (Howard et al., 1991; Lee and Dean, 1993a,b, 1994; Beckerman and Ebbole, 1996). The frequency of appressorium formation in *M. grisea* correlated strongly with the degree of surface hydrophobicity (Lee and Dean, 1993b). The barley powdery mildew pathogen *B. graminis* formed normal appressoria more efficiently on hydrophobic surfaces than on hydrophilic surfaces (Yamaoka and Takeuchi, 1999). In contrast, *C. gloeosporioides* was not influenced by hydrophobicity to induce appressorium formation and formed appressoria equally well on hydrophilic and hydrophobic surfaces (Lee and Dean, 1993b). *Uromyces* spp. failed to form appressoria on smooth hydrophilic or hydrophobic surfaces and required specific topographic signals for appressorium formation (Hoch and Staples, 1984; Hoch et al., 1987b). These observations suggest that plant pathogenic fungi may employ different mechanisms to sense plant signals to form appressorium.

1.4.5 Plant-Surface Wax Signals Appressorium Formation

The surface of each plant has a characteristic complex mixture of very hydrophobic materials containing very long-chain aliphatic compounds collectively called waxes (Kolattukudy et al., 1987). Fungal spores that land on plants first meet these surface waxes. Plant cuticular components induce germination and appressorium formation by fungal pathogens (Kolattukudy et al., 1995). The plant-surface wax may provide signals for the differentiation of fungal infection structures such as appressorium and penetration hypha from the extending germ tubes. The structure of surface wax may vary from plant to plant and the wax of the compatible host may selectively induce spore germination and appressorium formation. Germination and appressorium formation of spores of *Colletotrichum gloeosporioides* were induced selectively by the surface wax of its host (Podila et al., 1993). Other plant waxes could not induce differentiation of fungal infection structure in *C. gloeosporioides*, and avocado wax could not induce such developmental processes in other *Colletotrichum* species that are pathogenic to other plants. The fatty alcohol fraction of the wax was found to be most active. Synthetic aliphatic *n*-fatty alcohols with 24 or more carbons were found to induce germination and appressorium formation by *C. gloeosporioides* (Podila et al., 1993). Very long chain alcohols of the size that induce appressorium formation are known to be present in many plant waxes (Kolattukudy et al., 1995). Such waxes do not induce appressorium formation, probably because they contain inhibitors of appressorium formation. Addition

of other plant waxes to avocado wax inhibited the ability of avocado wax to induce appressorium formation by *C. gloeosporioides*. These inhibitory molecules have not been identified (Kolattukudy et al., 1995).

Terpenoid polar components in avocado wax induced appressorium formation. Urosolic acid and oleanolic acid, the two pentacyclic triterpenes found in fruit waxes, were potent inducers of germination and appressorium formation by *C. gloeosporioides* (Kolattukudy et al., 1995). It is suggested that plant-surface lipids contain inducers and inhibitors of germination and appressorium formation, and the balance may be responsible for the selective signaling by the host wax.

The plant-surface wax in the incompatible host may prevent intimate contact between the pathogen and the plant tissue, which may be necessary for parasitism (Vidhyasekaran, 1997, 2004). In the absence of this intimate contact with the plant cells, the pathogen may be unable to cause infection. The rust pathogens *Puccinia hordei*, *P. triticina*, *P. recondita*, and *P. agropyrina* could not infect the perennial wild barley *Hordeum chilense* and the fungal germ tubes were found to overgrow the stomata without forming appressorium and penetrating the stomata to invade the leaf tissue (Rubiales and Niks, 1996). This avoidance of intimate contact was associated with an extensive wax covering of the stomatal guard cells, which inhibited the induction of appressorium formation by the rust fungi (Vaz Patto et al., 2003).

Degradation of wax layer is common in compatible host–pathogen interactions, releasing cutin monomers to facilitate adhesion and induction of infection structures. Areas without wax platelets underneath hyphae removed from plant surfaces have been observed in several fungus–leaf interactions (Jansson and Åkesson, 2003). These wax-free areas would have been formed by a dissolution or utilization of wax platelets in contact with the fungus (Staub et al., 1974), or simply by an adherence of the platelets to the hyphae (Lewis and Day, 1972). A clearing zone of the wax surface was observed around the appressoria indicating enzymatic activity. Hydrolytic enzymes such as cutinases and esterases may be involved in surface wax degradation (Jansson and Åkesson, 2003). Changes in the wax layer of barley leaves were observed after treatment with an esterase preparation from *Blumeria graminis* (Kunoh et al., 1990). Cutinases and other esterases have been localized to the ECM (Jansson and Åkesson, 2003). This would probably be important for the prepenetration events.

1.4.6 Cutin Monomers as Signal Molecules

Fungal spores exude cutinase before and during germination (Pascholati et al., 1992; Nicholson et al., 1993). The cutinase may release cutin monomer by degrading the host cuticle (Nicholson et al., 1993; Francis et al., 1996). When cutin monomers were coated onto glass microscope slides or plastic coverslips, the percentage of appressorial germ tube formation in germinating conidia of *Blumeria graminis* f. sp. *hordei* dramatically increased (Figure 1.3; Francis et al., 1996). It suggests that cutin monomers may act as signal molecules in triggering appressorial formation.

1.4.7 Ethylene Signals Appressorium Formation

Ethylene is commonly produced in ripening climacteric fruits (the fruits that continue to ripen after harvest). Ethylene has been shown to act as a signal, inducing fungal spore germination and appressoria formation. On normally ripening tomato fruits, known to be producing ethylene, *Colletotrichum gloeosporioides* formed multiple appressoria. On the other hand, on transgenic tomato fruits incapable of producing ethylene the spores did not germinate. On exposure to exogenous ethylene the spores germinated, formed multiple appressoria, and produced infection lesions. These results suggest that ethylene may act as a signal to induce spore germination and appressoria formation (Kolattukudy et al., 1995).

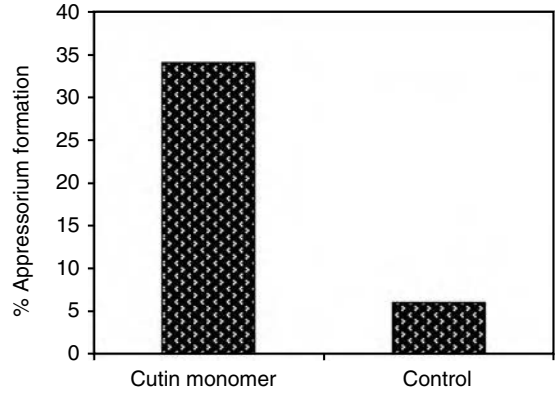

FIGURE 1.3 Effect of cutin monomer on induction of appressorium formation in *Blumeria graminis* f. sp. *hordei*. (Adapted from Francis, S.A., Dewey, F.M., and Gurr, S.J., *Mol. Plant Pathol.,* 49, 201, 1996.)

Ethylene induced both germination and appressorium formation in *C. gloeosporioides* and *C. musae* that attack climacteric fruits, but not in other *Colletotrichum* species that normally infect nonclimacteric fruits (Flaishman and Kolattukudy, 1994). Norbornadiene is an inhibitor of ethylene effect in plants. It inhibits ethylene effect on fungal spores. The inhibitory effect of norbornadiene can be overcome by higher ethylene concentration (Kolattukudy et al., 1995). It suggests that ethylene may be involved as a signal molecule in plants.

1.4.8 Fungal Signals in Induction of Appressorium Formation

Some fungal signals may trigger appressorium formation. Hydrophobins are the cell wall proteins found in many fungal species (Wessels et al., 1991; Wessels, 1994). A hydrophobin of the rice blast fungus *M. grisea* has been shown as a signal in inducing appressorium formation in this fungus. A gene (*MPG1*) coding for a type of cysteine-rich hydrophobic protein in the hydrophobin class has been cloned from *M. grisea* (Talbot et al., 1993, 1996) and it was found to be expressed during appressoria formation (Talbot et al., 1993). *mpg1*$^{-}$ mutants of the fungus were generated by a one-step gene replacement and these mutants showed reduced efficiency in forming appressorium (Talbot et al., 1993). It was suggested that secretion and self-assembly of a hydrophobin protein encoded by the *MPG1* gene may provide a sensing mechanism for surface hydrophobicity and appressorium formation (Talbot et al., 1993, 1996; Beckerman and Ebbole, 1996). *MPG1* is required for efficient induction of appressoria in response to a host surface or highly hydrophobic artificial substates. However, Beckerman and Ebbole (1996) identified several artificial substrates that can support efficient appressorium formation of *mpg1* mutants. Appressorium formation of *mpg1* mutant was rescued in trans by coinoculation with wild-type cells. This finding suggests that Mpg1 protein is not specifically required for appressorium formation, but is involved in the interaction with, and recognition of, the host surface (Beckerman and Ebbole, 1996).

The hydrophobins are secreted proteins. *MPG1* encodes a polypeptide containing a typical secretion signal peptide and Mpg1 protein is secreted into the medium (Beckerman and Ebbole, 1996). When the germlings of wild-type *M. grisea* (with the ability to form appressoria) and *mpg1* deletion mutants (defective in appressorium formation) were mixed and incubated, the *mpg1* mutants also formed appressoria in the presence of wild-type strains, probably due to the signal provided by the secreted Mpg1 protein from the wild-type isolates (Beckerman and Ebbole, 1996). These studies indicate that the fungal hydrophobins may act as a signal in inducing appressorium formation. The hydrophobin rodlets assembled at the

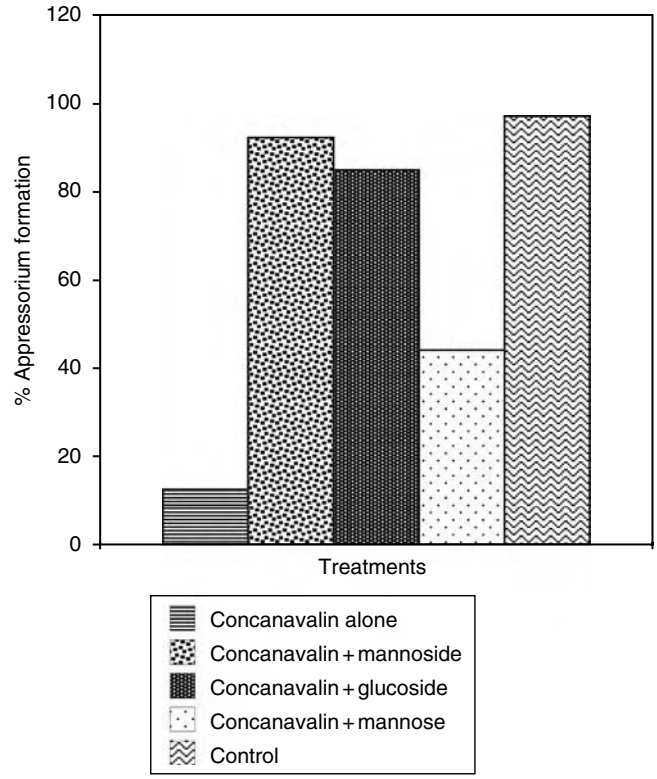

FIGURE 1.4 Effect of concanavalin A in appressorium formation in *M. grisea*. (Adapted from Xiao, J.Z., Oshima, A., Kamakura, T., Ishiyama, T., and Yamaguchi, I., *Mol. Plant Microbe Interact*., 7, 639, 1994a.)

hyphae–substrate interface may signal the presence of a "host" surface. The hydrophobin assembly may assist in adhesion of germling to host hydrophobic surfaces in such a way that acts as a conformational cue for appressorium differentiation (Wösten et al., 1994; Beckerman and Ebbole, 1996; Kershaw and Talbot, 1998).

Extracellular glycoproteins released from germinating conidia of some fungi would have signaled appressorium formation. Abundant mucilaginous substances have been observed around germ tubes of *M. grisea* (Xiao et al., 1994a). The mucilage disappeared when the conidia were germinated in the presence of protease, α-mannosidase and α-glucosidase, indicating that the extracellular mucilage contained glycoproteins. Concanavalin A, a lectin binding to α-D-mannose and α-D-glucose, specifically suppressed appressorium formation (Xiao et al., 1994a). Concanavalin A inhibition of appressorium was blocked by the potential ConA competitors, methyl α-D-mannoside, methyl-α-D-glucoside, and D-mannose (Figure 1.4; Xiao et al., 1994a). These results suggest that extracellular glycoprotein may be involved in sensing and transmission of information about appressorium induction (Xiao et al., 1994a).

1.5 SIGNAL TRANSDUCTION IN FUNGAL PATHOGENESIS

1.5.1 Transmembrane Receptor for Extracellular Signals

Plant signals such as topographic signals (Jones et al., 2001), plant wax (Kolattukudy et al., 1995), cutin monomers (Francis et al., 1996), which could be released by ECM cutinase

(Kunoh et al., 1988; Nicholson et al., 1988, 1993; Pascholati et al., 1992; Fric and Wolf, 1994; Francis et al., 1996; Fan and Koller, 1998; Rumbolz et al., 2000), ethylene (Kolattukudy et al., 1995, 2004), and possibly cellulose degradation products (Suzuki et al., 1998; Carver et al., 1999) are involved in triggering the development of infection structures in fungi. The secreted fungal hydrophobin encoded by *MPG1* has also been shown to play an upstream role in activating appressorium differentiation (Talbot et al., 1996). These extracellular signals may be recognized by cell membrane receptors (DeZwaan et al., 1999).

A receptor for inductive substrate cues has been detected in the rice blast fungal cell membrane (DeZwaan et al., 1999). The gene *PTH11* encodes a transmembrane protein that is an upstream effector of appressorium differentiation. The protein was localized to the cell membrane. The *pth11* mutants failed to progress beyond the recognition phase of appressorium differentiation. Exogenous cAMP and diacylglycerol suppressed defects associated with *pth11* mutants. cAMP restored both appressorium formation and pathogenicity, whereas diacylglycerol only restored appressorium formation. These findings suggest that the protein encoded by *PTH11* (Pth11p) plays an upstream role in activating appressorium signaling, possibly by acting as a receptor for inductive substrate cues (DeZwaan et al., 1999).

The extracellular signals may be relayed within the fungal cell to induce the developmental changes that lead to formation of appressorium and other infection structures (Flaishman et al., 1995; Wang and Nuss, 1995; Uhm et al., 2003). The relaying of the signal is referred to as signal transduction and is mediated by second messengers including Ca^{2+} (David, 1995; Lee and Lee, 1998; Uhm et al., 2003), cyclic nucleotides (Hoch and Staples, 1984; Garril et al., 1992; Lee and Dean, 1993, 1994; Lee and Lee, 1998; Takano et al., 2001), inositol phosphate (Berridge, 1993; Devecha and Irvine, 1995), and diacylglycerol, ceramides, and sphinomyelin (Thines et al., 1997).

1.5.2 G-Proteins

Fungal pathogens after perceiving plant signals may transduce these signals using heterotrimeric guanine nucleotide-binding (G) proteins (Liu and Dean, 1997). The cell membrane-associated heterotrimeric G-proteins are key components in the signal transduction pathways. They belong to GTPase superfamily and are involved in transducing extracellular signals from activated membrane receptors to a variety of intracellular targets (effectors) through a cascade of interacting proteins (Neer, 1995). G-proteins act as transducers and signal amplifiers (Gilman, 1987; Gudermann et al., 1997).

G-proteins are heterotrimers composed of α, β, and γ subunits (Neer, 1995). G-proteins bind guanine nucleotides and generally couple the activation of seven transmembrane domain receptors to downstream effector molecules leading to appropriate changes in gene expression (Dohlman et al., 1987, 1991). In the inactive state, the α subunit ($G\alpha$) binds GDP with high affinity, whereas the $\beta\gamma$ dimer ($G\beta\gamma$) stabilizes the complex. Binding of signal ligands to cell-surface receptors promotes an exchange of GDP for GTP on the $G\alpha$ subunit, which then triggers a reciprocal conformational change and dissociation of the three subunits into GTP-$G\alpha$ and $G\beta\gamma$ heterodimer. Both $G\alpha$ and $G\beta\gamma$ subunits interact with appropriate target effectors, such as enzymes, ion channels, and transporters (e.g., phosphodiesterase, protein kinases, adenylyl cyclases, phospholipases, and calcium ion channels) (Simon et al., 1991; Neer, 1995; Clapham and Neer, 1997; Hamm, 1998) to trigger downstream signaling pathways for cellular responses. The activation of G-proteins results in rapid alterations of concentrations of second messengers, such as cAMP, inositol phosphates, diacylglycerol, and cytosolic ion (Gudermann et al., 1997). The activated G-proteins are later desensitized by the GTPase activity of the $G\alpha$ subunit, followed by reassociation with the $G\beta\gamma$ complex, priming the system for another round of activation (Neer, 1995; Fang and Dean, 2000).

Gα subunits are grouped into four families based on amino acid sequence identity: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ (Simon et al., 1991). The $G\alpha_i$ (inhibitory) family consists of $G\alpha_i$, $G\alpha_o$, $G\alpha_z$, and $G\alpha_t$ (transducin) subfamilies. Genes encoding G-proteins have been cloned from various fungi (Choi et al., 1995; Gao and Nuss, 1996; Ivey et al., 1996; Baasiri et al., 1997; Kasahara and Nuss, 1997; Regenfelder et al., 1997; Fang and Dean, 2000; Nishimura et al., 2003). Genes encoding heterotrimeric G-protein α (Gao and Nuss, 1996, 1998; Liu and Dean, 1997; Regenfelder et al., 1997; Bölker, 1998) or β (Kasahara and Nuss, 1997; Nishimura et al., 2003) subunits have been shown to be required for the development of fungal infection structures. The G-protein α-subunit gene, *CGA1*, was found essential for appressorium formation in *C. heterostrophus* (Horwitz et al., 1999). Of the three Gα-protein-encoding genes cloned from the rice blast pathogen *M. grisea*, one of them, *MAGB* is required for appressorial formation (Liu and Dean, 1997). Disruption of *MAGB* led to defects in appressorium formation (Liu and Dean, 1997; Fang and Dean, 2000). A Gβ subunit encoding gene *mgb1* was found to be essential for appressorium formation in *M. grisea* (Nishimura et al., 2003). Conidia from *mgb1* mutants were defective in appressorium formation and failed to penetrate or grow invasively on rice leaves (Nishimura et al., 2003). The gene *cpg-1* encoding Gα subunit has been cloned from the chestnut blight pathogen *Cryphonectria parasitica* and it was found to be involved in signaling in the infection process (Choi et al., 1995).

A gene encoding a Gα subunit homolog, designated *ctg-1*, was isolated from the alfalfa anthracnose pathogen *Colletotrichum trifolii* (Truesdell et al., 2000). *Ctg-1* transcripts accumulate in germinating conidia, suggesting that the protein plays a regulatory role during this developmental stage. Replacement of *ctg-1* with a null allele resulted in fungal transformants whose conidia failed to germinate (Figure 1.5; Truesdell et al., 2000). The results demonstrate the requirement of *ctg-1* for an early stage in the pathogenic life cycle of *C. trifolii* (Truesdell et al., 2000). A Gα protein encoded by *bcg1* gene was found to be important in pathogenicity of *Botrytis cinerea* (Gronover et al., 2001).

G-protein signaling appears to be very important in the fungal infection process (Nishimura et al., 2003). Inactivation of Gα-encoding gene results in reduction in virulence of pathogens. Gα-deficient mutants of *Ustilago maydis* are nonpathogenic on corn plants (Regenfelder et al., 1997). Disruption of the Gα-encoding *cpg-1* gene in *Cryphonectria*

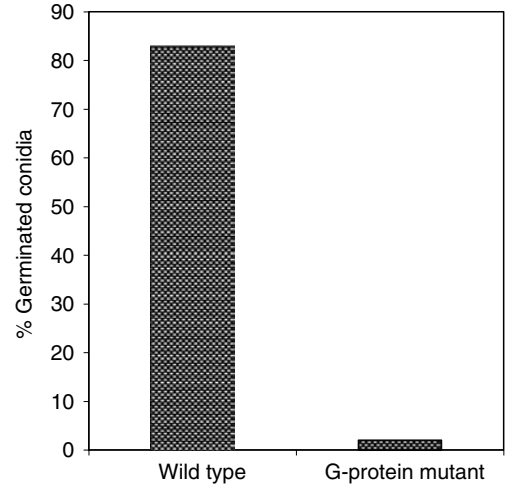

FIGURE 1.5 Conidial germination of *Colletotrichum trifolii* wild type and the *ctg-1* disruption mutant. (Adapted from Truesdell, G.M., Yang, Z., and Dickman, M.B., *Physiol. Mol. Plant Pathol.*, 56, 131, 2000.)

parasitica caused loss of conidiation and pathogenicity on chestnut (Gao and Nuss, 1996). Inactivation of *Gna1*, a gene encoding a Gα subunit in the wheat pathogen *Stagonospora nodorum* resulted in a defect in direct penetration (Solomon et al., 2004). Disruption in *magB* gene in *M. grisea* reduces virulence and appressorium formation (Liu and Dean, 1997; Fang and Dean, 2000). *C. trifolii* strains in which the Gα gene *ctg-1* was insertionally inactivated by gene replacement were obtained. These mutants showed decreased percentage of conidial germination and appressorial formation on the host plant alfalfa leaves (Truesdell et al., 2000). The bean gray mold fungus *Botrytis cinerea* mutants of Gα subunit BCG1 were able to penetrate host leaves, and produced small primary lesions. However, in contrast to the wild type, the mutants completely stopped invasion of plant tissue at that stage; secondary lesions were never observed (Gronover et al., 2004).

All these observations suggest that G-protein signaling plays a key role in the signal transduction system in the fungal infection process. In the downstream of G-protein signaling, calcium ion-dependent signaling system, cAMP/protein kinase signaling pathway and MAP kinase (MAPK) cascade may operate.

Adenylyl cyclase is a membrane-bound enzyme that catalyzes the production of cAMP from adenosine triphosphate (ATP). The adenylate cyclase gene *MAC1* was cloned from *M. grisea* (Choi and Dean, 1997). Transformants lacking *MAC1* gene were unable to form appressoria on an inductive surface and were unable to penetrate susceptible rice leaves. Appressorium formation was restored in the presence of exogenous cAMP derivatives (Choi and Dean, 1997). It suggests that *MAC1* gene may function upstream of cAMP. The Gβ subunit has been demonstrated to regulate cellular levels of cAMP (Nishimura et al., 2003) by direct interaction with adenylate cyclases (Chen et al., 1995; Yan and Gautam, 1996). Adenylyl cyclase may be a downstream target of the activated G-proteins and exogenous cAMP can rescue some defects in Gα mutants (Alspaugh et al., 1997; Choi and Dean, 1997; Krüger et al., 1998; Ivey et al., 1999; Loubradou et al., 1999). The Gα subunit protein encoded by the *MAGB* gene (MagBp) in *M. grisea* stimulates adenylate cyclase (Liu and Dean, 1997) activates cAMP-mediated signaling (Liu and Dean, 1997). Exogenous cAMP application suppressed the appressorium defect of *magB* mutants (Liu and Dean, 1997). These studies suggest that adenylate cyclase-mediated cAMP-signaling pathway operates downstream of G-protein signaling system.

Additional evidence for a relationship between fungal G-proteins and adenylate cyclase comes from studies on *C. parasitica* strains where wild-type *cpg-1* (the gene encoding Gα subunit) was replaced with a null allele (Gao and Nuss, 1996). These strains displayed an increase in the level of cAMP, suggesting that either CPG-1 inhibits adenylate cyclase or negatively regulates Gβγ, which stimulates the enzyme (Gao and Nuss, 1996). In contrast, MagB (the protein encoded by the Gα gene *magB*) stimulates adenylate cyclase (Liu and Dean, 1997). Some of the Gα genes may also operate through a pathway other than cAMP-dependent pathway. Most of the *Botrytis cinerea* Gα subunit BCG1-controlled genes were still expressed in adenylate cyclase mutants in planta, suggesting that BCG1 is involved in at least one additional signaling cascade in addition to the cAMP-depending pathway (Gronover et al., 2004). In *M. grisea*, exogenous cAMP also supports appressorium differentiation in the absence of inductive cues (hydrophobic surfaces and cutin monomers), and *mac1* adenylyl cyclase mutants have a defect in appressorium differentiation similar to that of *magB* mutants (Choi and Dean, 1997; Adachi and Hamer, 1998). However, *magB* mutants fail to differentiate on hydrophobic surfaces but continue to respond to soluble cutin monomers (Liu and Dean, 1997), whereas *mac1* mutants fail to differentiate in response to either inductive cue (Choi and Dean, 1997). Thus, parallel pathways of sensory input appear to converge at or before the *MAC1*-encoded adenylate cyclase protein (Mac1p) in *M. grisea* (Choi and Dean, 1997).

1.5.3 Calcium/Calmodulin-Dependent Signaling

Cytosolic calcium ion is regarded as an important cation for hyphal tip growth and appressorial development in many organisms (Jackson and Heath, 1993). The Ca^{2+} signaling pathway in cells is initiated through conformational changes of the GTP-binding protein (G-protein) linked to a membrane receptor (Larson et al., 1992). Phospholipase C is activated by the G-protein and hydrolyzes phosphatidyl inositol-1,4-biphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacyl glycerol (Su et al., 2001). IP_3 in turn causes the release of endogenous Ca^{2+} from intracellular calcium stores (Belde et al., 1993). Increments of intracellular Ca^{2+} result in the activation of calmodulin, the Ca^{2+} receptor protein. Ca^{2+} binding induces conformational changes in calmodulin, resulting in the activation of many enzymes, including the multifunctional calmodulin-dependent protein kinase and protein phosphatases (Van Edick and Waterson, 1995; Kim et al., 1998).

Involvement of Ca^{2+} signaling systems in the appressorium formation of *C. trifolii* (Warwar and Dickman, 1996) and *C. gloeosporioides* (Kim et al., 1998) has been demonstrated. Several inhibitors thought to act on various steps in the Ca^{2+}-signaling system were found to inhibit appressorium formation in *C. gloeosporioides* (Figure 1.6; Uhm et al., 2003). Intracellular Ca^{2+} concentration may be regulated by the release of Ca^{2+} from intracellular stores or by Ca^{2+} channels located in the cell membrane (Cornelius et al., 1989). An inhibitor of calcium release from intracellular calcium store, TMB-8 [3,4,5-trimethoxybenzoic acid 8-(diethyl-amino)octyl ester] inhibited appressorium formation in *C. gloeosporioides* (Figure 1.6; Uhm et al., 2003). A calcium channel blocker located in the plasma membrane, methoxy verampamil, also inhibited appressorium formation. The results suggest that calcium channels may be involved in the signal transduction system to induce appressorium formation in fungal pathogens (Uhm et al., 2003). The Ca^{2+} release from internal calcium stores appears to be more important than Ca^{2+} influx mediated through the plasma membrane gate on the appressorium formation in *M. grisea* (Lee and Lee, 1998) and *C. gloeosporioides* (Uhm et al., 2003).

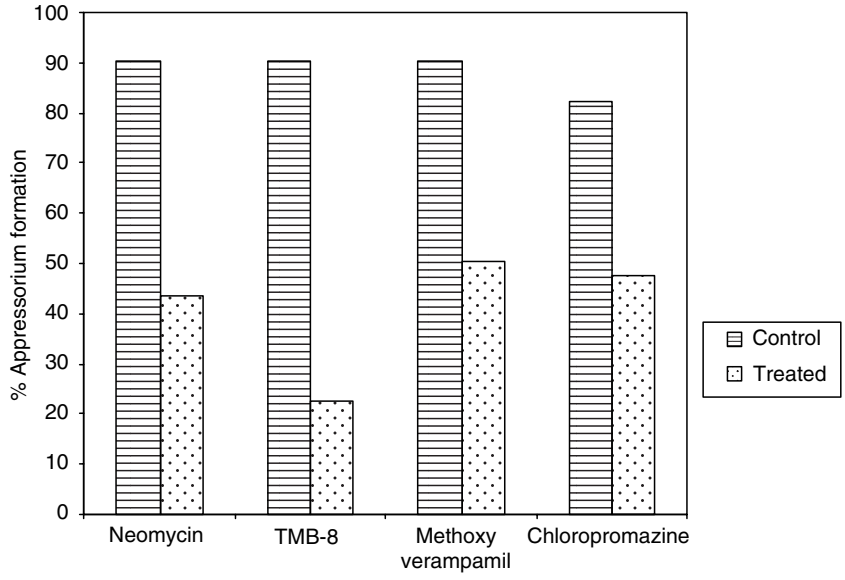

FIGURE 1.6 Effect of inhibitors of calcium/calmodulin signaling system in appressorium formation in *Colletotrichum gloeosporioides*. (Adapted from Uhm, K.-H., Ahn, I.-P., Kim, S., and Lee, Y.-H., *Phytopathology*, 93, 82, 2003.)

The action of Ca^{2+} in cells is mainly mediated by calmodulin, the Ca^{2+}-binding protein. Calmodulin was found to be involved in appressorium formation in *C. gloeosporioides* (Kim et al., 1998) and *C. trifolii* (Buhr and Dickman, 1997). Chloropromazine is a calmodulin antagonist, which competes with Ca^{2+} for binding to calmodulin and consequently inhibits Ca^{2+}/calmodulin signaling. Chloropromazine inhibited appressorium formation in *C. gloeosporioides*, indicating the role of Ca^{2+}/calmodulin signaling in appressorium formation (Figure 1.6; Uhm et al., 2003). Other calmodulin antagonists also inhibited appressorium formation in this fungus (Uhm et al., 2003). Inhibition of appressorium formation by calmodulin antagonists has been reported in *C. trifolii* (Dickman et al., 1995; Warwar and Dickman, 1996; Buhr and Dickman, 1997), *M. grisea* (Lee and Lee, 1998), and *Phyllosticta ampelicida* (Shaw and Hoch, 2000).

Calmodulin (*cam*) genes have been cloned from *C. gloeosporioides* (Kim et al., 1998), *C. trifolii* (Warwar et al., 2000), and *M. grisea* (Liu and Kolattukudy, 1999). These genes are highly conserved and nearly identical in their nucleotide sequences (Kim et al., 1998; Liu and Kolattukudy, 1999; Warwar et al., 2000). A calmodulin gene was cloned from *C. trifolii* (Dickman et al., 1995). Antisense strategy was used to reduce the expression of this calmodulin gene and this strategy reduced the frequency of appressorium formation (Dean, 1997; Warwar et al., 2000). The mucilage from *M. grisea* conidia contributed for the attachment of conidia to a surface (Hamer et al., 1988) and the attachment contributed for appressoria formation. The lectin ConA inhibited surface attachment of conidia, *cam* gene expression, and appressorium formation without affecting germination (Liu and Kolattukudy, 1999). These results revealed the importance of Ca^{2+}/calmodulin signaling in fungal appressorium formation.

The phosphoinositide signaling system is related to calcium signaling through IP_3, an intracellular calcium activator. IP_3 is formed from PIP_2 by activated phospholipase C and released into the cytosol (Devecha and Irvine, 1995). Neomycin, a phospholipase C inhibitor, inhibited appressorium formation in *C. gloeosporioides*, suggesting the importance of phosphoinositide signaling system in appressorium formation (Figure 1.6; Uhm et al., 2003).

1.5.4 cAMP/Protein Kinase Signaling Pathway

cAMP is an important second messenger in the signal transduction system. cAMP signaling pathway may operate downstream of G-protein signaling (Nishimura et al., 2003). A well-characterized intracellular target of cAMP in eukaryotic cells is the regulatory subunit of PKA (Taylor et al., 1990). PKA is a tetrameric holoenzyme that is composed of two regulatory subunits and two catalytic subunits. Binding of cAMP to the regulatory subunits releases the catalytic kinase subunits to phosphorylate target proteins involved in cAMP-regulated processes (Adachi and Hamer, 1998). cAMP signaling has been reported to be important in fungal pathogenesis (Kronstad, 1997).

Increases in the intracellular concentration of cAMP may act as part of an early signaling event in appressorium formation (Lee and Dean, 1993). The germ tube tip differentiates into an appressorium when the conidia of *M. grisea* germinate on a hydrophobic surface. However, on hydrophilic surfaces, the emerging germ tube does not differentiate into appressorium and continues to grow vegetatively. When cAMP was added to germinating conidia or vegetative hyphae appressorium formation was observed even on hydrophilic surfaces (Figure 1.7; Lee and Dean, 1993). It suggests that the need for an inductive surface can be bypassed by the addition of cAMP to the germinating conidia. Lee and Dean (1993b) suggested that the interaction of cell wall protein in the germ tube with the host surface results in the increased production of cAMP and initiation of the developmental program of appressorium formation.

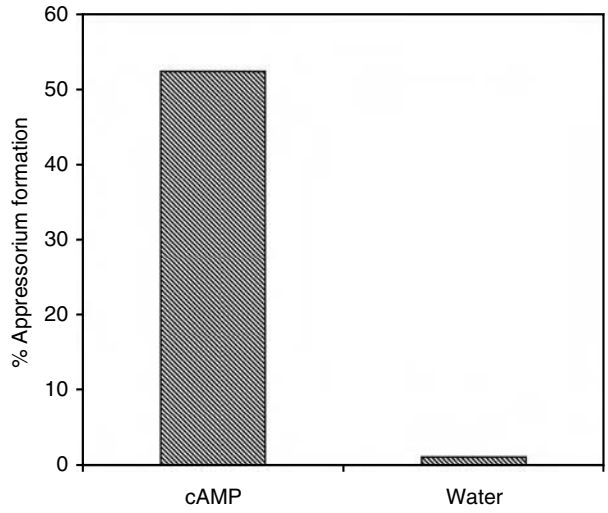

FIGURE 1.7 Effect of cAMP in induction of appressorium formation in *M. grisea* on hydrophilic surface. (Adapted from Lee, Y.-H. and Dean, R.A., *Plant Cell*, 5, 693, 1993b.)

Appressorium formation in *M. grisea* is inhibited by yeast extract, probably by perturbing signaling mechanisms that trigger appressorial formation (Beckerman and Ebbole, 1996). When cAMP was added along with yeast extract, the germ tubes differentiated into appressorium (Figure 1.8; Beckerman and Ebbole, 1996).

M. grisea mac1 (the gene encoding adenylate cyclase) null mutants were defective in forming appressorium. When cAMP was added to the germinating conidia of these mutants, normal appressorium formation was observed (Figure 1.9; Adachi and Hamer, 1998). All these studies have suggested that cAMP is an important second messenger in the signal transduction to induce appressorium formation.

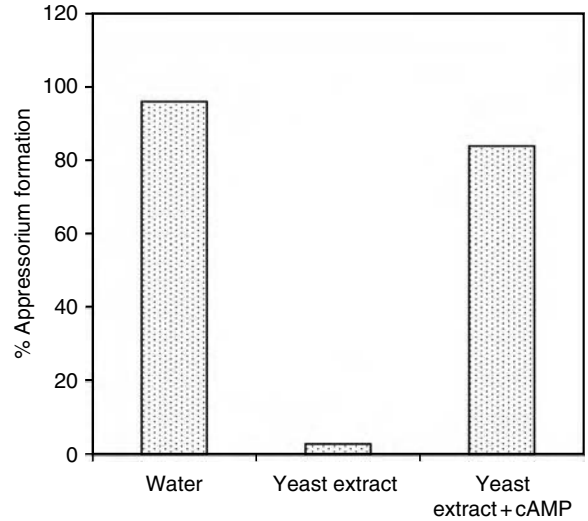

FIGURE 1.8 Effect of cAMP in induction of appressorium formation in *M. grisea* on hydrophobic surface. (Adapted from Beckerman, J.L. and Ebbole, D.J., *Mol. Plant Microbe Interact*., 9, 450, 1996.)

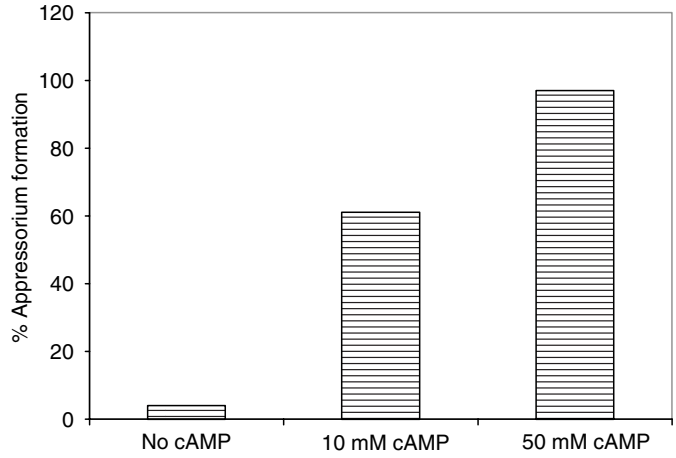

FIGURE 1.9 cAMP rescues the appressorium formation defect in *mac1* mutants of *M. grisea*. (Adapted from Adachi, K. and Hamer, J.E., *Plant Cell*, 10, 1361, 1998.)

The cAMP signaling system is conserved in diverse fungal pathogens (Lee et al., 2003) causing smuts (Gold et al., 1997), blast (Mitchell and Dean, 1995; Choi et al., 1998), blights (Kronstad, 1997), and powdery mildews (Hall and Gurr, 2000). cAMP signaling has been shown to be required to trigger initiation of appressorial development in *B. graminis* f. sp. *hordei* (Hall and Gurr, 2000). cAMP-regulated morphogenesis in *Ustilago maydis* (Gold et al., 1994) and the regulatory subunit of a cAMP-dependent protein kinase were required for gall (tumor) symptom development by *U. maydis* in maize (Gold et al., 1997). cAMP-regulated protein kinase has been shown to be involved in appressorium formation in *Colletotrichum trifolii* (Yang and Dickman, 1997, 1999a,b). In *U. maydis*, mating of two haploid is a prerequisite for the formation of infection structures. Pathogenicity depends on the transition from the haploid, noninfectious, yeast-like form to the infectious, filamentous dikaryon. The successful formation of the filamentous form occurs when there is mating between the haploid cells bearing different alleles at both the *a* and *b* mating type loci. The genes involved in the mating and development process have been shown to be regulated by the cAMP-signaling cascade (Hartmann et al., 1996; Krüger et al., 1998; Müller et al., 1999). The components of the cAMP pathway are the activating Gα subunit, Gpa3 (Regenfelder et al., 1997; Krüger et al., 1998), the adenylate cyclase, Uac1, and the regulatory (Ubc1) and catalytic subunit of the PKA (Adr1) (Gold et al., 1994, 1997). The cAMP pathway is also needed for subsequent development of the fungal pathogen in planta. Null mutants in any of these genes (*gpa3*, *uac1*, *ubc1*, and *adr1*) were unable to form galls (tumors) (Gold et al., 1997). Moderate activation of the cAMP pathway conferred either by mutation in the Gα subunit or by mutation in the regulatory subunit of the PKA altered the corn smut tumor morphology (Krüger et al., 2000). It suggests that cAMP pathway acts downstream of G-proteins in *U. maydis*.

The cAMP would have mediated its effect on appressorium formation in *M. grisea* through PKA. Adachi and Hamer (1998) identified *M. grisea* strains in which the *Mac1*$^{-}$ phenotype is unstable. Among the bypass suppressors of the *mac1* mutation, a specific mutation in the regulatory subunit of PKA was identified. This mutation allowed appressorium formation on nonhydrophobic surfaces and in the presence of yeast extract. These findings suggest that cAMP mediates its effect on appressorium formation through PKA, most likely through a catalytic subunit. The cAMP-dependent protein kinase catalytic subunit has been shown to be required for appressorium formation and pathogenesis by the rice blast pathogen *M. grisea* (Mitchell and Dean, 1995). *CPKA* gene, which encodes a catalytic subunit of cAMP-dependent PKA, is also involved in functional appressorium formation in *M. grisea*

(Mitchell and Dean, 1995; Xu and Hamer, 1996; Xu et al., 1997). *cpkA* mutants are delayed in appressorium formation but form appressoria at the same level as wild-type strains. Appressoria formed by *cpkA* mutants are fully melanized but are defective in penetrating plant cells. These findings suggest that *CPKA* gene is involved in appressorial penetration process (Xu et al., 1997).

A cAMP-dependent PKA catalytic subunit gene (*pka1*) has been shown to be involved in the infection process of *Sclerotinia sclerotiorum* (Jurick et al., 2004). Increased cAMP levels raised oxalate production in *S. sclerotiorum* (Jurick et al., 2004). High intracellular cAMP levels increased oxalic acid levels in the fungus (Rollins and Dickman, 1998) and oxalate-deficient mutants are nonpathogenic (Godoy et al., 1990). These results suggest that cAMP pathway may be involved in signaling oxalate production, which is involved in the infection process of *S. sclerotiorum* (Jurick et al., 2004).

1.5.5 Mitogen-Activated Protein Kinase Signaling Cascades

MAPK in fungal cells are well known for transducing a variety of extracellular signals to regulate growth, differentiation, and pathogenesis in fungal pathogens (Xu and Hamer, 1996; Xu et al., 1998; Lev et al., 1999; Mayorga and Gold, 1999; Müller et al., 1999; Takano et al., 2000; Xu, 2000; Park et al., 2004). MAPKs homologous to the yeast Fus3/Kss1, Slt2, and Hog1 MAPKs have been identified in several fungal pathogens and found to be important for appressorium formation, invasive hyphal growth, and fungal pathogenesis (Xu, 2000). The MAPK cascades generally consist of three functionally linked kinases: a MAPK; MAP kinase kinase (MAPKK); and a MAP kinase kinase kinase (MAPKKK). After stimulation by extracellular stimuli, MAPKKK activates MAPKK by the phosphorylation of serine and serine/threonine residues in the SXXXS/T motif. As a dual-specificity kinase, MAPKK then activates MAPK through the phosphorylation of threonine and tyrosine residues in the TXY motif located between kinase subdomains VII and VIII. Activated MAPK then modulates cellular responses by the phosphorylation of various enzymes or transcription factors. In this way, extracellular stimuli are transduced into intracellular responses (Blumer and Johnson, 1994; Herskowitz, 1995; Liu et al., 2000).

MAPK signaling cascade may operate at downstream of cAMP signaling pathway. In *M. grisea*, two MAPK genes, *PMK1* and *MPS1*, have been identified (Xu and Hamer, 1996; Xu et al., 1998). Gene disruption resulted in impaired appressorium formation or appressorium penetration. Both mutants were able to respond to exogenous cAMP for the early stages of appressorium development, indicating potential interactions between the cAMP–PKA pathway and the MAPK signaling cascades (Kronstad et al., 1998). In *Ustilago maydis*, a MAPK gene, *ubc3* (*kpp2*), homologous to *PMK1* gene of *M. grisea* has been characterized (Mayorga and Gold, 1999; Müller et al., 1999). Mutants in which this MAPK gene was deleted had no growth or morphological defects but were dramatically reduced in mating, formation of filamentous dikaryons, and virulence (Mayorga and Gold, 1999; Müller et al., 1999). In plants inoculated with Δ*kpp2* mutants of solo pathogenic haploid strains, tumor formation was observed only on leaves and tumor diameter did not exceed 1 mm (Kahmann et al., 1999; Müller et al., 1999). Two other MAPK genes, *ubc4* and *ubc5*, were shown to be needed in filamentous growth and pathogenicity of *U. maydis*. The *ubc4* gene encoded a MAPKKK and *ubc5* encoded a MAPKK (Andrews et al., 2000). In the smut fungus, the signal is transmitted through a MAPK module to the high mobility-group (HMG) domain transcription factor *Prf1*, leading to its activation. This triggers pathogenic development (Brefort et al., 2005).

Another MAPK encoded by *kpp6* gene has been identified to be important in the infection process of *U. maydis* (Brachmann et al., 2003). Transcription of the *kpp6* gene yields two transcripts differing in length, but encoding proteins of identical mass. *kpp6* mutants carrying a nonactivatable allele of *kpp6* are attenuated in pathogenicity. These mutants can still form appressoria, but are defective in the subsequent penetration of the plant cuticle. The *kpp6* may

respond to a plant signal and regulate the genes necessary for efficient penetration of the plant tissue (Brachmann et al., 2003). The importance of cAMP pathway in pathogenesis of *U. maydis* has been already discussed (see earlier) and an interplay of the cAMP and MAPK signal transduction pathways seems to be important in the infection process of the corn smut pathogen *U. maydis*.

In the rice blast fungus, *M. grisea*, two MAPK (Pmk1 and Mps1) pathways play important role in pathogenesis. Pmk1 is a member of extracellular signal-regulated kinase (ERK) family of the MAPK superfamily, whose main function in other eukaryotes is the transduction of extracellular signals. Pmk1 is functionally related to the yeast Fus3 MAPK, which is essential for mating responses. The *PMK1* gene has been cloned. Δ*pmk1* mutants do not produce appressoria and are nonpathogenic even when introduced directly into plant tissue by injection. Furthermore, Δ*pmk1* mutants are not complemented by exogenous cAMP but do undergo the early hooking stages of appressorium formation in its presence (Xu and Hamer, 1996). Inactivation of the Pmk1 MAPK resulted in a defect in both appressorium formation and invasive growth in plants but showed no other effect on development, including mating reaction (Xu and Hamer, 1996).

Another MAPK, Mps1, belongs to the yeast and fungal ERK (YERK2) subfamily (Lee et al., 1993; Xu et al., 1998) and related to the yeast Slt2 (Mpk1) kinase. It is required for plant penetration (Lee and Dean, 1993a; Xu et al., 1998). MAPK may also be involved in the development of functional appressorium. The appressorium of *M. grisea* generates enormous cellular turgor, which allows the fungus to send narrow penetration peg through the rice leaf cuticle (Howard et al., 1991). During turgor generation, transfer of storage carbohydrate and lipid reserves to the appressorium occurs under the control of the *PMK1* MAPK pathway (Thines et al., 2000).

A MAPK gene (*BMP1*; *Botrytis* MAPK required for pathogenesis 1), which is highly homologous to the *M. grisea Pmk1*, has been isolated from *Botrytis cinerea*, a necrotropic pathogen of gray mold disease of several plants (Zheng et al., 2000). The MAPK pathway appears to be involved in the penetration process of *B. cinerea*. Conidia from *bmp1* mutants germinated on plant surfaces but failed to penetrate plant tissues. The MAPK was also found essential in the development of the fungal growth. *bmp1* gene replacement *B. cinerea* mutants showed reduced growth rate (Figure 1.10; Zheng et al., 2000). Reintroduction of the wild-type

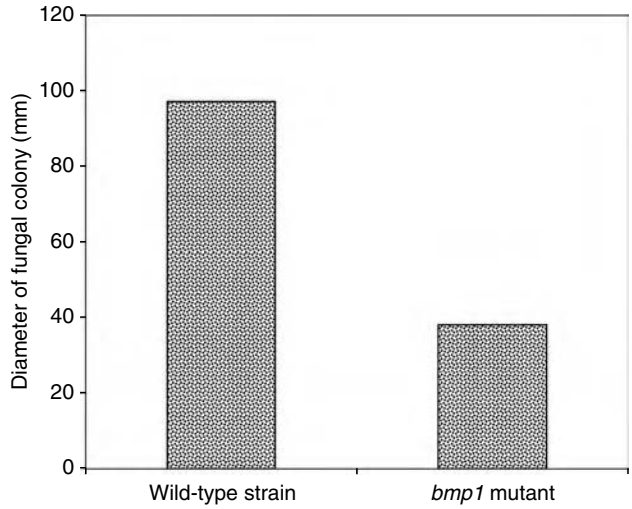

FIGURE 1.10 Function of *BMP1* MAPK gene in induction of growth of *Botrytis cinerea*. (Adapted from Zheng, L., Campbell, M., Murphy, J., Lam, S., and Zhu, J.-R., *Mol. Microbe Interact.*, 13, 724, 2000.)

allele *BMP1* allele into the *bmp1* mutant restored normal growth. *bmp1* mutants were nonpathogenic on carnation flowers and tomato leaves (Zheng et al., 2000). These results suggest that MAP pathway is a key component in the signal transduction system in the infection process of *B. cinerea*.

The infection process of the cucumber anthracnose pathogen *Colletotrichum lagenarium* involves spore germination, formation of melanized appressoria, appressorial penetration, and subsequent invasive growth in host plants. The MAPK signaling pathway plays a central role in these steps (Takano et al., 2000). Two MAPK genes designated *CMK1* and MAF1 have been isolated from *C. lagenarium* (Takano et al., 2000; Kojima et al., 2002). Conidia of the wild-type isolate of *C. lagenarium* adhered to hard surface, whereas *cmk1* mutants showed very much less attachment to the substrate (Table 1.1; Takano et al., 2000). It suggests that the physical contact signals are transduced through MAPK signaling cascade to initiate the infection process. The wild-type strain germinated well in the water on perception of the hard-surface signals, whereas the *cmk1* mutants hardly germinated on the hard surface (Table 1.1). The germinated wild-type isolate formed appressoria, whereas the *cmk1* mutants even after germination in the presence of yeast extract failed to form appressoria. The *cmk1* mutants did not penetrate the plant surface and when the mutants were inoculated on wounded sites of leaves, they hardly extended vegetative hyphae. These results indicate that *CMK1* is required for invasive growth in cucumber leaves (Takano et al., 2000). *cmk1* mutants could produce melanins during vegetative growth. The three melanin biosynthesis genes, *PKS1*, *SCD1*, and *THR1*, were expressed in both wild-type and *cmk1* mutant strains during mycelial melanization. During prepenetration events in the wild-type *PKS1*, *SCD1*, and *Thr1* transcripts were not detected in preincubated conidia, but accumulated at 3 h post inoculation in water when conidia germinated. In contrast, when the *cmk1* mutant was incubated in water where conidia could not germinate, transcripts of all three genes were not detected even after long incubation of 16 h. In case of incubation in yeast extract solution, when the conidia of *cmk1* mutant germinated, all three melanin genes were highly expressed. These results suggest that the transcription of melanin genes were tightly linked to the conidial germination process. For *cmk1* mutants, melanization was not observed in germinating conidia, melanin genes were highly expressed. These findings suggest that CMK1 MAPK is needed for melanization and development of functional appressoria in *C. lagenarium* (Takano et al., 2000). Another MAPK gene in *C. lagenarium* is *MAF1*. The *maf1* gene replacement mutants did not form appressoria (Kojima et al., 2002). It seems that *MAF1* was required for the early differentiation phase of appressorium formation, whereas *CMK1* was involved in the maturation process of appressoria (Kojima et al., 2002). All these

TABLE 1.1
Role of *CMK1* Gene Encoding MAP Kinase on Conidium Attachment, Conidial Germination, Appressorial Formation, and Invasive Growth of *Colletotrichum lagenarium*

C. lagenarium Strain	Conidium Attachment (%)	Conidial Germination (%)	Formation of Appressoria	Formation of Invasive Growth
Wild type	87.1	98.4	+	+
cmk1 mutant	22.4	1.5	−	−

Source: Adapted from Takano, Y., Kikuchi, T., Kubo, Y., Hamer, J.E., Mise, K., and Furusawa, I., *Mol. Plant Microbe Interact*., 13, 374, 2000.

+ Abundant formation; − No formation.

studies clearly indicate that MAPK signaling system is needed for spore adhesion, spore germination, functional appressorium formation, and invasive growth in plants.

A gene encoding MAPK has been isolated from the Southern corn leaf blight pathogen *C. heterostrophus* and the gene was designated *CHK1*. This gene belongs to the ERK family of the MAPK superfamily, whose main function in other eukaryotes is the transduction of extracellular signals. Chk1 is related to the YERK1 (yeast and fungal ERK) subfamily of the ERK family (Kültz, 1998). Deletion of this gene showed that it is required for the development of infection structures (Lev et al., 1999). A MAPK gene, *PTK1* has been isolated from the barley net blotch pathogen *Pyrenophora teres* (Ruiz-Roldan et al., 2001). Mutants, carrying an interrupted copy of the gene did not form appressoria, could not colonize host tissues following artificial wounding, and lost their ability to infect barley leaves (Ruiz-Roldan et al., 2001).

MAPK cascade may be involved in signaling pathogenesis of fungal pathogens infecting floral tissues. *Fusarium graminearum* infects wheat flowers and causes ear blight disease. A gene encoding a MAPK (*MAP1* gene) has been cloned from the floral pathogen. The *MAP1* gene has been shown to be essential for pathogenicity. *F. graminearum map1* mutants were nonpathogenic on wheat flowers (Urban et al., 2003). These results suggest that MAPK pathway provides necessary signals for the pathogenesis of even floral tissue pathogen, similar to foliar and root pathogens.

Claviceps purpurea is a common pathogen of a wide range of grasses and cereals causing ergot disease. It does not form special penetration structures such as appressoria. The signal transduction system in the fungal infection process in rye ovarian tissue involved a MAPK (Mey et al., 2002a). A gene encoding the MAPK, *cpmk1*, was cloned from *C. purpurea*. The gene shows significant homology to *Fus3* of *Saccharomyces cerevisiae* and to *pmk1* of *M. grisea*. A *cpmk1* mutant of *C. purpurea* was obtained (Mey et al., 2002a). Loss of *CPMK1* had no obvious effect on growth rate or conidial formation in *C. purpurea*. However, the mutant was unable to colonize rye ovarian tissue. Complementation of the mutant with a wild-type copy of *cpmk1* fully restored its pathogenicity, confirming that this MAPK is essential for infection of rye by *C. purpurea* (Mey et al., 2002a).

A gene encoding protein kinase, *pmk1*, has been cloned from the cereal blast fungus *M. grisea* (Mey et al., 2002a). Transformation of the *pmk1* mutant of *M. grisea* with a copy of *cpmk1* fully restored its ability to form appressoria and its pathogenicity on barley. *C. purpurea* penetrates by producing penetration hypha, without forming any appressorium, whereas *M. grisea* penetrates the host tissue by forming appressoria. However, the MAPK gene from *C. purpurea* (*cpmk1*) could perform the function of infection hypha formation as well as appressoria formation in the two different pathogen systems. It suggests that the signaling pathway involving CPMK1 is highly conserved (Mey et al., 2002a). Another MAPK, CPMK2, an SLT2-homologous MAPK, has been shown to be essential for pathogenesis of *C. purpurea* on rye (Mey et al., 2002b).

The *fmk1* gene encoding a MAPK that belongs to the yeast and fungal extracellular signal-regulated kinase (YERK1) subfamily has been isolated from the soil-borne vascular wilt fungus *Fusarium oxysporum* (Di Pietro et al., 2001). Mutants of *F. oxysporum* f. sp. *lycopersici* carrying an inactivated copy of *fmk1* lost pathogenicity on tomato plants. The mutants showed greatly impaired adhesion to the tomato root surface. Colonies of the *fmk1* mutants were easily wettable, and hyphae were impaired in breaching the liquid–air interface, suggesting defects in surface hydrophobicity. The mutants failed to develop penetration hypha. *fmk1* mutants also showed reduced invasive growth on tomato and drastically reduced transcript levels of *pl1* encoding the cell wall–degrading enzyme pectate lyase (Di Pietro et al., 2001). These results suggest that MAP signal cascade is involved in signaling spore adhesion to root surface, development of penetration hypha, invasive growth development, and in production of pectate lyase, which is needed for cell wall penetration and colonization of *F. oxysporum* f. sp. *lycopersici*

(Di Pietro et al., 2001). A MAPKKK gene, *CZK3*, was isolated from *Cercospora zeae-maydis*, the maize leaf spot pathogen. This gene was found to signal the fungal development, the fungal toxin cercosporin production, and disease development (Shim and Dunkle, 2003).

The MAPK pathway appears to be conserved in foliar fungal pathogens such as *Cochliobolus heterostrophus* (Lev et al., 1999), *Colletotrichum lagenarium* (Takano et al., 2000; Kojima et al., 2002), and *Pyrenophora teres* (Ruiz-Roldan et al., 2001), root pathogens such as *Fusarium oxysporum* (Di Pietro et al., 2001) and *Gaeumannomyces graminis* (Dufresne and Osbourn, 2001), floral parts-infecting pathogens such as *Claviceps purpurea* (Mey et al., 2002a,b) and *Fusarium graminearum* (Urban et al., 2003), and fruits-infecting pathogens such as *Botrytis cinerea* (Zheng et al., 2000). The *FUZ7* gene of the corn smut fungus *Ustilago maydis* encodes an MAPKK homolog that is required for the infection process (Banuett and Herskowitz, 1994). MAPK genes also appear to be conserved. The MAPK gene *fmk1* cloned from the soil-borne pathogen *F. oxysporum* (Di Pietro et al., 2001), *cpmk1 and cpmk2* genes from *Claviceps purpurea* (Mey et al., 2002a,b), *MAP1* gene from *F. graminearum* (Urban et al., 2003), *BMP1* gene from *B. cinerea* (Zheng et al., 2000), *pmk1*-like gene from the root pathogen *G. graminis* (Dufresne and Osbourn, 2001), *Cmk1* gene from the anthracnose pathogen *C. lagenarium* (Takano et al., 2000), and *CHK1* gene from foliar blight pathogen *Cochliobolus heterostrophus* were homologous to *pmk1* gene cloned from the foliar blast pathogen *M. grisea* (Lev et al., 1999). In fact, the orthologous MAPK gene *Pmk1* from *M. grisea* complemented defect in root attachment, surface hydrophobicity, host penetration, and pathogenicity found in *fmk1* mutant of the wilt pathogen *FF. oxysporum* (Di Pietro et al., 2001). *Colletotrichum lagenarium Cmk1* gene was shown to complement appressorium formation defects in *M. grisea pmk1* mutants (Takano et al., 2000). Transformation of *pmk1* mutant of *M. grisea* with complete copy of *cpmk1* (Mey et al., 2002a) or *cpmk2* (Mey et al., 2002b) from *Claviceps purpurea* fully restored its ability to form appressoria and its pathogenicity on barley.

1.5.6 Lipid-Induced Protein Kinase Signaling

Another group of protein kinases is lipid-induced protein kinases (LIPK). A LIPK gene has been isolated from *Colletotrichum trifolii*, causal agent of alfalfa anthracnose (Dickman et al., 2003). The gene was induced specifically by purified plant cutin or long-chain fatty acids, which are monomeric constituents of cutin. Gene replacement of *lipk* yielded strains which were unable to develop appressoria and which were unable to infect intact host plant tissue (Dickman et al., 2003). These results suggest that the fungus senses the host surface, releasing cutin monomer, which induces LIPK-mediated pathway that is required for appressorium development.

1.5.7 PAK Signaling

P21-activated kinase (PAK)/STE20 is a family of protein kinases in the OPK (other protein kinases) group. PAK family of protein kinases plays central roles in cell signaling as effectors of Rho-type p21 GTPases. It is known that MAPKs are regulated by the Ste20 PAK kinase in yeast. Two PAK genes, *CHM1* and *MST20*, were isolated from *M. grisea* (Li et al., 2004). *MST20* was not involved in the infection process of the pathogen. However, in *chm1* mutants appressorium formation was reduced and the developed appressoria were defective in penetration. The *chm1* deletion mutant was phenotypically different from *pmk1* (MAPK) mutant that is defective in appressorium formation. *CHM1* did not play any critical role in activating the PMK1 MAPK pathway during appressorium formation and infectious hyphae growth. Although *CHM1* appears to be essential for appressorial penetration, *CHM1* may have redundant functions in *M. grisea* (Li et al., 2004).

1.5.8 Phosphorylation and Dephosphorylation Cascades

Phosphorylation of proteins may be involved in signaling. Protein kinases are involved in protein phosphorylation, which plays a key role in diverse biological signal transduction systems (Cohen, 1982; Hardie, 1990; Flaishman et al., 1995). Protein kinase inhibitor genistein, which inhibited phosphorylation of proteins, also inhibited induction of appressorium formation in *Colletotrichum gloeosporioides* (Flaishman et al., 1995). It suggests that the increased phosphorylation due to activation of protein kinase in fungal cell may be involved in signal transduction system inducing fungal infection structures.

Nutrient-rich conditions do not usually allow appressorium formation in fungi. In fact, yeast extract that promotes germination and vigorous hyphal growth of *C. gloeosporioides* does not allow appressorium formation (Flaishman et al., 1995). Phosphorylation of the 29 and 43 kDa proteins associated with appressorium formation could not be detected in the *C. gloeosporioides* conidia germinating in yeast extract. On the other hand, the addition of calcyculin A, a phosphatase inhibitor to germinating conidia resulted in phosphorylation of the same 29 and 43 kDa proteins as those phosphorylated by the host wax and ethylene (the plant signals) and this phosphatase inhibitor induced appressorium formation. It is known that biological signals cause transient phosphorylation resulting in accumulation of certain phosphorylated components for short duration after treatment with the signal. The level of such phosphorylated components is normally controlled by the relative rates of phosphorylation and dephosphorylation. Thus, not only stimulation of the relevant kinase but also inhibition of phosphatase should be able to cause accumulation of the phosphorylated protein and appressorium formation in *C. gloeosporioides* (Flaishman et al., 1995).

1.5.9 P-Type Adenosine Triphosphatase Signaling

P-type ATPases are fueled ion pumps with a single catalytic subunit, and have a phosphorylated reaction cycle intermediate (Palmgren and Axelsen, 1998). P-type ATPases are so named because of the phosphoenzyme intermediate formed during their activation. P-type ATPases pump a variety of charged substrates such as H^+, Ca^{2+}, K^+, Na^+, Mg^+, Cu^{2+}, Cd^{2+}, and phospholipids. The best-known members of the group are H^+-ATPases (Balhadère and Talbot, 2001). All P-type ATPases share a core structure, including the presence of a phosphorylation site, an ATP binding domain, and three other consensus sequences. They have been implicated in a number of transport and signaling processes (Catty et al., 1997; Palmgren and Axelsen, 1998).

P-type ATPases have been suggested to be involved in signaling penetration hypha development in fungal pathogens. *PDE1* gene encoding a P-type ATPase has been isolated from *M. grisea* (Balhadère and Talbot, 2001). A *pde1* mutant of the pathogen was unable to cause plant infection (Balhadère et al., 1999). In *pde1* mutants, the cuticle penetration was reduced, and the only penetration structures observed were nonpolarized hyphae that were limited to the initial epidermal cell. The results suggest that *PDE1* encodes a protein involved in the elaboration of penetration hyphae from the base of the appressorium during plant infection (Balhadère and Talbot, 2001).

The P-type ATPase encoded by *PDE1* belongs to the DRS2 family of aminophospholipid translocases. The aminophospholipid translocases generate phospholipid asymmetry in membranes. The regulated disruption of phospholipid asymmetry consequently provides a pathway for cellular signaling (Bevers et al., 1999). The maintenance of membrane asymmetry by a plasma membrane-localized *PDE-1* encoded ATPase may be required for the rapid alteration in membrane conformation that occurs during formation of the penetration hypha (Balhadère and Talbot, 2001). The functions of P-type ATPases in signaling the infection processes in other fungal pathogens are yet to be studied.

1.6 GENES INVOLVED IN FORMATION OF INFECTION STRUCTURES

Various physical and/or chemical signals may be transduced across fungal cell membrane and induce transcription of some specific fungal genes involved in the formation of infection structures.

Treatment with cAMP induces appressorium formation in *M. grisea*. The cAMP induced a number of genes involved in the infection process. These genes included *MPG1* encoding hydrophobin (Irie et al., 2003), *GAS1* (*MAS3*) and *GAS2* (*MAS1*) which are *Magnaporthe* appressoria specific proteins (Xue et al., 2002; Irie et al., 2003), *PTH11* and *MAC1* encoding adenylate cyclase gene (Irie et al., 2003). *GAS1* or *GAS2* or both are dispensable for mycelial growth, conidiation, and sexual reproduction, but they are important for appressorial penetration and lesion development (Xue et al., 2002). Mutants disturbed in *MPG1* were very much reduced in appressorium formation and plant infection (Beckerman and Ebbole, 1996). Two genes, *NPR1* and *NPR2*, were found to regulate the *MPG1* expression (Lau and Hamer, 1996). For full expression of *MPG1*, the MAPK-encoding gene *PMK1* and the wide domain regulators of nitrogen source utilization, *NPR1* and *NUT1*, were required (Soanes et al., 2002). During appressorium morphogenesis, high-level *MPG1* expression was found to require the *CPKA* (encoding the catalytic subunit of PKA) and *NPR1* genes (Soanes et al., 2002). The *pth11* and *abc1* deletion mutants were reduced in virulence, but still they form appressoria (DeZwaan et al., 1999; Urban et al., 1999). The *pth11* can activate appressorium differentiation in response to inductive cues (DeZwaan et al., 1999).

Another gene, *MST12*, was isolated from *M. grisea* (Park et al., 2002). *mst12* mutants produced typical melanized appressoria. However, the appressoria formed by *mst12* mutants failed to penetrate plant tissues. These results indicate that MST12 may function downstream of the MAPK PMK1 to regulate genes involved in infectious hyphae growth (Park et al., 2002). Wang et al. (2003) isolated *ICL1*, a gene that encodes isocitrate lyase, one of the principal enzymes of the glyoxylate cycle from *M. grisea. ICL1* showed elevated expression during the development of infection structures and cuticle penetration. In the *ICL1* mutant, the prepenetration stage of development, before entry into plant tissue, was affected by loss of the glyoxylate cycle (Wang et al., 2003). *APF1* gene isolated from *M. grisea* was found to be involved in appressorial formation of the fungus (Siluè et al., 1998). The mutant *apf*-phenotypes were deficient in appressorium formation. *APF1* appears to act independently from the cAMP signaling pathway, known to mediate appressorium formation in *M. grisea*. Either the product of *APF1* acts downstream of cAMP-dependent protein kinase in the signaling pathway, representing signaling component, or it acts independently of this pathway (Siluè et al., 1998). Another gene, *PLS1*, encodes a putative integral membrane protein of 225 amino acids (Pls1p) in *M. grisea*. This protein was detected only in appressoria and was localized in plasma membranes and vacuoles (Clergeot et al., 2001). Pls1p is structurally related to the tetraspanin family. In animals, these proteins are components of membrane signaling complexes, controlling cell differentiation and adhesion (Gourgues et al., 2002). The *pls1* mutant differentiates appressoria that fail to breach the leaf epidermis. It appears that *PLS1* controls an appressorial function essential for the penetration of the fungus into host leaves (Clergeot et al., 2001). Another gene required for pathogenesis of *M. grisea* is *ABC1* (ATP-binding cassette 1) and it may be involved in protection of the pathogen against plant defense mechanisms (Urban et al., 1999).

Two genes *cap3* and *cap5* were cloned from *Colletotrichum gloeosporioides* in appressorium-forming spores. They code for 26- and 27-amino acid cysteine-rich polypeptides. These genes were developmentally regulated to express during appressorium formation (Kolattukudy et al., 1995). Another gene (*cap22*) uniquely expressed during appressorium formation by *C. gloeosporioides* encodes a 22 kDa protein that showed a limited homology to a variety of surface glycoproteins. This protein contained two consensus N-glycosylation sites (Hwang

and Kolattukudy, 1995). Immunogold electron microscopic examination of appressorial fungal spores showed that CAP22 protein was associated with appressorial wall. Thus, CAP22 may be a glycoprotein secreted into the appressorial walls (Kolattukudy et al., 1995).

Another gene uniquely expressed during appressorium formation in *C. gloeosporioides* is *cap20*, which encodes a 20 kDa protein (Hwang et al., 1995). CAP20 protein was produced mainly during appressorium formation in *C. gloeosporioides* starting at about 4 h of exposure to the signal that triggers the differentiation of hyphae into appressorium. Immunogold electron microscopy revealed that CAP20 protein is associated with the appressorial walls. Spores of *C. gloeosporioides* mutants in which *cap20* was disrupted germinated and formed normal-looking appressoria. However these mutants failed to produce disease lesions on avocado and tomato fruits; some growth of mycelia on the surface was sometimes observed, but no penetration of mutants into the fruit could be detected. Thus, *cap20* gene expression may be necessary for functional appressoria formation (Kolattukudy et al., 1995).

A gene, *ClaPEX6*, has been shown to be required for the cucumber anthracnose pathogen *Colletotrichum lagenarium* to infect plants (Kimura et al., 2001). The *PEX* genes encode peroxins, which are required for peroxisome biogenesis. Peroxisomes are single-membrane-bound organelles possessing multiple metabolic functions, including β-oxidation of fatty acids, glyoxylate metabolism, and metabolism of reactive oxygen species. Peroxisomal metabolic function has been demonstrated to be essential for appressorium-mediated infection processes of *C. lagenarium* (Kimura et al., 2001). The disruption of *ClaPEX6* impairs peroxisomal metabolism. The *clapex6* mutants grew poorly on fatty acids indicating a defect in fatty acid β-oxidation in peroxisomes. The *clapex6* mutants formed small appressoria with severely reduced melanization that failed to form infectious hyphae. The results suggest that *ClaPEX6* gene is essential for the development of infectious penetration hyphae from the appressorium in *C. lagenarium* (Kimura et al., 2001).

Another gene *CST1* was also found essential for the development of infectious hyphae in *C. lagenarium* (Tsuji et al., 2003). The *CST1* gene is a homologue of *Stel 2* gene of the yeast *Saccharomyces cerevisiae*, which is known to be a transcriptional factor downstream of MAPK. Conidia of the *cst1* mutant strains could germinate and form melanized appressoria on the leaf surface, but could not produce infectious hyphae from appressoria. It suggests that *CST1* gene is essential in the development of penetration hypha. In addition, matured appressoria of the *cst1* mutants contained an extremely low level of lipid droplets (Tsuji et al., 2003). Lipid droplets were abundant in conidia of the mutants, but rapidly disappeared during appressorium formation. This misscheduled lipid degradation might be related to the failure of appressorium penetration in the mutant strain (Tsuji et al., 2003). The results suggest that the *CST1* gene may encode a transcriptional factor functioning downstream of MAPK pathway and may be involved in signaling the infection process.

Görnhardt et al. (2000) have cloned three genes specific for germinating cysts and appressoria of *Phytophthora infestans*, the potato late blight pathogen. They were activated shortly before the onset of invasion of the host tissue. The three genes isolated belong to a small polymorphic gene family. The genes encoded proteins were structurally similar to human mucins and they were named Car (cyst-germination-specific acidic repeat) proteins. Car proteins were transiently expressed during germination of cysts and formation of appressoria and were localized at the surface of germlings. It was suggested that Car proteins may assist in adhesion of the fungus to the leaf surface (Görnhardt et al., 2000). Adhesion may provide signals for formation of infection structures.

Chk1, a MAPK, functions in the infection process of the maize pathogen *Cochliobolus heterostrophus*. Two fungal genes encoding cellulolytic enzymes, a cellobiohydrolase, *CBH7*, and an endoglucanase, *EG6* were found to be induced on the host plant during the infection process downstream of the *chk1*-mediated MAPK pathway (Lev and Horwitz, 2003). Induction of both genes began at the onset of invasive growth. Disruption of MAPK CHK1 resulted

in a delay in the penetration of hyphae into the leaf and a concomitant delay in the induction of expression of both cellulase genes (Lev and Horwitz, 2003). Cellulases might have helped in the penetration process (Vidhyasekaran, 2007).

1.7 SIGNALS IN FUNGAL INFECTION PROCESS

Several signaling systems take part in the fungal infection process. To illustrate these complex signaling systems in fungal pathogenesis, the signals involved in some fungal pathogen models are presented here. The selected models include different types of diseases such as blast (*M. grisea*), anthracnose (*Colletotrichum gloeosporioides*), powdery mildew (*Blumeria graminis*), smut (*Ustilago maydis*), and wilt (*Fusarium oxysporum*).

1.7.1 *MAGNAPORTHE GRISEA*

M. grisea causes blast disease in rice and barley. When the conidium lands on the host plant, the conidia adhere to the plant surface. The conidium germinates and forms a germ tube that differentiates into appressorium. Appressorium differentiation occurs in two phases. First, the germ tube apex hooks and swells, and apical vesicles polarize toward the substratum. These events represent a ''recognition phase'' (Bourett and Howard, 1990). During the second phase, continued swelling of the germ tube apex yields a symmetrical appressorium, a septum forms between the appressorium and the remainder of the germling, and appressorial melanization occurs (DeZwaan et al., 1999). Once mature, the appressorium generates enormous hydrostatic pressure that mediates direct penetration of a narrow penetration peg throughout the host cuticle and epidermal layer (Howard et al., 1991). Intracellular hyphae then ramify throughout the host tissue and form conidiophores that erupt through the host surface and release conidia into the environment (DeZwaan et al., 1999).

Several signaling systems are involved in this infection process (Figure 1.11). Hydrophobic surfaces and *MPG1* gene encoded hydrophobins secreted by the pathogen provide signals for attachment of spores to hydrophobic surfaces. The conidium releases a presynthesized material, which facilitates adhesion of the conidium to the plant surface (Hamer et al., 1988). Adhesion of the conidium provides signals for inducing germination (Liu and Kolattukudy, 1999). The conidium produces cutinase and release cutin monomers (Choi and Dean, 1997). On perception of physical and chemical cues after close contact with plant's hydrophobic surface, the conidium germinates to produce a short germ tube. The germlings also adhere to the plant surface and hydrophobins (Wösten et al., 1994) and glycoprotein mucilage (Xiao et al., 1994a,b) may be involved in adhesion of the germlings. The close contact provides cues for appressorium formation (Bourett and Howard, 1990; Xiao et al., 1994a). A gene of *M. grisea*, *CBP1*, encoding a putative extracellular chitin-binding protein, may be involved in the hydrophobic surface sensing of the fungus leading to appressorium formation (Kamakura et al., 2002). *MPG1* gene-encoded hydrophobin (Kershaw and Talbot, 1998) and the ECM glycoprotein (Xiao et al., 1994a) also provide necessary signals in inducing differentiation of appressorium. The signaling system involved in expression of *MPG1* gene includes a mitogen-activated kinase gene, *PMK1*, two wide domain regulators of nitrogen source utilization genes, *NPR1* and *NUT1*, and a PKA gene, *CPKA* (Soanes et al., 2002).

The generated signals may be transduced through the transmembrane G-protein-coupled receptors that detect surface cues. The receptor-coding gene, *pth11* is involved in appressorium formation in response to inductive cues (DeZwaan et al., 1999). The signaled information may be transduced to one or more downstream signaling pathways. The Gα subunit protein encoded by the *MAGB* gene (Fang and Dean, 2000) and the Gβ subunit encoding *mgb1* gene (Nishimura et al., 2003) may be the important components in the G-protein signaling system

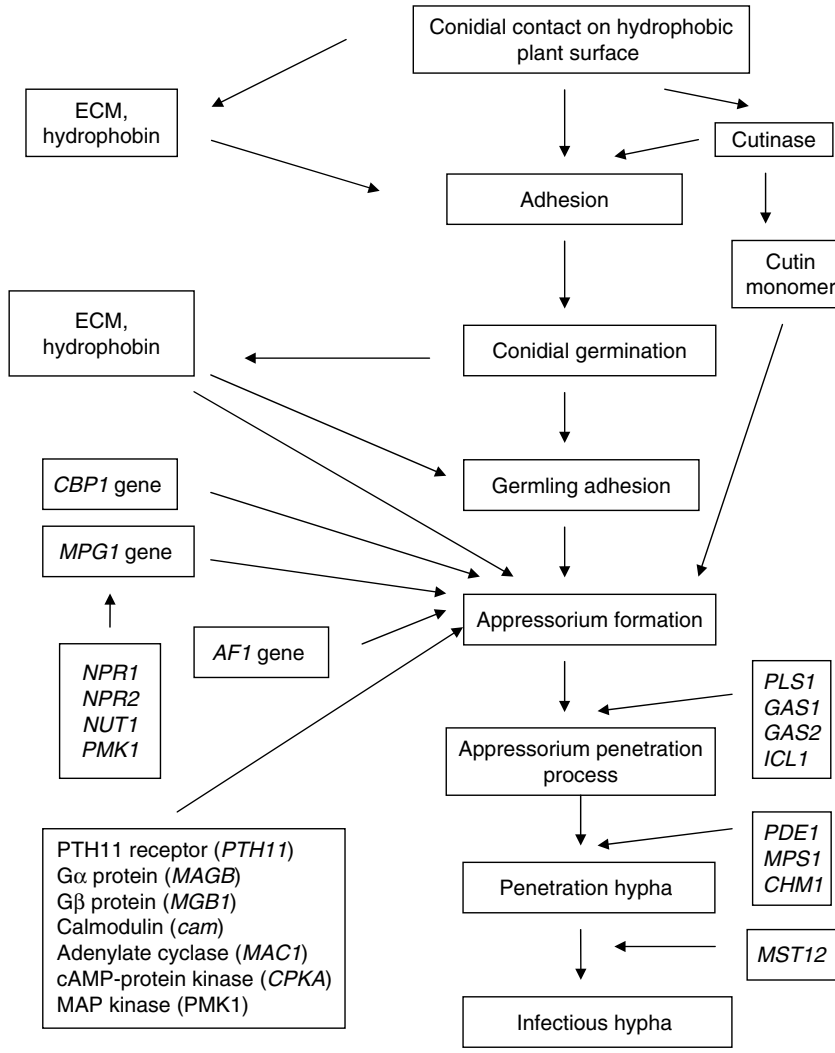

FIGURE 1.11 Signals in the infection process of *M. grisea*.

involved in the appressorium formation. In the downstream of G-protein signaling system, calcium/calmodulin-dependent signaling (Liu and Kolattukudy, 1999) and cAMP-mediated signaling cascade (Yan and Gautam, 1996) may operate in *M. grisea*. The G-protein regulates cellular levels of cAMP by direct interaction with adenylate cyclase encoded by *MAC1* gene (Choi and Dean, 1997; Nishimura et al., 2003). Parallel pathways of sensory input (cues from hydrophobic surfaces and soluble host cutin monomers) appear to converge at or before the *MAC1*-encoded adenylate cyclase protein. cAMP-dependent PKA may act in the signal transduction system (Adachi and Hamer, 1998). *CPKA* gene encoding PKA is involved in functional appressorium formation (Xu et al., 1997). MAPK signaling cascade may operate downsteam of cAMP signaling (Xu and Hamer, 1996). *APF1* gene isolated from *M. grisea* triggers appressorium formation and acts independently from the cAMP signaling pathway (Siluè et al., 1998).

PMK1 encodes a MAPK and was found essential to produce appressorium. Transfer of storage carbohydrate and lipid reserves to the appressorium occurs under control of the *PMK1*

MAPK pathway (Thines et al., 2000). The differentiated appressorium is also tightly attached to the host tissue (Howard et al., 1991) and seems to be important in the penetration process. The genes, *PLS1*, *GAS1*, *GAS2*, and *ICL1*, have been shown to be involved in the function of appressorium in the penetration process. P-type ATPase encoded by *PDE1* gene is involved in signaling penetration hypha development (Balhadère and Talbot, 2001). A PAK kinase gene *CHM1* is involved in appressorial penetration (Li et al., 2004). Another gene involved in the development of penetration hypha is a MAPK gene *MPS1* (Xu et al., 1998). *MST12* gene regulates infectious hypha development (Park et al., 2002). *ABC1* gene is required for pathogenesis of *M. grisea*, mostly to protect itself against plant defense mechanism (Urban et al., 1999).

1.7.2 *Blumeria graminis*

B. graminis is a highly specialized obligate biotropic fungal pathogen that causes powdery mildew of cereals. Conidia of the fungus on coming contact with plant leaf surface release ECM within seconds after contact (Carver et al., 1999; Fujita et al., 2004a) (Figure 1.12). This ECM facilitates adhesion of conidia to leaf surface (Wright et al., 2002a) and it is involved in recognizing the site of substratum contact (Fujita et al., 2004a). This recognition appears to involve hydrophobic interactions (Wright et al., 2000) and the action of constitutive cutinase in degrading the plant cuticle to release factors that are quickly taken

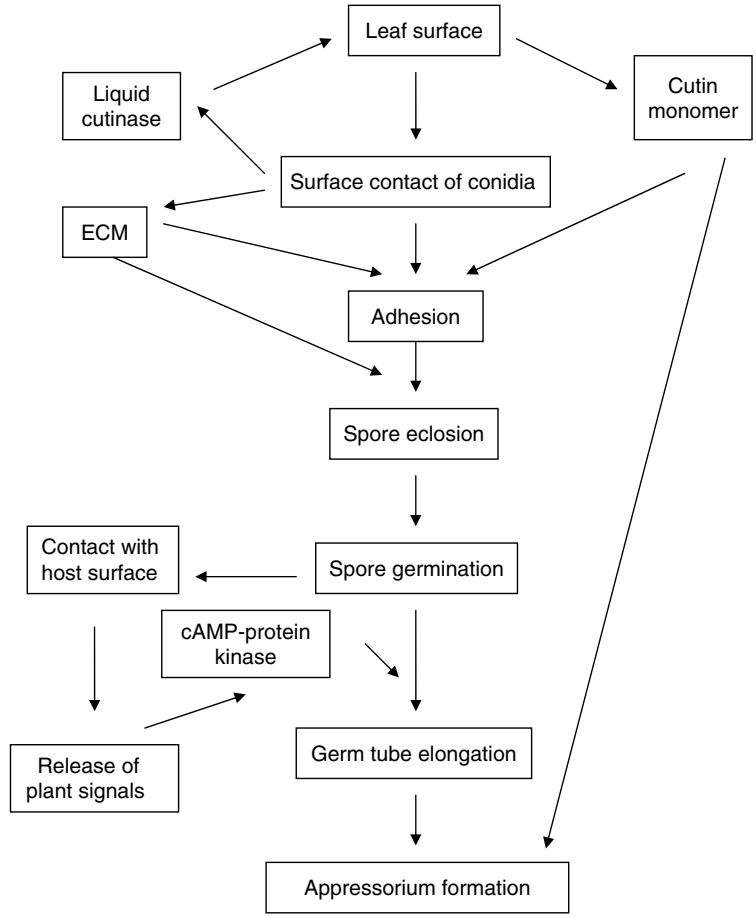

FIGURE 1.12 Signals in the infection process of *Blumeria graminis*.

up by conidia (Nielsen et al., 2000). This interaction provides signals for conidial adhesion and subsequent spore germination (Meguro et al., 2001). During the germination process, the fungal spores undergo major structural changes within 5 to 10 min after adhesion (Kunoh et al., 1988). The germinated spores produce first a short primary germ tube before a second appressorial germ tube emerges, elongates, and differentiates an appressorium. The primary germ tube produces adhesive extracellular materials that rapidly secure the young germling to the host leaf surface (Wright et al., 2000). On perception of host surface characteristic signals, germ tube elongation occurs through intracellular signaling (Carver et al., 1996). This signaling may involve cAMP–PKA pathway (Hall and Gurr, 2000). Cutin monomer released from the host plant by the action of the exuded fungal cutinase, provides signals for induction of appressorium formation (Francis et al., 1996). Infection arises from a hooked appressorial lobe that differentiates at the tip of the appressorial germ tube (Figure 1.12). A penetration peg emerges from beneath the appressorium (Carver et al., 1995a). It penetrates the host cuticle and epidermal cell wall and forms haustoria (Pryce-Jones et al., 1999).

1.7.3 *Colletotrichum gloeosporioides*

C. gloeosporioides causes anthracnose diseases on more than 197 plant species. For infection to take place, a series of signaling systems operate. The sequence of infection process involves spore adhesion to host surface, spore germination, germ tube elongation, appressorium formation, and penetration by an infection peg. Conidium senses hard-surface signals when it lands on host plant surface and the genes *chip1*, *chip2*, and *chip3* were induced within 2 h after contact with the hard surface (Figure 1.13). The hard-surface contact may signal a chain

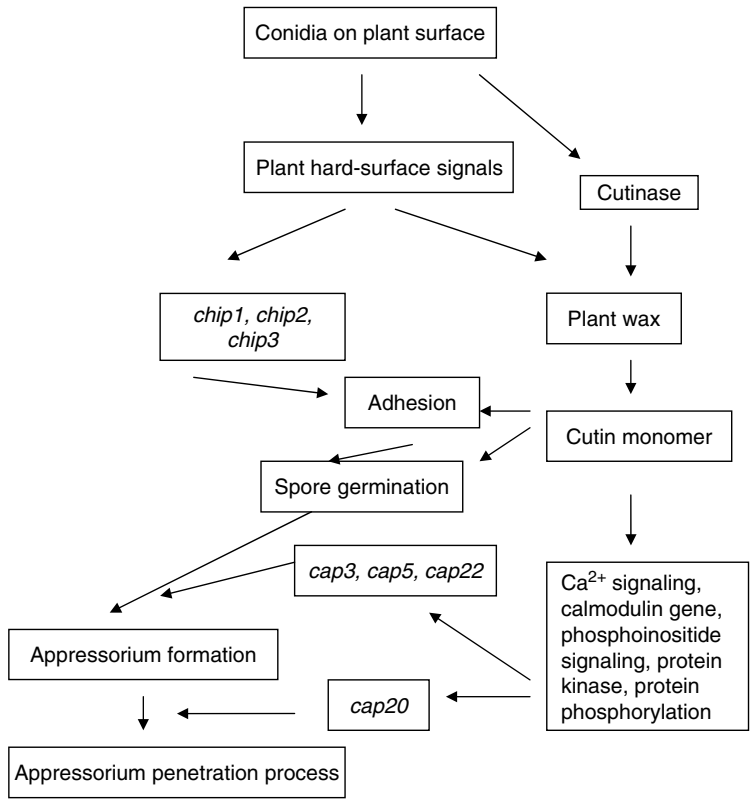

FIGURE 1.13 Signals in infection process of *Colletotrichum gloeosporioides*.

of molecular events needed for conidial germination and appressorium formation. Conidial adhesion to plant surface may trigger the signaling system. Appressorium formation is induced by thigmo-signals of the contact surface such as hydrophobicity and rigidity, and chemicals including surface wax and ethylene (Kolattukudy et al., 1995). The signals are transduced by Ca^{2+} signaling system (Uhm et al., 2003). Calmodulin gene (*cam*) may trigger the signaling process (Kim et al., 1998), resulting in the activation of protein phosphorylation induced by protein kinase (Flaishman et al., 1995; Uhm et al., 2003). Phosphoinositide signaling system, activated by phospholipase C is also involved in the signal transduction in this fungus (Uhm et al., 2003). Several genes were activated by the transduced signals. The genes *cap3* and *cap5* are involved in the appressorium formation (Kolattukudy et al., 1995). Another gene, *cap22* is associated with the appressorial wall. The gene *cap20* may be necessary for the function of appressorium penetration process (Kolattukudy et al., 1995) (Figure 1.13).

1.7.4 *Ustilago maydis*

U. maydis causes corn smut disease and the disease is characterized by tumor (galls) formation on ears, tassels, leaves, and shoots from nodes. In this fungus, mating of haploid sporidia is a prerequisite for the formation of infection structures. Plant signals may activate a chain of signaling events in the infection process (Figure 1.14). G-protein signaling plays a key role in pathogenesis of *U. maydis* (Regenfelder et al., 1997). cAMP-signaling pathway operates

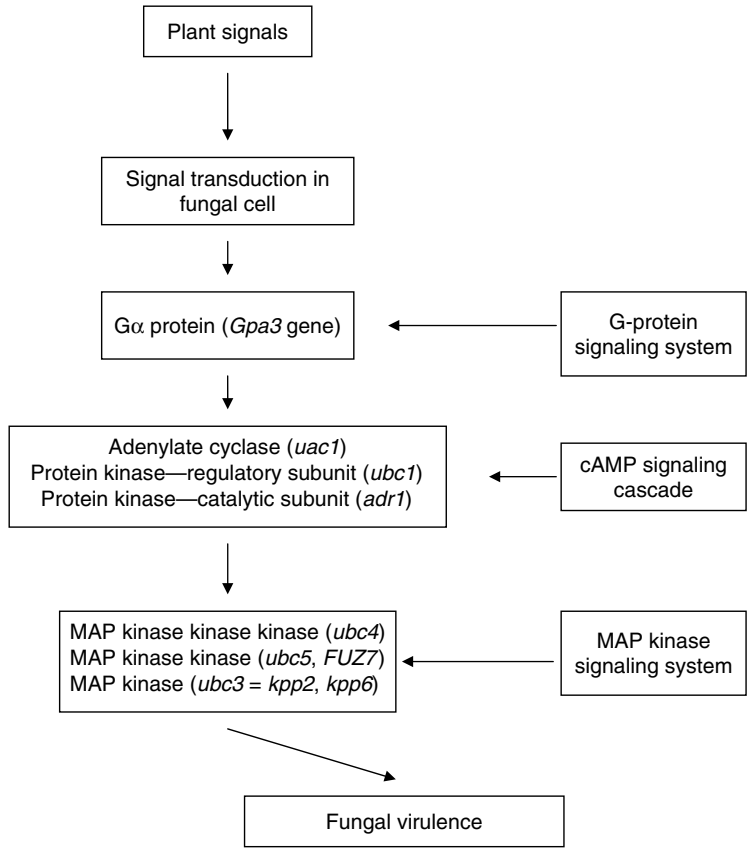

FIGURE 1.14 Signal transduction system in pathogenesis of corn smut pathogen *Ustilago maydis*.

downstream of G-protein. The cAMP pathway includes the adenylate cyclase (*Uac1* gene), and the regulatory (*ubc1* gene) and catalytic subunits of the PKA (*Adr1* gene) (Gold et al., 1994, 1997). MAPK-signaling pathway also is involved in the infection process. MAPKKK (*ubc4* gene), MAPKK (*ubc5* gene), and two MAPKs encoded by *ubc3* and *kpp6* form important components of the MAPK-signaling system (Müller et al., 1999; Andrews et al., 2000; Brachmann et al., 2003).

1.7.5 *Fusarium oxysporum*

Role of signaling and signal transduction systems has been extensively studied in foliar fungal pathogens. Similar signaling systems also may operate in soil-borne fungal pathogens. In contrast to many foliar pathogens, soil-borne fungi tend to penetrate the host roots directly without producing fully differentiated infection structures (Mendgen et al., 1996). *F. oxysporum* is a soil-borne facultative parasite that causes wilt diseases in many crops. The fungus enters the roots directly through penetration hyphae and colonizes the cortex by intra- and intercellular growth. Once it reaches the vascular tissue, the fungus spreads rapidly upward through the xylem vessels provoking wilt symptoms.

The spores of *F. oxysporum* f. sp. *lycopersici* germinate in the root surface in response to stimuli exuded by host roots. The germlings adhere to tomato root surface (Di Pietro et al., 2001). The attachment of germlings to the host plant surface was similar to that observed in foliar pathogens. The gene *fmk1* encoding a MAPK was involved in the signal transduction resulting in germling adhesion (Di Pietro et al., 2001). The developed germ tubes differentiate into infection hyphae (Mendgen et al., 1996). The infection hyphae penetrate directly epidermal walls (Rodríguez-Gálvez and Mendgen, 1995). FMK1 has been shown essential for invasive growth of the fungus. It is essential for differentiation of infection hyphae, attachment to roots and penetration (Di Pietro et al., 2001). MAPK-signaling system appears to be essential in the infection process of the wilt pathogen *F. oxysporum*.

1.8 CONCLUSION

Fungal pathogens perceive plant signals and the signals are transduced intracellulary to initiate the infection process. The recognition process is initiated almost at the first touch of the plant surface by pathogen. Initiation of the signaling process has been demonstrated even within 20 s of the first touch. Adhesion or close contact of the fungal spores with plant surface appears to be important in sensing the plant signals. Fungal pathogens sense the mechano-sensitive thigmotropic signals arising from the first touch. Although the role of plant-surface topography in directing fungal germ tube growth and the differentiation of infection structures has been well documented, it is still not known how this topography is transduced into signals within the fungal cell (Heath, 2000). However, the fact that contact-stimulated appressorium formation can be inhibited by Arg–Gly–Asp (RGD)-containing peptides suggests a role for integrin-like proteins that, like integrins in mammals, mediate interactions between extracellular materials and the cytoskeleton (Corrêa et al., 1996). Nevertheless, contact stimulus may not be the only trigger of infection structure formation, as host topography alone is not sufficient to explain the high efficiency of appressorium induction seen *in vivo* on cereal rust fungi (Collins and Read, 1997). Several genes specifically involved in the hard-surface contact signaling process have been detected in fungal pathogens. These include *INF24*, *INF56*, *CBP1*, *MPG1*, *chip1*, *chip2*, and *chip3*. However, the exact role of these genes in the signal transduction is not known.

Plant wax may contain components to trigger the signaling process in fungal cell. Urosolic acid and oleanolic acid, the two pentacyclic triterpenes found in fruit waxes induce the signaling systems. The cutin monomers, which are the wax degradation products, are

the potent signal inducers in fungal pathogenesis. Ethylene appears to be another plant signal, which induces spore germination and appressorium formation. Flavonoids exuded by roots and seeds signal fungal spore germination. The cAMP-protein kinase pathway may be involved in the flavonoid-stimulated signaling system in the fungal infection process.

On contact with host plant surface, the fungal spores release ECM and this release has been visualized within few minutes of contact. The ECM has been shown to contain cutinases and other esterases. The cutinases are involved in the signal transduction for inducing adhesion of spores, germlings, and appressoria. The appressorium produces infection peg that penetrates the preformed defensive barriers, such as the cuticle and the underlying pectinaceous layer, probably using turgor-generated physical force (Howard et al., 1991; De Jong et al., 1997; Bechinger et al., 1999), with the assistance from the enzymes secreted in response to host signals (Podila et al., 1993; Wattad et al., 1997). The ECM released from fungal spores upon contact with the host cell has been reported to contain several cuticle and cell wall–degrading enzymes including cutinases, pectinases, cellulases, and xylanases (Kunoh et al., 1990; Pascholati et al., 1992; Fric and Wolf, 1994; Suzuki et al., 1998, 1999; Komiya et al., 2003). These enzymes would aid the pathogen to penetrate the host cell and cause infection. The plant signal induced ECM contains toxins which may aid colonization of the fungus in the host tissues (Jansson and Åkesson, 2003). Thus, the plant signals may trigger the production of ECM, which may be involved in the development of different stages of infectious structure development.

The extracellular signals may be relayed within the fungal cell that lead to the formation of appressorium and other infection structures. The signals may be relayed through G-proteins, calcium ion-dependent signaling system, cAMP-protein kinase signaling pathway, and MAPK cascade. This type of signal transduction system appears to be conserved in fungal pathogens causing varied types of disease symptoms such as blast, blight, powdery mildew, smut, anthracnose, ergot, fruit rot, wilt, and root rot. However, the activation of well-structured signaling system in pathogens alone may not lead to disease development. The plants also perceive pathogen signals and these signals are transduced in a well-structured signal transduction system in plants. The interplay of these signals in plant–pathogen interactions determines the disease development. The delivery and perception of pathogen signals in plants will be described in the next chapter.

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2 Perception and Transduction of Pathogen Signals in Plants

2.1 INTRODUCTION

Molecular communications between plant and pathogen commence almost immediately after the pathogen makes contact with the plant surface (Fujita et al., 2004). On perception of the plant signals, the pathogen prepares to invade the host tissues by using their various toxic weapons. On the other hand, plants perceive the pathogen signals (Fliegmann et al., 2004; Huang et al., 2005) and prepare to defend themselves by producing several antimicrobial compounds to ward off the pathogens (Mengiste et al., 2003). In this battle, susceptibility or resistance is determined by the winner; if the pathogen is able to overcome the toxic environment in the plant tissue, the disease develops and if the plant is able to ward off the pathogen's toxic weapons, disease resistance develops.

Plant cells recognize presence of spores of fungal pathogens on their surface, and initiate defense-associated responses even within 30 min of their contact with the spores of pathogens (Fujita et al., 2004). Recognition of potential pathogens is central to plants' ability to defend themselves against pathogens (Montesano et al., 2003). Phenylalanine ammonia-lyase (PAL) mRNA levels increased by 30 min in barley leaves after inoculation with either the compatible pathogen *Blumeria graminis* f. sp. *hordei* or with the incompatible pathogen *Erysiphe pisi* (Shiraishi et al., 1995). PAL is the key enzyme involved in biosynthesis of several defense compounds (Vidhyasekaran, 2004, 2007). Less than 1 h exposure to conidia of *B. graminis* induced expression of defense genes on barley leaves (Cho and Smedegaard-Petersen, 1986). Felle et al. (2004) showed apoplastic H^+ and Ca^{2+} efflux within 2 h of inoculation with *B. graminis* f. sp. *hordei* in barley leaves. It suggests that the first encounters of fungal surface material with the host-plasma membrane occur within 2 h of contact between the plant and the pathogen (Felle et al., 2004). The chitin oligomer induced similar apoplastic alkalinization. It suggests that the release of elicitor such as chitin oligomer from the fungal cell wall containing chitin may occur much before the 2 h period to induce apoplastic pH signaling (Felle et al., 2004).

Plant cells may respond to the conidial extracellular material (ECM) secreted much before conidial germination on the plant surface. A rise in *PAL* gene transcription could be detected within 0.5–1 h after inoculation of the pathogen *B. graminis* f. sp. *hordei* in barley leaves, and germination of the conidia was observed only after 1–1.5 h of inoculation. It suggests a host plant response to factors released before conidial germination (Fujita et al., 2004). Plant cells recognize fungal cell wall components (Boller, 1995). When the pathogen penetrates host tissues, plant secretes chitinases (Collinge et al., 1993), chitosanases (Grenier and Asselin, 1990), and β-1,3-glucanases (Mauch et al., 1988) extracellularly. These enzymes partially degrade fungal cell wall polysaccharides and produce soluble and diffusible fragments that may act as signals detecting the presence of a potential pathogen to the plant tissues (Ham et al., 1991; Ryan and Farmer, 1991).

Plants are able to recognize pathogen-derived elicitor molecules that trigger a number of induced defenses in plants. The recognition of a potential pathogen results in activation of intracellular signaling events including ion fluxes, phosphorylation/dephosphorylation cascades, kinase cascades, and generation of reactive oxygen species (ROS) (Radman et al., 2003). Intercellular signaling system involves ROS, nitric oxide (NO), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). Two major pathways in defense signaling, one SA-dependent and the other SA-independent but involving JA and ET, are recognized (Kunkel and Brooks, 2002). These signaling events lead to reinforcement of plant cell walls and the production of defense proteins and phytoalexins. These events proceed in both susceptible and resistant interactions, probably with different speed and intensity. The pathogens also produce suppressor molecules to counteract the action of elicitors, resulting in susceptibility. These complex signaling systems are described in this chapter.

2.2 WHAT ARE ELICITORS?

The term elicitor was first used to describe the molecules that are capable of eliciting the production of phytoalexins (Keen et al., 1972; Vidhyasekaran, 1988), but it is now commonly used for compounds eliciting any type of plant defense (Ebel and Cosio, 1994; Boller, 1995; Hahn, 1996; Nürnberger, 1999; Nürnberger and Brunner, 2002; Montesano et al., 2003). The term elicitor includes both substances of pathogen origin (exogenous elicitors; microbial elicitors) and compounds released from plants (endogenous elicitors; host plant elicitors) (Boller, 1995; D'Ovidio et al., 2004).

Microbial elicitors can be classified into two groups, such as general elicitors and race-specific elicitors. General elicitors are those microbial elicitors that are able to trigger defense responses both in host and nonhost plants (Kruger et al., 2003; Vidhyasekaran, 2004). Race-specific elicitors induce defense responses leading to disease resistance only in specific host cultivars (Bonas and Lahaye, 2002; Montesano et al., 2003). A complementary pair of genes in a particular pathogen race and a host cultivar determines this cultivar-specific (gene-for-gene) resistance. Thus, a race-specific elicitor encoded by or produced by the action of an avirulence (*avr*) gene present in a particular race of a pathogen elicits resistance only in a host plant variety carrying the corresponding resistance (*R*) gene (Lu and Higgins, 1998; Cohn et al., 2001; Luderer and Joosten, 2001; Nimchuk et al., 2001; Nürnberger and Scheel, 2001).

General elicitors signal the presence of potential pathogens to both host and nonhost plants (Brady et al., 1993; Côté and Hahn, 1994; Nürnberger, 1999). Among the general elicitors, some elicitors show restricted specificity in recognizing plants; they are active only in specific hosts (Yamaguchi et al., 2000a; Shibuya and Minami, 2001). The oligosaccharide elicitor isolated from the rice blast pathogen *Magnaporthe grisea*, which induced phytoalexin synthesis in rice cells, could not induce phytoalexin synthesis in soybean cotyledon cells (Yamaguchi et al., 2000b). In contrast, a β-glucan elicitor from *Phytophthora sojae* induced defense responses in both the host soybean and in several nonhosts (Brady et al., 1993; Côté and Hahn, 1994). The race-specific elicitor may be involved in *R* gene-mediated signaling pathway, whereas general elicitors may take part in general resistance signaling pathway (Hu et al., 2005).

Fungal elicitors do not have any common chemical structure and they belong to different kinds of chemical classes, such as oligosaccharides, proteins, peptides, glycoproteins, and lipids. Elicitors are produced by necrotrophic (Desjardins and Plattner, 2000), hemibiotrophic (Yamaoka et al., 1990), and biotrophic (obligate) (Ryerson and Heath, 1992) pathogens. Elicitor molecules also have been detected in some saprophytes (Dean and Anderson, 1991) and yeasts (Klüsener and Weiler, 1999).

2.3 OLIGOSACCHARIDE ELICITORS

2.3.1 Chitooligosaccharide Elicitors

Chitin is a linear polysaccharide composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) residues (Vander et al., 1998). It is a polymer of *N*-acetyl-D-glucosamine and is an insoluble component found in fungal cell walls. The enzyme chitinase degrades chitin into soluble components. Chitinase is commonly present in plants (Lawrence et al., 2000). The chitin monomer and dimer were inactive, whereas the trimer possessed very slight elicitor activity in inducing lignification in wheat leaves. In contrast, the chitin tetramer, pentamer, and hexamer all possessed significant elicitor activity inducing lignification (Figure 2.1; Barber et al., 1989). The smaller chitooligosaccharides (COSs) with degree of polymerization (DP) between 3 and 6 showed lower elicitor activity, whereas pentameric and hexameric COSs could induce HR (hypersensitive response)-related cell death in rice with efficiency similar to the COS mix (DP 5–20) (Ning et al., 2004). In suspension-cultured rice cells, *N*-acetylchitooligosaccharides larger than hexaose (DP = 7–8) showed very high elicitor activity at the nanomole range but those smaller than tetraose showed only negligible activity even at a very high concentration (Yamaguchi et al., 2000b). The chitin pentamer was the smallest active oligomer in inducing an oxidative burst in suspension-cultured wheat cells (Ortmann et al., 2004). Several researchers have shown that di- and tri-*N*-acetylchitomers were not effective for eliciting cellular responses (Felix et al., 1993a; Inui et al., 1997; Ortmann et al., 2004).

COS induces various defenses in plants and it induced resistance against blast disease in rice (Figure 2.2; Ning et al., 2004). COS strongly induced the expression of several defense-related genes in rice suspension cells (Minami et al., 1996; Nishizawa et al., 1999; Day et al., 2001; Ning et al., 2004). COS induces transient depolarization of membranes (Kuchitsu et al., 1993; Kikuyama et al., 1997), extracellular alkalinization and ion efflux (Felix et al., 1993a; Kuchitsu et al., 1997; Muller et al., 2000), changes in protein phosphorylation (Felix et al., 1993b), generation of ROS (Kuchitsu et al., 1995; Tsukada et al., 2002; Ning et al., 2004), increased

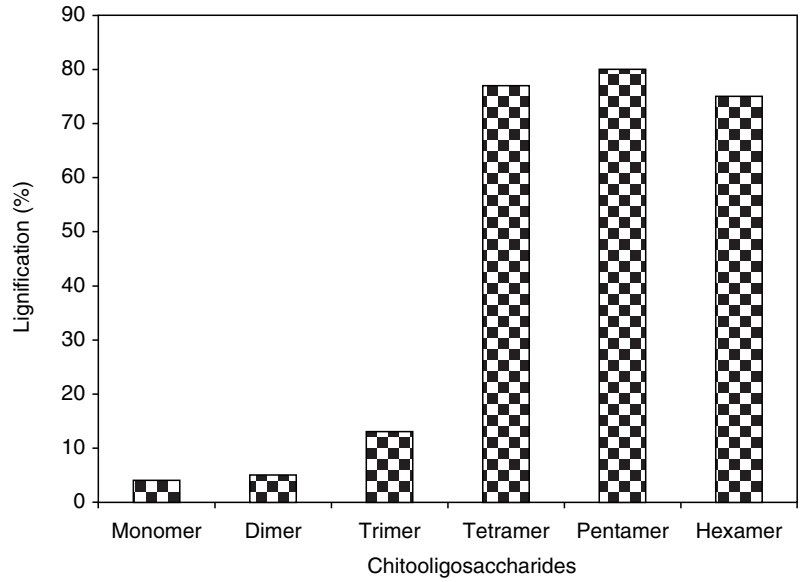

FIGURE 2.1 Efficacy of chitooligosaccharides in induction of lignification in wheat leaves. (Adapted from Barber, M.S., Bertram, R.E., and Ride, J.P., *Physiol. Mol. Plant Pathol.*, 34, 3, 1989.)

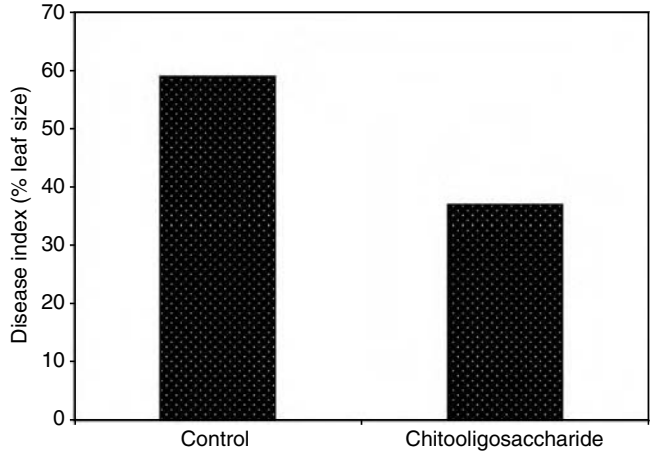

FIGURE 2.2 Induction of blast disease resistance by chitooligosaccharide in rice. (Adapted from Ning, W., Chen, F., Mao, B., Li, Q., Liu, Z., Guo, Z., and He, Z., *Physiol. Mol. Plant Pathol.*, 64, 263, 2004.)

accumulation of chitinases (Inui et al., 1997) and PAL (Inui et al., 1997) and their gene transcripts (Ning et al., 2004), production of phytoalexins (Ren and West, 1992; Kuchitsu et al., 1993; Yamada et al., 1993), induction of hypersensitive cell death (Vander et al., 1998; Ning et al., 2004), and deposition of lignin (Barber et al., 1989; Vander et al., 1998).

2.3.2 Chitosan Elicitors

Chitosan is a partially water-soluble derivative of chitin composed of (1→4)-linked GlcNAc and 2-amino-2-deoxy-β-D-glucopyranose (GlcN). It is a deacetylated polymer of β-1,4-glucosamine and is found in many fungal cell walls. Fungal chitosan may be biosynthesized from chitin by the action of chitin deacetylase, converting chain GlcNAc residues to GlcN residues (Araki and Ito, 1975). Fungal cell walls may contain chitosans with degrees of acetylation (DA) ranging from 0% to 100%. Chitosan has been reported to be an active elicitor inducing defense-related responses (Moerschbacher et al., 1986a,b; Gotthardt and Grambow, 1992). Elicitor activity of chitosan molecules may vary depending on their DA (Barber and Ride, 1988; Ning et al., 2004). The activity of chitosan is likely to reside in the acetylated regions of the molecule, since fully deacetylated chitosan is inactive (Barber and Ride, 1988) and chitosan with DA of 80% was highly active (Mitchell et al., 1994). Chitosan molecules with intermediate degrees of deacetylation showed strong elicitor activity on wheat leaves (Figure 2.3; Vander et al., 1998).

Chitosan elicited phytoalexin synthesis in pea (Kendra and Hadwiger, 1984), proteinase inhibitor in tomato (Walker-Simmons and Ryan, 1984), and lignin synthesis in wheat leaves (Vander et al., 1998).

2.3.3 Oligoglucan Elicitors

β-1,3-Glucans are important components of cell walls of fungal pathogens. β-1,3-Glucans are degraded into soluble fragments by the host enzyme β-1,3-glucanase (Yoshikawa et al., 1990; Ham et al., 1991). β-1,3-Glucanases have been detected in several plants (Høj and Fincher, 1995). β-1,3-Glucanase was found to be constitutively expressed in tomato (Lawrence et al., 2000) and rice plants (Akiyama et al., 1996, 1997).

Yoshikawa et al. (1981) showed the release of a soluble phytoalexin elicitor from mycelial walls of *Phytophthora sojae* by two isoenzymes of β-1,3-endoglucanase contained in soybean

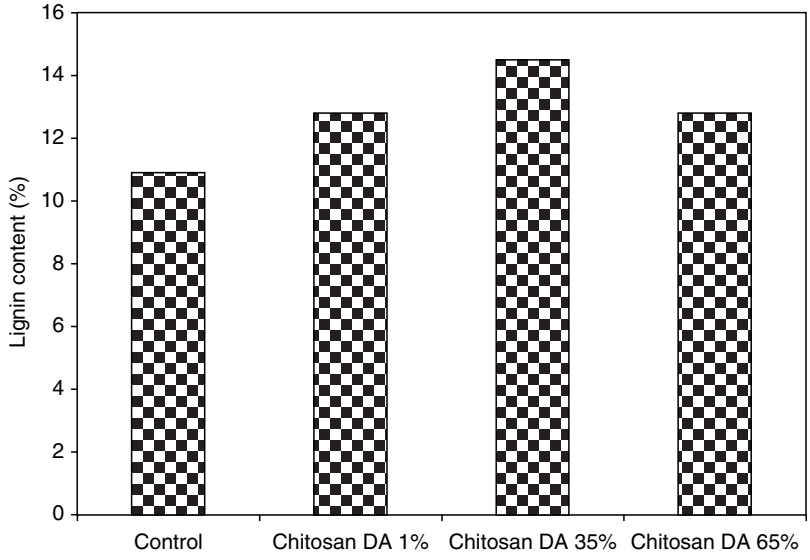

FIGURE 2.3 Efficacy of polymeric chitosans with different degrees of acetylation (DA) in inducing lignin synthesis in wheat leaves. (Adapted from Vander, P., Vårum, K.M., Domard, A., Gueddari, N.E.E., and Moerschbacher, B.M., *Plant Physiol.*, 118, 1353, 1998.)

tissues. The purified enzymes hydrolyzed several β-1,3-glucans in a strictly random manner (Keen and Yoshikawa, 1983). The enzyme activity also increased in the infected tissues (Jutidamorongphan et al., 1991). Nichols et al. (1980) showed that β-1,3-glucanase activity increased within 6 h in pea pod tissue inoculated with *Fusarium solani* f. sp. *pisi*. β-1,3-Glucanase has been reported to accumulate extracellularly based on its occurrence in intercellular washing fluids (IWFs) collected from infected leaves (Kauffman et al., 1987; Kombrink et al., 1988). Mauch and Staehelin (1989) used immunocytochemical methods to locate the glucanase. Antiglucanase antibodies labeled the expanded middle lamella region of the cell wall, indicating that glucanase is present extracellularly. When fungal pathogens initially grow in the intercellular space of their host plants, they may make contact with the β-1,3-glucanase localized in the middle lamella. Upon contact, the β-1,3-glucanase may release oligosaccharide fragments from the β-1,3-glucan-containing fungal cell wall. These oligosaccharides released by β-1,3-glucanase from fungal cell walls may act as elicitors of phytoalexin production.

The importance of the host enzyme β-1,3-glucanase in production of oligoglucan elicitor molecules from the fungal cell wall glucans has been demonstrated in soybean–*P. sojae* interaction. Exogenous application of purified β-1,3-glucanase to the fungus-inoculation sites resulted in induction of host defense mechanism, particularly accumulation of the phytoalexin glyceollin in soybean. Conversely, laminarin, which has been shown to inhibit the release of elicitors from fungal cell walls *in vitro* partially, suppressed glyceollin synthesis (Yoshikawa et al., 1990). ET increased 50-fold to 100-fold the level of β-1,3-endoglucanase mRNA (Takeuchi et al., 1990), and exogenous ET increased the level of β-1,3-endoglucanase and triggered the accumulation of the phytoalexin glyceollin in soybean (Yoshikawa et al., 1990). All these results suggest that the process of elicitor release mediated by host β-1,3-endoglucanase is an important process leading to phytoalexin synthesis.

Several β-glucan fragments released from the fungal cell wall by the action of β-1,3-glucanase have been characterized as elicitors. Linear β-1,3-glucans have been reported to be

efficient elicitors of defense responses in tobacco (Klarzynski et al., 2000). A glucan-related glucoheptaose elicitor molecule, 1,6-β-linked and 1,3-β-branched heptaglucoside (hepta-β-glucoside, HG), has been purified from the soybean pathogen *P. sojae* (Sharp et al., 1984). It has a structure with β-1,6-linked backbone and glucosyl stubs attached through 1,3-linkage. It could induce phytoalexin biosynthesis in soybean cotyledon cells at 10^{-8}–10^{-9} M (Sharp et al., 1984).

Several β-1,3-glucan fragments obtained from cell walls of the rice blast pathogen *M. grisea* by the action of β-1,3-glucanase strongly induced phytoalexin synthesis in rice suspension-cultured cells. The elicitor activity was distributed in the oligosaccharide fractions larger than tetraose and the activity increased in proportion to the size of the oligosaccharides. A highly elicitor-active glucopentaose induced phytoalexin synthesis at 10 nM (Yamaguchi et al., 2000a). The glucopentaose had a structure with a 1,3-linked backbone and glucosyl stub attached through 1,6-linkage (Yamaguchi et al., 2000a). β-1,3-Glucan oligomers with a DP of over 4 were shown to elicit chitinase activity, and DP6 also acted as an elicitor of PAL activity in rice cells (Inui et al., 1997). A related branched β-1,3-glucan with 30 glucose residues, mycolaminaran, has also been shown to have elicitor activity, but only at higher concentrations than that reported for the glucan (Keen et al., 1983).

2.3.4 Other Carbohydrate Elicitors

Keen et al. (1983) purified various elicitors from cell walls of *P. sojae*. Among them, glucomannans were the most active elicitors and were approximately 10 times more active than β-1,3-glucan elicitor (Keen et al., 1983). A glycan elicitor consisting of β-1,3- and β-1,6-linked glucose has been detected in germinating zoospores of *P. sojae* (Waldmueller et al., 1992). A carbohydrate elicitor has been isolated from *Blumeria graminis* f. sp. *tritici*, the wheat powdery mildew pathogen (Schweizer et al., 2000). Analysis of the carbohydrate moiety revealed mostly glucose, with smaller amounts of xylose and mannose. The glucosyl residues of the elicitor were found to be linked (1→2), (1→4), and (1→6), with (1→4, 1→6)-branch point residues. Mixed-linkage (1→4, 1→6)-mannosyl residues appeared to be important for elicitor activity (Schweizer et al., 2000). The elicitor induced the thaumatin-like proteins in barley, oat, rye, rice, and maize (Schweizer et al., 2000).

2.4 PROTEIN/PEPTIDE ELICITORS

2.4.1 Elicitins

Several *Phytophthora* and *Pythium* spp. secrete a family of structurally related proteins called elicitins (Huet and Pernollet, 1989; Ricci et al., 1992; Yu, 1995; Kamoun et al., 1997a; Ponchet et al., 1999; Vauthrin et al., 1999; Bourque et al., 2002; Baillieul et al., 2003; Qutob et al., 2003). All the elicitins share a conserved elicitin domain from amino acids 1 to 98. The primary structure of elicitins has been determined after sequencing of cloned genes and cDNAs (Ricci et al., 1989; Kamoun et al., 1993a, 1997; Pernollet et al., 1993; Huet et al., 1994; Panabières et al., 1995; Mao and Tyler, 1996). The elicitins can be grouped into five classes based on their primary structure (Table 2.1; Kamoun et al., 1997a; Baillieul et al., 2003). Class I-A and Class I-B comprise 10 kDa elicitins that display only the elicitin domain with 98 amino acid-long proteins. Class I-A elicitins are called α-elicitins and they have an acidic pI. The Class I-B elicitins have basic pI and are called β-elicitins. Class II contains highly acidic elicitins, which possess a short (5–6 amino acids long), hydrophilic C-terminal tail. Class III comprises elicitins with a long (65–101 amino acids long) amino acid C-terminal domain rich in Ser, Thr, Ala, and Pro, an amino acid composition and distribution that suggests potential O-glycosylation sites. Elicitins from *Pythium* spp.

TABLE 2.1
Classification of Elicitins

Class	Elicitin	Fungus
I-A	Cacto (Cag-α)	*Phytophthora cactorum*
	Infestin 1 (INF1)	*Phytophthora infestans*
	Para A1	*Phytophthora parasitica*
	α-Megaspermin PmH20 (Meg-α PmH20)	*Phytophthora megasperma*
	α-Megaspermin Pmm (Meg-α Pmm)	*Phytophthora megasperma* f. sp. *megasperma*
	α-Cinnamomin (Cin-α)	*Phytophthora cinnamomi*
	α-A1-Cryptogein (Cry-α A1)	*Phytophthora cryptogea*
	α-Capsicein (Cap-α)	*Phytophthora capsici*
	Soj1	*Phytophthora sojae*
	Soj2	*Phytophthora sojae*
I-B	Dre-α	*Phytophthora drechsleri*
	Dre-β	*Phytophthora drechsleri*
	β-Megaspermin PmH20 (Mega-β PmH20)	*Phytophthora megasperma*
	β-Megaspermin Pmm (Mega-β Pmm)	*Phytophthora megasperma* f. sp. *megasperma*
	β-Cryptogein (Cry-β)	*Phytophthora cryptogea*
	β-Cinnamomin (Cin-β)	*Phytophthora cinnamomi*
II	Cryptogein-HAE26 (Cry-HAE26)	*Phytophthora cryptogea*
	Cryptogein-HAE20 (Cry-HAE20)	*Phytophthora cryptogea*
	Cinnamomin-HAE (Cin-HAE)	*Phytophthora cinnamomi*
III	INF2A	*Phytophthora infestans*
	INF2B	*Phytophthora infestans*
	INF4	*Phytophthora infestans*
	INF5	*Phytophthora infestans*
	INF6	*Phytophthora infestans*
	γ-Megaspermin (Meg-γ PmH20)	*Phytophthora megasperma*
Py	Vex1	*Pythium vexans*
	Vex2	*Pythium vexans*

are classified as a distinct group called the Py class (Kamoun et al., 1997b) or as a subgroup of Class I (Ponchet et al., 1999).

The Class I-A and Class I-B elicitins are holoproteins with molecular masses of 10 kDa (Huet et al., 1992; Nespoulos et al., 1992; Ponchet et al., 1999). The holoproteins are devoid of side chain modification (Terce-Laforgue et al., 1992). γ-Megaspermin isolated from *Phytophthora megasperma* H20 (PmH20) shows unusual features of elicitins (Baillieul et al., 2003). The C-terminal domain of γ-megaspermin, from amino acids 99 to 154, is rich in Ser, Thr, Ala, and Pro. γ-Megaspermin carries an oligosaccharide moiety (Baillieul et al., 1995). Sequence analysis revealed that γ-megaspermin is a glycoprotein belonging to Class III elicitin (Baillieul et al., 2003; Cordelier et al., 2003). This glycoprotein elicitor contained an elicitin domain from amino acids 1 to 98 with the six conserved Cys residues at positions 3, 27, 51, 56, 71, and 95 (Baillieul et al., 2003). The γ-megaspermin cDNA sequence encodes a 174 amino acid protein with a signal peptide of 20 amino acids. The calculated molecular mass and pI of the mature protein are 14,920 Da and 3.80, respectively (Baillieul et al., 2003). γ-Megaspermin homologs are secreted by *P. cinnamomi*, *P. capsici*, *P. cryptogea*, and *P. parasitica* (Baillieul et al., 1996). Other Class III elicitins include INF2A, INF2B, INF5, and INF6 (Baillieul et al., 2003). INF2A and INF2B contain a putative hydrophobic signal peptide of 20 amino acids, followed by a conserved 98 amino acid elicitin domain. However, in contrast to other classes of elicitins, INF2A and INF2B bear additional C-terminal

domains of 67 and 71 amino acids, respectively. In both proteins, these C-terminal domains are rich in serine, threonine, alanine, and proline. This amino acid composition and the distribution of the four residues suggest the presence of clusters of O-linked glycosylation sites (Wilson et al., 1991). Probably INF2A and INF2B may be glycoproteins (Kamoun et al., 1997a). The Class III elicitins have an increased content in acidic amino acids.

These elicitins induce HR in particular plant species, specifically *Nicotiana* spp. in the Solanaceae and *Raphanus* and *Brassica* spp. in the Cruciferae (Ricci et al., 1989; Kamoun et al., 1993b; Bonnet et al., 1996). Elicitins may be the major determinants of the resistance response of *Nicotiana* against most *Phytophthora* spp. (Yu, 1995; Grant et al., 1996). When applied to tobacco plants, elicitins induce systemic distal leaf necrosis after migration toward the leaf tissue from the inoculation site (Zanetti et al., 1992). The elicitins induce cell death (Keller et al., 1996; Levine et al., 1996; Dorey et al., 1998), production of ET, and synthesis of phytoalexins (Milat et al., 1991a,b) in tobacco. They induce the accumulation of pathogenesis-related (PR) proteins in tobacco leaves (Bonnet et al., 1986). The elicitins induce rapid protein phosphorylation (Viard et al., 1994), Ca^{2+} influx (Tavernier et al., 1995; Lecourieux et al., 2002), oxidative burst (Sasabe et al., 2000), hydrogen peroxide (H_2O_2) production (Pugin et al., 1997; Simon-Plas et al., 1997; Sasabe et al., 2000), defense gene expression (Suty et al., 1995; Sasabe et al., 2000), stimulation of PAL (Dorey et al., 1999), production of phytoalexins (Milat et al., 1991b), SA accumulation (Dorey et al., 1999), disruption of microtubular cytoskeleton (Binet et al., 2001), cell wall modifications (Kieffer et al., 2000), and rapid cell death (Dorey et al., 1999) in cell suspension cultures.

Phytophthora drechsleri produces both α- and β-elicitins (Huet et al., 1992). *P. parasitica* strain 6H-11A produces the elicitin named parasiticein 6H-11A α-elicitin (Kamoun et al., 1993b). Cryptogein, the elicitor from *P. cryptogea*, is synthesized as a preprotein with a signal peptide removed cotranslationally before the secretion. It accumulates in the mycelium in its mature form (Terce-Laforgue et al., 1992). Cryptogein binds to high-affinity sites in the plasma membrane of tobacco cells (Wendehenne et al., 1995; Bourque et al., 1999) and induces, in the first few minutes, many events (Figure 2.4).

It induces protein phosphorylation (Viard et al., 1994; Lebrun-Garcia et al., 1998; Zhang et al., 1998; Lecourieux-Ouaked et al., 2000). Application of cryptogein to tobacco cells induces plasma membrane depolarization and Cl^- efflux, both of which occur after a similar lag period of about 5 min (Pugin et al., 1997). It induces ionic exchange modifications (Blein et al., 1991), including calcium ion influx (Lecourieux-Ouaked et al., 2000) and an anionic efflux (Tavernier et al., 1995; Pugin et al., 1997). It also induces plasma membrane NADPH oxidase activity responsible for production of ROS (Bottin et al., 1994; Pugin et al., 1997), cytosol acidification and extracellular medium alkalinization (Pugin et al., 1997), pentose phosphate pathway (Pugin et al., 1997), mitogen-activated protein kinase (MAPK) activation (Lebrun-Garcia et al., 1998, 2002), disruption of the microtubular cytoskeleton (Binet et al., 2001), NO production (Foissner et al., 2000; Lamotte et al., 2004), production of ET (Blein et al., 1991), and induction of defense-related genes (Suty et al., 1995).

The exact sequence and relationships between these early events are not yet known. These early events were inhibited by staurosporine, a protein kinase inhibitor (Viard et al., 1994; Tavernier et al., 1995; Lecourieux-Ouaked et al., 2000), indicating that phosphorylation reactions occurred upstream from these effects. Tavernier et al. (1995) identified protein phosphorylation followed by Ca^{2+} influx as the earliest steps. The late reactions include phytoalexin synthesis (Blein et al., 1991; Tavernier et al., 1995) and cell death (Binet et al., 2001). The protein elicitor migrates for long distances in tobacco plants (Devergne et al., 1992).

A 32 kDa glycoprotein-elicitin elicitor has been isolated from the culture filtrate of *Phytophthora megasperma* H20 (PmH20), a pathogen of Douglas fir (Baillieul et al., 1995). This glycoprotein elicitin induces cell death, expression of defense genes, production of SA,

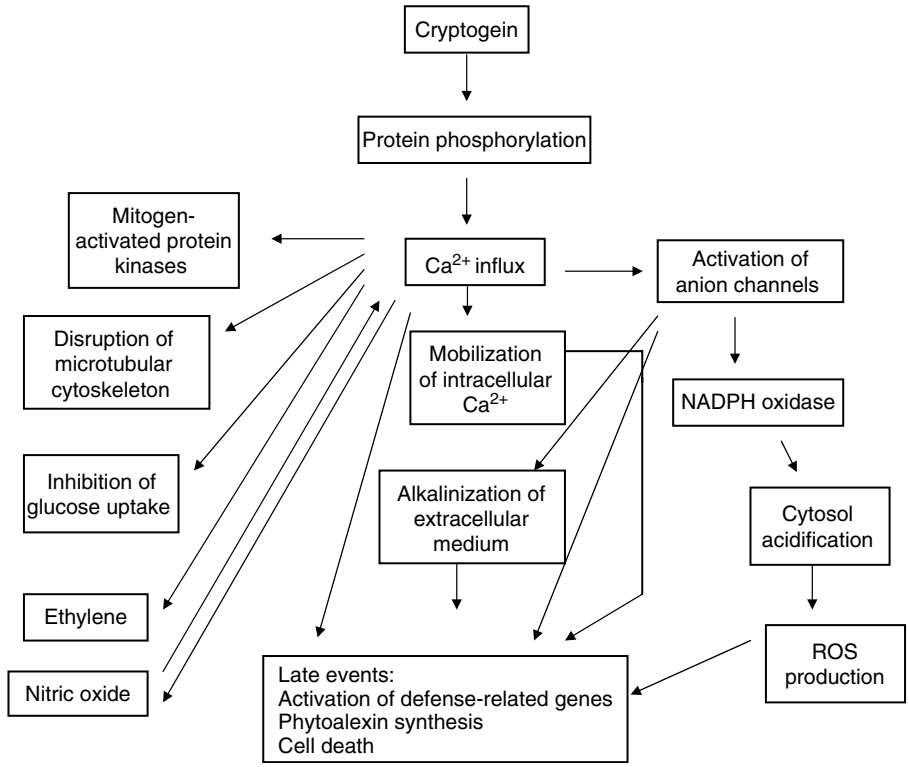

FIGURE 2.4 Functions of cryptogein elicitor in induction of various signal transduction systems in tobacco.

and induction of disease resistance in tobacco (Baillieul et al., 1995). When infiltrated into tobacco leaves, the elicitor induced PR protein expression (Dorey et al., 1997; Cordelier et al., 2003) and accumulation of the phytoalexin scopoletin (Costet et al., 2002).

Elicitins are structurally similar to lipid-transfer proteins of plant cells (Blein et al., 2002). Elicitins behave like sterol carrier proteins (Mikes et al., 1997, 1998). Elicitins have one binding site with a strong affinity for dehydroergosterol and they catalyze sterol transfer between phospholipidic artificial membranes (Mikes et al., 1998). They are able to pick up sterols from plasma membranes (Vauthrin et al., 1999). Elicitins exhibit differences with regard to the kinetics of loading and the rates of sterol exchanges between liposomes or micelles (Mikes et al., 1998; Vauthrin et al., 1999). The most efficient elicitin is cryptogein (from *P. cryptogea*), and the less efficient are parasiticein (from *P. parasitica*) and capsicein (from *P. capsici*). A correlation between the elicitor activities of elicitins and their ability to load and transfer sterols has been demonstrated (Rustérucci et al., 1996; Bourque et al., 1998; Osman et al., 2001). Cryptogein induces stronger cell responses than do parasiticein and capsicein (Rustérucci et al., 1996; Bourque et al., 1998).

The link between elicitor and sterol-loading properties was assessed with the use of site-directed mutagenesis of the 47 and 87 cryptogein residues, which are suggested to be involved in sterol binding (Osman et al., 2001). The mutated cryptogeins were strongly altered in their sterol-binding efficiency, specific binding to high-affinity sites, and activities on tobacco cells (Osman et al., 2001). It appears that the formation of a sterol–elicitin complex is a prerequisite step before elicitins fasten to specific binding sites. The first event involved in elicitin-mediated cell responses may be the protein loading with a sterol molecule (Osman et al., 2001).

2.4.2 Xylanase Elicitor

A proteinaceous elicitor has been isolated from *Fusarium oxysporum* f. sp. *pisi* and *Macrophomina phaseolina* (Dean et al., 1989). Similar elicitor has been isolated from a saprophytic fungus *Trichoderma viride* (Dean et al., 1989; Dean and Anderson, 1991). The elicitor has been identified as endoxylanase (Dean and Anderson, 1991). The elicitor induces ET biosynthesis, and hence it is called ET-inducing xylanase (EIX). It is a potent elicitor of plant defense response in specific cultivars of tomato and tobacco (Ron and Avni, 2004). The elicitor isolated from *T. viride* has been purified and identified as a 22 kDa protein. The amino acid composition of the 22 kDa polypeptide is enriched by glycine, serine, threonine, tyrosine, and tryptophan, but depleted in alanine, leucine, glutamine, and lysine. The protein lacks sulfur-containing amino acids. The protein is glycosylated and synthesized as a 25 kDa precursor protein that is processed to 22 kDa during secretion (Dean and Anderson, 1991). The elicitor induces ET (Fuchs et al., 1989; Dean and Anderson, 1991), PR proteins (Lotan and Fluhr, 1990), phytoalexin production (Farmer and Helgeson, 1987), tissue necrosis (Bailey et al., 1990), lipid peroxidation (Ishii, 1988), electrolyte leakage (Bailey et al., 1990), and cell death (Bucheli et al., 1990; Ron et al., 2000; Elbaz et al., 2002). EIX mutants lacking enzymatic activity (β-1,4-endoglucanase) retained elicitation activity. It suggests that xylanase activity is not required for the HR elicitation process and the protein per se functions as the elicitor (Enkerli et al., 1999; Furman-Matarasso et al., 1999; Rotblat et al., 2002).

2.4.3 PaNie$_{213}$ Elicitor

An elicitor protein, PaNie$_{213}$ (*Pythium aphanidermatum* necrosis-inducing elicitor with 213 amino acids), has been isolated from *Pythium aphanidermatum* (Veit et al., 2001). A cDNA encoding the elicitor protein (PaNie$_{234}$) was isolated. This protein contained 234 amino acids and had a putative eukaryotic secretion signal harboring a proteinase cleavage site. The mature elicitor 25 kDa protein without the secretion signal (PaNie$_{213}$) consisted of 213 amino acids (Veit et al., 2001). PaNie$_{213}$ protein triggered cell death and de novo formation of 4-hydroxybenzoic acid in cultured cells of carrot, callose formation on the cell walls of leaves of *Arabidopsis*, and necroses in tobacco and tomato leaves (Koch et al., 1998; Veit et al., 2001). Elicitor protein similar to PaNie of *P. aphanidermatum* has been isolated from *Phytophthora megasperma* f. sp. *glycinea*, *P. infestans*, *P. parasitica*, and *Fusarium oxysporum* f. sp. *erythroxyli* (Veit et al., 2001).

2.4.4 Nep1 Elicitor

A 24 kDa Nep1 (Necrosis- and ET-inducing protein-1) has been isolated from culture filtrates of *F. oxysporum* f. sp. *erythroxyli*. It induces necrosis and ET production in leaves of many dicot plant species (Jennings et al., 2001). It induces accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) in tobacco leaves. Tobacco cells treated with Nep1 induced extracellular alkalinization, ROS production, and cell death (Jennings et al., 2001).

2.4.5 NIP1 Elicitor

A necrosis-inducing peptide called NIP1 was purified from culture filtrate of *Rhynchosporium secalis*, the barley scald pathogen. The peptide strongly induced accumulation of PRHv-1, a thaumatin-like PR protein in resistant barley varieties. In susceptible varieties, similar induction was not observed. Protease treatment of NIP1 completely destroyed elicitor activity suggesting that the elicitor activity resides in the peptide (Hahn et al., 1993).

2.4.6 PB90 Elicitor

PB90 is a novel protein elicitor with a molecular mass of 90 kDa isolated from culture filtrate of the cotton blight pathogen *Phytophthora boehmeriae* (Wang et al., 2003b,c). Infiltration of this elicitor into leaves of tobacco, which is a nonhost for *P. boehmeriae*, triggers HR, induces H_2O_2 synthesis, and elicits increases in activities of peroxidase and PAL (Wang et al., 2003c). It induces accumulation of SA in tobacco leaves (Zhang et al., 2004b).

2.5 GLYCOPROTEIN ELICITORS

2.5.1 Carbohydrate Moiety in the Glycoprotein Elicitor May Confer Elicitor Activity

Elicitors isolated from several pathogens have been identified as glycoproteins. A peptidogalactoglucomannan elicitor has been identified in *Cladosporium fulvum*, the leaf mold pathogen of tomato. The carbohydrate moiety of the glycoprotein had mannose, galactose, and traces of glucose. The protein moiety was rich in alanine, aspartic acid/asparagine, glutamic acid/glutamine, proline, serine, and theronine (DeWit and Kodde, 1981a). There was a strong positive correlation between the mannose and galactose content of the peptidogalactoglucomannan and its phytoalexin-eliciting activity (DeWit and Kodde, 1981b).

Three elicitors have been purified from the cell wall and culture filtrate of *Colletotrichum lagenarium*, a pathogen of melon (*Cucumis melo*) (Toppan and Esquerré-Tugayé, 1984). All three elicitors were glycoproteins containing amino acid, sugar, and phosphate residues. Elicitor 1 was richer in aspartic and glutamic residues, elicitor 2 in the basic residues lysine and arginine, and elicitor 3 in serine, threonine, and glycine. The three elicitors also contained the same sugar residues: mannose, galactose, and glucose. Mannose was the most abundant in elicitor 1, rhamnose was detected in elicitor 2, and glucosamine in elicitor 3. The elicitor activity appeared to remain in the carbohydrate moiety rather than in the protein moiety (Toppan and Esquerré-Tugayé, 1984).

A crude extract from the germ tube wall of uredosporelings as well as mycelium of *Puccinia graminis* f. sp. *tritici* elicited lignification (Moerschbacher et al., 1986a,b; Kogel et al., 1988). The elicitor was purified and identified as a glycoprotein with a molecular weight of about 130,000. The predominant sugars in the polysaccharide moieties of the fungal cell wall glycoproteins were mannose (50%), galactose (47%), and glucose (3%). The carbohydrate and protein contents were in the ratio of 9:1. Digestion with pronase or trypsin had no effect on elicitor activity but complete loss of activity was observed after periodate treatment (Kogel et al., 1988). The results suggest that the carbohydrate moiety contains the active site of the elicitor. In the glycoprotein elicitor, two types of glycomoieties were linked to the protein core (Beissmann and Reisener, 1990). A mannan residue was linked N-glycosidically to asparagine and an α-galactan residue was O-glycosidically linked to serine or threonine (Beissmann and Reisener, 1990). The mannan residue could be removed from the protein core by endo-β-*N*-acetylglucosaminidase H (endo-H) cleavage without loss of elicitor activity, suggesting the importance of α-galactan for the elicitor activity (Beissmann et al., 1992).

An elicitor has been isolated from *Magnaporthe grisea* (Schaffrath et al., 1995). The elicitor is a glycoprotein with a molecular weight of 15.6 kDa. The elicitor activity was not lost when the elicitor was treated with pronase but the activity was completely abolished when it was treated with sodium periodate, indicating that carbohydrate is the active moiety (Schaffrath et al., 1995). Many elicitor fractions have been identified in culture filtrates of *Colletotrichum lindemuthianum* (Hamdan and Dixon, 1987). All fractions were predominantly polysaccharides but they contained about 5% protein also. Galactose and mannose were the major monosaccharide components (Hamdan and Dixon, 1987). The protein moiety has been

shown to be not necessary for elicitor activity (Hamdan and Dixon, 1986). A glycoprotein elicitor was isolated from *Colletotrichum falcatum*, the sugarcane (*Saccharum officinarum*) pathogen, and activity of the elicitor was found to reside in the carbohydrate moiety (Ramesh Sundar et al., 2002).

2.5.2 Protein Moiety in Glycoprotein Elicitors May Confer Elicitor Activity

In some glycoprotein elicitors, only the protein moiety has been found to be the active fraction. A glycoprotein elicitor was isolated from culture fluids of *Verticillium dahliae* and it induced phytoalexin formation in cotton cell suspension cultures (Davis et al., 1998). Elicitor activity of the elicitor was completely abolished by protease treatment but not by periodate treatment, indicating that only protein components are responsible for elicitation of phytoalexins in cotton cells. Glycoproteins extracted from isolated cell walls and culture filtrates of *Phytophthora sojae* elicited phytoalexin in soybean hypocotyls (Keen and Legrand, 1980). The glycoprotein was purified and its molecular weight was found to be 42 kDa. Proteinase digestion and deglycosylation treatments of the pure glycoprotein were performed to determine which portion was responsible for elicitor activity. Activity of the 42 kDa glycoprotein was unaffected by autoclaving the elicitor. Treatment with proteinases, pronase, and trypsin destroyed the activity in parsley (*Petroselinum crispum*) cells, whereas deglycosylation had no effect. The results suggest that the elicitor activity is conferred by the protein portion (Parker et al., 1991).

Two endoglycanases were used to test if this elicitor activity is due to N-linked glycopeptides (Basse and Boller, 1992). Endo-H hydrolyzes N-linked glycans between the two GlcNAc residues that are linked to the peptide, releasing oligosaccharides with one GlcNAc residue at its reducing end. *N*-Glycanase hydrolyzes various classes of N-linked oligosaccharides between asparagine and GlcNAc and produces oligosaccharide fragments with two GlcNAc residues at the reducing end. Both endo-H and *N*-glycanase did not affect the *P. sojae* elicitor activity (Basse and Boller, 1992), indicating that the union of carbohydrate and the peptide part is not important for elicitor activity. Periodate is known to degrade glycosyl residues with adjacent unsubstituted hydroxyl groups, and therefore is expected to attack terminal or 1,6- or 1,4-linked glycosyl residues. Periodate treatment did not affect the elicitor activity (Basse and Boller, 1992). The elicitor was completely inactivated by boiling in 20 mM β-mercaptoethanol, a treatment known to reduce S–S bridges in peptides. It indicates that S–S bridges are involved in the activity of the *P. sojae* elicitor (Basse and Boller, 1992). These results show that the elicitor-active component of crude *P. sojae* elicitor consists of polypeptides without essential carbohydrate parts.

The cell wall of *P. sojae* contains a 42 kDa glycoprotein that induces the activation of defense-related genes in parsley (Nürnberger et al., 1994) and potato (Halim et al., 2004). The active part of this glycoprotein is an internal peptide of 13 amino acids (Pep-13; Hahlbrock et al., 1995). Pep-13 elicitor shows some host-specificity. It is active in potato and parsley cells (Brunner et al., 2002), whereas it is inactive in soybean, carrot, tobacco, and *Arabidopsis* (Nürnberger et al., 1995). The Pep-13-containing glycoprotein of *P. sojae* has been identified as transglutaminase (Brunner et al., 2002). The Pep-13 motif is highly conserved in different members of the *Phytophthora* species, including *P. infestans* (Brunner et al., 2002). Mutations in Pep-13 abolished elicitor activity and transglutaminase activity (Brunner et al., 2002).

The 32 kDa glycoprotein elicitor of *Phytophthora capsici* showed phospholipase A2 (PLA2) activity (Nespoulos et al., 1999). It was structurally similar to γ-megaspermin elicitin from *P. megasperma* PmH20 (Baillieul et al., 2003). Glycoprotein fragments with elicitor activity could be isolated from the mycelial cell wall of *P. parasitica* var. *nicotianae* (Roux et al., 1994). A purified glycoprotein with M_r 34,000 has been obtained from the pathogen and it was named as GP34 (Sèjalon-Delmas et al., 1997).

2.5.3 Functions of Glycoprotein Elicitors

The glycoprotein elicitors are known to induce several types of host plant defense responses. The 42 kDa glycoprotein elicitor from *P. sojae*, Pep-13, induced efflux of K^+ and Cl^- ions and influx of H^+ and Ca^{2+} (Zimmermann et al., 1997), oxidative burst, the accumulation of transcripts encoding 4-coumarate:CoA ligase (4CL), and hypersensitive-like cell death in potato cells (Halim et al., 2004). Infiltration of Pep-13 into potato leaves induces the accumulation of JA and SA, suggesting that both the JA and SA pathways may operate in Pep-13-treated tissues. Pep-13 induced necrosis formation, H_2O_2 formation, and accumulation of JA, but not activation of a subset of defense genes in *NahG* mutant potato plants, which are not able to accumulate SA. In wild-type plants, SA accumulated and defense genes were activated, and necrosis formation, H_2O_2 formation, and accumulation of JA were observed. It suggests that Pep-13 is able to elicit SA-dependent and -independent defense responses (Halim et al., 2004). Receptor site for Pep-13 has been characterized in plasma membrane of parsley cells (Nürnberger et al., 1994; Nennsteil et al., 1998).

The 46 kDa glycoprotein elicitor isolated from the culture filtrate of *Phytophthora parasitica* var. *nicotianae* elicited the synthesis of phytoalexins in tobacco callus (Farmer and Helgeson, 1987). When introduced through the root system, GP34 elicitor from *P. parasitica* var. *nicotianae* elicited lipoxygenase (LOX) activity (Sèjalon-Delmas et al., 1997), and the LOX activity was shown to be associated with disease resistance in this tobacco–*P. parasitica* var. *nicotianae* interaction (Vèronèsi et al., 1996). GP34 induced LOX activity even at nanomole amounts (Sèjalon-Delmas et al., 1997). The elicitor induced hydroxyproline-rich glycoprotein (HRGP) accumulation in tobacco seedlings (Sèjalon-Delmas et al., 1997).

The 65 kDa glycoprotein elicitor from *Verticillium dahliae* induced the phytoalexin formation in cotton cells (Davis et al., 1998). The glycoprotein elicitor of *Cladosporium fulvum* induced electrolyte leakage, callose deposition, necrosis, and phytoalexin accumulation when applied to tomato leaves (DeWit and Roseboom, 1980). The glycoprotein elicitor isolated from *Mycosphaerella pinodes* triggered a plethora of defense responses in pea, including induction of phytoalexin pathway genes leading to phytoalexin accumulation (Yamada et al., 1992; Wada et al., 1995; Toyoda et al., 2000), lytic enzymes, such as endochitinase and endo-β-1,3-glucanase (Yoshioka et al., 1992b), and superoxide generation (Kiba et al., 1995). It induced pea PAL gene 1 (Kato et al., 1995). It also induced protein phosphorylation events and a rapid change of phosphoinositide metabolism (Shiraishi et al., 1990; Toyoda et al., 1992, 1993b, 2000). Treatment of pea tissues with the elicitor rapidly increased activation of two myelin basic protein-dependent kinases p44 and p48 within 15–30 min upon elicitation (Uppalapati et al., 2004).

2.6 LIPID ELICITORS

2.6.1 Sphingolipids

Cerebrosides, compounds categorized as glycosphingolipids, were found to occur in a wide range of fungal pathogens as novel elicitors (Umemura et al., 2002, 2004). Cerebrosides A and C were isolated from the rice blast fungus *Magnaporthe grisea* (Umemura et al., 2000). Cerebroside C was detected in *Cochliobolus miyabeanus*, *Cercospora solani-melongenae*, and *Mycosphaerella pinodes* (Umemura et al., 2000). *Pythium graminicola* and diverse strains of *Fusarium oxysporum* produce cerebroside C (Umemura et al., 2004). *Rhizoctonia* sp. mycelia contained cerebroside B (Umemura et al., 2000). *Glomerella cingulata* and *Sclerotium cepivorum* produce cerebroside B, whereas *Botrytis allii* produces both cerebrosides A and B (Umemura et al., 2004). Some type of cerebroside elicitor involving cerebroside A, B, or C was detected in *Pythium* and *B. allii*. These elicitors were identified as ceramide monohexosides. These ceramide monohexosides consist of a ceramide moiety containing 9-methyl-1-4,8-sphingadienine in

amidic linkage to 2-hydroxyoctadecenoic or 2-hydroxyhexadecenoic acid and a carbohydrate segment consisting of one residue of glucose (Maciel et al., 2002). These elicitors showed phytoalexin-inducing activity when applied to rice plants by spray treatment and also induced the expression of PR proteins in rice leaves (Umemura et al., 2000). Ceramides prepared from the cerebrosides by removal of glucose also showed elicitor activity (Umemura et al., 2000). Induction of phytoalexins by the treatment with cerebroside elicitor was markedly inhibited by Ca^{2+} channel blockers, suggesting that Ca^{2+} may be involved in the signaling pathway of elicitor activity of cerebroside (Umemura et al., 2002).

2.6.2 Arachidonic and Eicosapentaenoic Acids

Highly active lipid elicitors isolated from the potato late blight pathogen *P. infestans* have been identified as arachidonic acid (AA) and eicosapentaenoic acid (EPA). EPA and AA were abundant in sporangia, zoospores, and cystospores of *P. infestans* (Creamer and Bostock, 1988). Bromine treatment completely eliminated the unsaturated fatty acids in lyophilized sporangia, zoospore, and cystospore tissue and abolished their sesquiterpenoid phytoalexin elicitor activity. Addition of brominated spore tissue to pure AA significantly enhanced the phytoalexins levels (Creamer and Bostock, 1988). These observations indicate that lipids play an important role in elicitation of defense responses.

AA and EPA are present primarily in esterified form in the fungus (Creamer and Bostock, 1986). However, free AA elicits a faster response than do esterified forms. Free carboxyl group is required for optimal elicitor activity (Preisig and Kuc, 1985). Potato LOX enhances the activity of AA. Salicylhydroxamic acid is an inhibitor of LOX and it effectively inhibited AA-elicited HR (Preisig and Kuc, 1987). When potato tissue was treated with radiolabeled free AA, most of the fatty acid was incorporated without further modification into potato acyl lipids. A low level of radiolabel from AA was recovered continually in the region of hydroxy fatty acids, suggesting that oxidation of the fatty acid molecule was occurring. AA incorporated into acyl lipids was recovered primarily from phospholipids. Over 90% of the radiolabel in polar lipids was in two lipid classes, such as phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). AA was released from potato tuber by potato lipid acyl hydrolase and lipase (Preisig and Kuc, 1985, 1987). Thus, release of AA from esterified form, the form in which it occurs in the fungus, appears to be important for the elicitation activity (Preisig and Kuc, 1988).

Glucans from *P. infestans* enhanced metabolism of AA to compounds comigrating with oxidized fatty acids. Calcium chloride also enhanced the elicitor activity and it also enhanced oxidation of AA. Salicylhydroxamic acid inhibited oxidative metabolism of AA and it inhibited HR (Preisig and Kuc, 1988). All these results revealed that AA has to be oxidized by LOX. The importance of LOX in AA-elicited defense mechanisms was demonstrated by using LOX-deficient potato cell lines. These cell lines showed diminished response to AA (Vaughn and Lulai, 1992).

2.6.3 Ergosterols

Ergosterol is the main sterol of most fungi. The ergosterol elicitor was isolated from spores of the tomato pathogen *Cladosporium fulvum* (Granado et al., 1995). When the suspension-cultured cells of tomato were treated with the ergosterol, a rapid, transient extracellular alkalinization in tomato cells was observed (Granado et al., 1995). The extracellular alkalinization is an important defense response event. Ergosterol interacted with tobacco suspension-cultured cells and triggered pH changes of extracellular medium, oxidative burst, and synthesis of phytoalexins (Kasparovsky et al., 2003). Ergosterol elicited mobilization of internal calcium stores mediated by inositol-1,4,5-trisphosphate (IP_3) and serine/threonine protein

kinases (Kasparovsky et al., 2003). Ergosterol elicitor strongly and rapidly induced the accumulation of nonspecific lipid-transfer protein (nsLTP) mRNAs in grapevine (Gomés et al., 2003). LTPs have been shown to play an important role in induction of disease resistance (Vidhyasekaran, 2004).

2.7 TOXINS AS ELICITOR MOLECULES

Some of the toxins produced by pathogens act as elicitors. Elicitors act as signal compounds at low concentrations, whereas toxins act at higher concentrations. The rice blast pathogen produces a toxin, α-picolinic acid. This toxin has been shown to elicit HR and enhance disease resistance in rice (Zhang et al., 2004). It induces H_2O_2 generation and cell death (Zhang et al., 2004). Fumonisin B1 is a toxin produced by the maize pathogen *Fusarium verticillioides* (Desjardins and Plattner, 2000) and it acts as an elicitor inducing active plant defense mechanism (Stone et al., 2000).

Victorin is the host-specific toxin produced by *Helminthosporium victoriae*. Treatment of oats (*Avena sativa*) susceptible to *H. victoriae* with low concentrations of victorin elicits production of the oat phytoalexin avenalumin, whereas treatment with higher concentrations greatly reduces the accumulation of avenalumin (Mayama et al., 1986). Victorin induced the phytoalexin avenalumin in oat varieties resistant to the crown rust pathogen *Puccinia coronata* f. sp. *avenae* (Mayama et al., 1995). It suggests that a toxin may also act as elicitor. Ca^{2+}-signaling system may be involved in the signal transduction of the victorin signal (Navarre and Wolpert, 1999). ET also may transduce the toxin/elicitor signal (Navarre and Wolpert, 1999).

2.8 PLANT CELL WALL–DEGRADING ENZYMES AS ELICITORS

Fungal pathogens produce enzymes, which degrade plant cell wall, to facilitate hyphal penetration to obtain nutrients from the plant cell. These enzymes act as virulence factors, but some of them may act as elicitors. Cellulase causes rapid accumulation of phytoalexins in *Nicotiana tabacum* cell cultures, an increase in the production of capsidiol and debneyol, and production of two previously unknown phytoalexins (Threlfall and Whitehead, 1988). Pectolyase is a cell wall–degrading enzyme and also an elicitor, which is a potent inducer of membrane depolarization in the cell membrane of *N. tabacum* (Zimmermann et al., 1998). The French bean pathogen *Colletotrichum lindemuthianum* produces an endopolygalacturonase (endo-PG), and the gene encoding this enzyme, *CLPG1*, has been cloned (Boudart et al., 2003). The CLPG1 endo-PG elicited defense responses when supplied to French bean seedlings (Lafitte et al., 1993; Boudart et al., 1998). CLPG1 induced ROS and β-1,3-glucanase activity before development of leaf necrosis in tobacco plants (Boudart et al., 2003). A functional catalytic site was required for the elicitor activity of the fungal endo-PG CLPG1 in tobacco (Boudart et al., 2003).

A glycoprotein elicitor isolated from *Botrytis cinerea* has been identified as endo-PG. It activates defense reactions in grapevine (*Vitis vinifera*) including calcium ion influx, production of ROS, MAPKs, defense gene transcript accumulation, and phytoalexin production (Poinssot et al., 2003). Most of these defense reactions were also activated in grapevine in response to purified oligogalacturonides with a DP of 9–20, released by the endo-PG activity on plant cells. However, the intensity and kinetics of events triggered by the oligogalacturonide were very different when compared with the endo-PG effects. Desensitization assays revealed that elicitor activity was not due to released oligogalacturonides. The results suggest that the endo-PG may act as an elicitor (Poinssot et al., 2003).

Pectolytic enzymes may also release pectic fragments (oligogalacturonides) that can act as endogenous elicitors (Ebel and Cosio, 1994; Shibuya and Minami, 2001). Elicitors of casbene synthetase, the key enzyme in the biosynthesis of the castor bean (*Ricinus communis*)

phytoalexin, were detected in culture filtrates from *Rhizopus stolonifer*, a common fungus found in castor bean seeds (Stekoll and West, 1978). Polygalacturonase activity was readily detected in the partially purified elicitor preparation (Lee and West, 1981a,b). Heat treatment of the enzyme preparations led to equivalent losses of both enzymic activity and elicitor ability suggesting that the elicitor abilities of these enzymes may be dependent on their catalytic activities (Lee and West, 1981b). The catalytic action could cause the breakdown of a normal plant constituent to one or more degradation products, which themselves could function as elicitors. Incubation of castor bean homogenate with the purified enzyme led to the isolation of soluble heat-stable elicitor components lacking enzyme. Analysis of the crude heat-stable elicitor fraction indicated that it was a pectic fragment containing galacturonic acid or its methyl ester. More complete digestion of the heat-stable elicitor by endo-PG at optimum pH conditions for prolonged period resulted in loss of activity (Bruce and West, 1982). Only partial digestion of polygalacturonic acid with polygalacturonase produced the heat-stable elicitor. The partial digestion produced a mixture of α-1,4-D-galacturonic oligomers, which elicited the defense responses in castor bean (Bruce and West, 1982).

Endo-PG from *Cladosporium cucumerinum*, the cucumber pathogen, elicited lignification in cucumber hypocotyls (Robertson, 1986). The enzyme released elicitors of lignification from polygalacturonic acid and cucumber cell wall (Robertson, 1986).

Endo-PG from *Aspergillus niger* elicited necrosis in *Vigna unguiculata* (Cervone et al., 1987). The enzyme elicited synthesis of proteinase inhibitors (Walker-Simmons et al., 1984) and lignification (Robertsen, 1986). The eliciting activity of the enzyme was by releasing oligogalacturonide elicitors from the pectic polysaccharide of plant cell walls (Walker-Simmons et al., 1984). Endo-PGs from different races of *Colletotrichum lindemuthianum* elicited phytoalexins in bean by forming elicitor-active oligogalacturonides (Lorenzo et al., 1990; Tepper and Anderson, 1990). Endo-pectin lyase (PL) produced by *Botrytis cinerea*, a pathogen of carrot, induced phytoalexin synthesis in carrot. Treatment of carrot cell walls with 10 units of endo-PL resulted in high elicitor activity (Movahedi and Heale, 1990). The carrot cells treated with pectinase released host wall fragments that elicited the production of the phytoalexin (Movahedi and Heale, 1990). A pectic lyase purified from the fungus *Fusarium solani* f. sp. *pisi* induced accumulation of proteinase inhibitor I in tomato leaves (Ryan, 1987). Movahedi and Heale (1990) reported that *B. cinerea* produced an aspartic proteinase both *in vitro* and in infected carrot tissue. When the enzyme was applied at low concentrations, it induced the phytoalexin 6-methoxymellein. The enzyme-released endogenous-elicitors (mixture of uronide and peptide) induced the phytoalexin.

2.9 RACE-SPECIFIC AND CULTIVAR-SPECIFIC ELICITORS

The gene-for-gene interactions between fungal pathotype and plant cultivars are controlled by the fungal avirulence genes and their matching plant disease resistance genes. The molecular explanation for such matching is that each avirulence gene encodes a specific elicitor, which recognizes and interacts with the product of the corresponding resistance gene, thus triggering defense responses. However, only very few race-specific elicitors have been purified. The α race of *Colletotrichum lindemuthianum* produces an extracellular galactose- and mannose-rich glycoprotein elicitor that induces phytoalexin accumulation in an α-race-resistant bean cultivar but not in a susceptible one. It suggests that this elicitor acts as an avirulence factor (Tepper and Anderson, 1986; Tepper et al., 1989).

Avr9 peptide encoded by the avirulence gene *avr9* is a race-specific elicitor isolated from *Cladosporium fulvum*, the tomato leaf mold pathogen (May et al., 1996). The *avr9* gene encodes a precursor protein that is processed upon secretion to mature protein of 28 amino acids that is capable of inducing specific hypersensitivity on *Cf9*-bearing tomato cultivars

(van Kan et al., 1991). Injection of elicitor preparations containing the *Avr9* gene product of *C. fulvum* race 4 into tomato carrying the *Cf9* gene induced leaf necrosis and production of ROS (Lu and Higgins, 1998). The Avr9 elicitor induced several early defense responses in transgenic tobacco carrying the *Cf9* gene. These responses include changes in ion fluxes (Piedras et al., 1998; Blatt et al., 1999), production of ROS (Piedras et al., 1998), and the activation of MAPKs and a Ca^{2+}-dependent protein kinase (CDPK) (Romeis et al., 1999, 2001). The intracellular fluid containing Avr9 induced a serine/threonine kinase called Avr9/Cf9 induced kinase 1 (ACIK1) in Cf9 tobacco cell cultures (Rowland et al., 2005). Another race-specific elicitor isolated from *C. fulvum* strain is the *Avr4* gene product. It encodes a precursor protein that is processed upon secretion into mature protein of 86 amino acids (Joosten et al., 1994).

A race-specific elicitor, IF_4 (intercellular fluid 4), has been obtained from *C. fulvum* race 4. The unpurified or partially purified IF_4 showed race-specificity. The IF_4 showed the necrotic effect on tomato *Cf5* plants and induced the host-plasma membrane Ca^{2+}-permeable channels, whereas the *avr5* gene product from *C. fulvum* race 5 did not induce necrosis or membrane Ca^{2+}-permeable channels on *Cf5* plants. The results demonstrated the race-specific effect of the fungal elicitor (Gelli et al., 1997). The increase in the host-plasma membrane H^+-ATPase, changes in the plasma membrane redox activities (Vera-Estrella et al., 1994a,b), the induction of a protein kinase cascade mediating the phosphorylation/dephosphorylation of the plasma membrane H^+-ATPase (Xing et al., 1996), and the activation of a stimulatory type of heterotrimeric G-proteins (Xing et al., 1997) have been shown to be specifically induced on *Cf5* tomato cells by the IF_4.

Vera-Estrella et al. (1992) showed that IF_4 but not IFs from races virulent on *Cf5* plants caused an oxidative burst and lipid peroxidation. *In vitro* treatment of plasma membranes isolated from *Cf5* cells with IFs from *Cf5*-incompatible but not from *Cf5*-compatible races of *C. fulvum* resulted in the inhibition of Ca^{2+}-ATPase (Lam et al., 1998). Ca^{2+} efflux is regulated by Ca^{2+}-ATPase, and inhibition of Ca^{2+}-ATPase by the race-specific elicitor may result in increase in cytosolic Ca^{2+} concentrations. Oscillations in cytosolic Ca^{2+} levels seem to be involved in elicitation of host defense responses (Blume et al., 2000; Klüsener et al., 2002).

Some elicitors may be cultivar-specific and trigger defense responses only in resistant cultivars. An avirulence gene *Avr1b-1* was cloned from the oomycete soybean pathogen *Phytophthora sojae* and it was required for avirulence of the pathogen on soybean plants carrying resistance gene *Rps1b*. When the Avr1b-1 protein was synthesized in the yeast *Pichia pastoris* and the secreted protein infiltrated into soybean leaves, it triggered hypersensitive resistance in host plants carrying the *Rps1b* gene (Shan et al., 2004). The Avr1b-1 protein was not active on susceptible plants. The results suggest that the Avr1b-1 protein is a cultivar-specific elicitor acting only on resistant plants triggering defense response. *Rhynchosporium secalis*, the barley leaf scald pathogen, produces a peptide elicitor, NIP1, which specifically acts on resistant barley cultivars (Hahn et al., 1993). The NIP1 is the product of the avirulence gene *NIP1* and the elicitor elicits a defense response on barley plants with the *Rrs1* resistance gene (Schürch et al., 2004).

Elicitors isolated from basidiospore germlings of *Uromyces vignae*, the cowpea rust fungus, showed cultivar-specific action in cowpea (Chen and Heath, 1990, 1993). Elicitors specific for the *Lr20* allele in wheat were detected in diffusates from wheat leaves infected with pathotypes of the leaf rust fungus (*Puccinia recondita* f. sp. *tritici*) avirulent with respect to this allele (Jones and Deverall, 1978). Other elicitors of *P. recondita* f. sp. *tritici* selective for a gene on chromosome 5A in wheat but not specific for any known resistance gene were found in the IFs (Sutherland et al., 1989) and they caused particularly strong necrosis in the cultivar Cappelle-Desprez (Deverall and Deakin, 1987). These elicitors were also obtained from germ tubes of uredospores of *P. recondita* f. sp. *tritici* (Sutherland et al., 1989). Extracts from leaves of wheat lines carrying the resistance gene *Lr9* or *Lr28* infected with avirulent

pathotypes of *P. recondita* f. sp. *tritici* showed the presence of elicitors specific for putative *Avr9/Lr9* and *Avr28/Lr28* interactions (Saverimuttu and Deverall, 1998).

IWFs were isolated from *Arabidopsis* leaves inoculated with two different pathotypes of *Peronospora parasitica*. When infiltrated into compatible and incompatible *Arabidopsis* genotypes, the IWFs induced necrosis on incompatible genotypes but not on compatible genotypes (Rethage et al., 2000). The results suggest that the IWFs contain cultivar-specific elicitors of *P. parasitica* pathotypes. However, the putative elicitors were not purified.

2.10 SPECIFICITY OF GENERAL ELICITORS

Some of the general elicitors also show host-specificity. The *Phytophthora megasperma* f. sp. *glycinea* elicitor hexa-β-glucoside, which elicited phytoalexin synthesis in soybean cells, could not induce biosynthesis of phytoalexins in rice cells. Similarly, the glucopentaose purified from cell walls of the rice pathogen *M. grisea* could not act as an elicitor for soybean cells (Yamaguchi et al., 2000b). The Pep-13 elicitor from *Phytophthora infestans* appears to be inactive as an elicitor in carrot, tobacco, soybean, and *Arabidopsis* (Nürnberger et al., 1995). However, Pep-13 was able to induce defense reactions in potato cells with a similar specificity as in parsley cells (Brunner et al., 2002).

The EIX elicitor induces HR in specific plant species and varieties, thus it shows some specificity in its action (Ron et al., 2000; Elbaz et al., 2002). EIX specifically binds to the plasma membrane of both tomato and tobacco responding cultivars (Hanania and Avni, 1997). PaNie elicitor protein isolated from *Pythium aphanidermatum* induced defense mechanisms in dicotyledonous plants, such as alfalfa, tobacco, and tomato, but not in monocotyledonous plants, such as maize, oat, and *Tradescantia zebrina* (Veit et al., 2001).

Elicitins show some host-specificity. Elicitin protein from *Phytophthora parasitica* did not induce any visible response in most plant species tested including several solanaceous plants, but elicited HR in all tested *Nicotiana* spp. and in some but not all *Raphanus sativus* and *Brassica campestris* cultivars (Table 2.2; Kamoun et al., 1993b). Thus, the elicitin protein is a

TABLE 2.2
Plant Species-Specific/Cultivar-Specific Activity of Parasiticein, the Elicitin Produced by *Phytophthora parasitica*

Plant Species	Cultivar	Response to Parasiticein Elicitor
Raphanus sativus	Daikon	++[a]
	Early Mino	++[a]
	Miura	+[b]
	Miyashige	+[b]
	White Icicle	−[c]
	Spanish Radish	−[c]
Brassica campestris	Turnip Just Right	+[b]
	Shogoin Turnip	−[c]
Nicotiana tabacum	Xanthi-nc	++[a]
	Havana	++[a]

Source: Adapted from Kamoun, S., Young, M., Glascock, C.B., and Tyler, B.M., *Mol. Plant Microbe Interact.*, 6, 15, 1993b.

[a] ++ indicates large necrotic spots.
[b] + indicates minute necrotic spots.
[c] − indicates no visible response.

genus-specific elicitor within the Solanaceae and cultivar-specific elicitor within the Cruciferae (Kamoun et al., 1993b). The other elicitin tested, cryptogein, also showed this type of species-/cultivar-specificity (Kamoun et al., 1993b). This type of specificity may be due to the presence of some specific binding sites in the specific plant species/cultivars. This possibility has not been studied, although elicitin-binding sites have been identified in *Nicotiana* species (Wendehenne et al., 1995).

2.11 ENDOGENOUS OLIGOGALACTURONIDE ELICITORS

Besides the fungal elicitors, elicitors of host origin are also known to induce defense responses in plants (Ridley et al., 2001; Boudart et al., 2003). Elicitors of host origin are called constitutive or endogenous elicitors, whereas elicitors of pathogen origin are called exogenous elicitors. The elicitor, which elicits the defense-related proteinase inhibitors in tomato, has been found to be a host cell wall–derived polygalacturonide with a DP of about 20 (Bishop et al., 1981, 1984). Elicitor-active components from French bean hypocotyls were identified as oligogalacturonides with nine galacturonosyl units (Dixon et al., 1989). Elicitor of phytoalexin accumulation in soybean has been identified as dodeca-α-1,4-D-galacturonide (Nothnagel et al., 1983). Oligogalacturonide elicitor has been detected in parsley cells (Davis and Hahlbrock, 1987). The elicitor-active substances found in carrot cells are a mixture of heterogenous molecules in which uronide and peptide are essential components (Kurosaki et al., 1985; Movahedi and Heale, 1990). A noncarbohydrate elicitor has also been identified in tomato (Farmer et al., 1989).

A mixture of α-1,4-D-galacturonic oligomers have been identified as elicitors of phytoalexin synthesis in castor bean (Bruce and West, 1982). The fractions corresponding to octomers and smaller oligomers showed no significant elicitor activity, whereas fractions corresponding to nonamers through pentadecamers were active, with the tridecamer eliciting the greatest response (Jin and West, 1984). Methyl esterification of the carboxylate groups greatly diminished the elicitor activity of the oligomers, suggesting a requirement for the polyanionic character of the oligomers for full activity (Jin and West, 1984).

The mixture of oligomers with 9–12 galacturonosyl units showed elicitor activity at concentration down to about 3 μg uronic acid unit/mL, and induced lignification in cucumber (Robertsen, 1986). The ability of oligogalacturonides to induce phytoalexin and lignin synthesis in *Vigna unguiculata* was strictly dependent upon their DP. Oligomers of galacturonic acid with a DP between 10 and 13 were effective, whereas shorter oligomers had little or no activity (Robertsen 1986; Ryan, 1987, 1988; Lorenzo et al., 1990).

A large pectic polysaccharide called rhamnogalacturonan I in sycamore (*Acer pseudoplatanus*) elicited proteinase inhibitor. Galacturonic acid was the major glycosyl residue in rhamnogalacturonan I. Arabinosyl, galactosyl, and rhamnosyl residues were also present (Ryan et al., 1981). α-1,4-Digalacturonic acid with 4,5-unsaturated nonreducing terminal galacturonosyl residue and the unsaturated trimer were effective inducers of the synthesis of proteinase inhibitor I when supplied to tomato plants through their cut petioles (Ryan, 1987). The galacturonosyl oligomers that elicit phytoalexin responses are larger than the oligomers that induce proteinase inhibitors. Although accumulation of phytoalexins and lignin (Davis et al., 1984; Robertsen, 1986) is elicited by oligomers of DP 10–13, proteinase inhibitor synthesis is even induced by dimers (Bishop et al., 1984; Ryan, 1987).

Oligogalacturonides induce the early responses such as plasma membrane depolarization, ion fluxes, and cytosol acidification in suspension-cultured cells (Mathieu et al., 1991). ROS are produced very rapidly after oligogalacturonides are supplied to plant cells and seedlings (Legendre et al., 1993a; Lee et al., 1999a; Orozco-Cardenas and Ryan, 1999).

Most of the studies have demonstrated that the endogenous (plant) elicitors may be released by pathogen-derived pectin-degrading enzymes. However, Bergey et al. (1999)

reported the induction of a plant endo-PG in response to oligosaccharide elicitors, and this enzyme may also be involved in release of oligogalacturonide elicitors from plants.

2.12 MULTIPLE ELICITORS MAY BE NEEDED TO ACTIVATE DEFENSE RESPONSES

2.12.1 Elicitor Complex

Defense responses may include activation of various defense genes, including several *PR* genes, phytoalexin synthesizing genes, defensin and thionin genes, and phenylpropanoid metabolic pathway genes. Fungal pathogens produce several types of elicitors, including oligosaccharides, proteins, glycoproteins, lipids, enzymes, and toxins. Individual pathogens may produce several elicitors. Several kinds of elicitors have been isolated and characterized from a single pathogen. *Phytophthora infestans*, the potato late blight pathogen, has been shown to contain at least six elicitins such as INF1, INF2A, INF2B, INF4, INF5, and INF6 (Kamoun et al., 1997a,b; Baillieul et al., 2003), AA, EPA, homo-γ-linolenic acid, nonanoic acid, linoleic acid, and β-glucan as elicitors (Bryan et al., 1985; Bostock et al., 1986; Ricci et al., 1989). *Phytophthora sojae*, the soybean pathogen, contains glycoprotein, β-1,3-glucan, glucomannan, mycolaminarin, hexa-β-glucosyl glucitol, and α and β classes of elicitins as elicitors (Ricci et al., 1989; Basse and Boller, 1992). *Cladosporium fulvum*, the tomato pathogen, contains a glycoprotein and a peptide as elicitors (DeWit and Roseboom, 1980). *Colletotrichum graminicola*, the corn pathogen, is known to contain two kinds of elicitors; one is a carbohydrate and another is a peptide (Yamaoka et al., 1990). Several glycoproteinaceous elicitors with different molecular weights have been isolated from cell walls of *Colletotrichum lindemuthianum*, the bean pathogen (Dixon, 1986; Hamdan and Dixon, 1987). Several chitin, chitosan, and β-1,3-glucan molecules have been detected in cell walls of several fungal pathogens and they also act as elicitors (Ren and West, 1992). Some of the toxins produced by the pathogens are also known to act as elicitors (Mayama et al., 1986, 1995). Endogenous elicitors of host origin are also involved in the signal transduction process.

It has been demonstrated that the elicitors may have specificity in inducing particular type of defense genes and hence, to induce a plethora of defense genes involved in defense response in a plant, different elicitors should act in coordination with each other. The defense-associated genes involved in conferring resistance to *Rhynchosporium secalis* in barley include genes encoding PR proteins PR-1, PR-5, PR-9, and PR-10, a germin-like protein (OxOLP), a LOX gene, *LoxA*, a gene encoding a putative protease inhibitor, *SD10*, and an unknown defense gene *pI2-4* (Steiner-Lange et al., 2003). The cultivar-specific elicitor NIP1 isolated from *R. secalis* triggered the induction of the four *PR* genes only. It has been suggested that additional elicitors are needed to induce OxOLP, LoxA, pI2-4, and SD10 in resistant barley leaves (Steiner-Lange et al., 2003).

2.12.2 Network of Elicitor Molecules

The multiple elicitor molecules may coordinate with each other and induce high expression of the defense genes. The cerebroside (Umemura et al., 2002), glycoprotein (Schaffrath et al., 1995), glucitol (Yamaguchi et al., 2002), glucan (Yamaguchi et al., 2000a), and chitin elicitors (Ning et al., 2004) have been detected in the rice blast pathogen *Magnaporthe grisea*. Treatment of rice cell suspension cultures with cerebroside and chitin oligomer resulted in a synergetic induction of phytoalexins (Umemura et al., 2002). A synergistic effect was observed for oligochitin and oligoglucan for the induction of phytoalexin biosynthesis in rice cells, suggesting the presence of cross talk between the signal transduction cascades downstream of these signal molecules (Yamaguchi et al., 2000b). The two oligosaccharide

elicitors derived from *Magnaporthe grisea* cell walls, *N*-acetylchitoheptaose and tetra-glucosyl glucitol, synergistically activated phytoalexin biosynthesis in cultured rice cells (Yamaguchi et al., 2002). The two elicitors were recognized by different receptors. These results suggest the presence of positive interaction between the signal transduction cascade downstream of each elicitor/receptor, which enhances resistance against pathogens (Yamaguchi et al., 2002).

Hexa-β-glucosyl glucitol is the elicitor isolated from the soybean pathogen *Phytophthora sojae*, whereas decagalacturonic is a pectic fragment released from plant polygalacturonic acid by action of endo-PG produced by the pathogen of soybean. The elicitor activity of combinations of the decagalacturonide and hexa-β-glucosyl glucitol showed a 35-fold stimulation above the calculated additive response (Davis et al., 1986a,b). A combination of the galactoglucomannan elicitor of *Colletotrichum lindemuthianum* with endogenous oligogalacturonide elicitor showed a greater effect on the accumulation of soluble and condensed phenolics including phytoalexins in bean (Tepper and Anderson, 1990).

The endogenous elicitor acted synergistically with the glucan elicitor from *Phytophthora sojae* in the induction of coumarin phytoalexins in parsley cells (Davis and Hahlbrock, 1987). About 10-fold stimulation in coumarin accumulation above the calculated additive response was observed in cell cultures treated with combinations of plant and fungal elicitors. The synergistic effect was also observed for the induction of PAL, 4CL, and *S*-adenosyl-L-methionine-xanthotoxol *O*-methyl transferase (XMT), the key enymes involved in phenyl propanoid metabolism and furanocoumarin biosynthetic pathway (Davis and Hahlbrock, 1987).

2.13 AVAILABILITY OF FUNGAL ELICITORS AT THE SITE OF FUNGAL INVASION IN PLANTS

Although most of the elicitors have been isolated from fungal cultures, a few studies have demonstrated that the elicitors may be available for action at the site of fungal invasion in plants. It has been reported that conidia of *Blumeria graminis* f. sp. *hordei*, *B. graminis* f. sp. *tritici*, and *Erysiphe pisi* rapidly released ECM-containing elicitors, and the ECM accumulated at their interface with barley coleoptile cells within minutes of landing (Fujita et al., 2004). The ECM was released from the few conidial surface projections that touched the curved surfaces of leaf epidermal cells. More amount of ECM was released when conidia made full contact than partial contact (Fujita et al., 2004). The ECM has been reported to contain elicitors (Schweizer et al., 2000). The ECM also contains elicitor-releasing enzymes such as xylanase (Komiya et al., 2003), cutinase (Pascholati et al., 1992), cellulase (Suzuki et al., 1998), pectinases (Suzuki et al., 1999), and hydrolytic enzymes (Fric and Wolf, 1994). These enzymes may also facilitate transfer of the a signaling molecule such as the soluble heteroglycan found in *B. graminis* f. sp. *tritici* conidial washings to the host surface (Schweizer et al., 2000).

Some of the fungal elicitors may be soluble in leaf surface dew. Surface washings of ungerminated *B. graminis* f. sp. *tritici* conidia contained a heteroglycan elicitor that induced defense-related gene expression in wheat (Schweizer et al., 2000). Elicitor activity has been shown to be present in surface washings of conidia of the barley pathogen *B. graminis* f. sp. *hordei* (Yukioka et al., 1997) and in washings of the pea powdery mildew pathogen *Erysiphe pisi* germlings (Toyoda et al., 1993a). Germinating zoospores of *Phytophthora sojae* released highly elicitor-active glucans (Waldmueller et al., 1992). The secreted elicitors in the host surface may trigger defense responses.

Some elicitors have been isolated from infected tissues. Kamoun et al. (1997a) have reported the cloning of the fungal elicitin elicitors, INF1, INF2A, and INF2B from potato leaves infected with *Phytophthora infestans*. They cloned an elicitin cDNA, *inf1*, selected from

a library constructed from RNA isolated from leaves of potato 3 days after inoculation with *Phytophthora infestans* strain 88069. The library yielded clones containing cDNA sequences of two other elicitin genes, *inf2A* and *inf2B* (Kamoun et al., 1997a). INF1 has been detected in potato leaves inoculated with *P. infestans* (Kamoun et al., 1997b).

A glycoprotein elicitor has been isolated from IWF obtained from wheat leaves inoculated with *Puccinia graminis* f. sp. *tritici* (Beissmann et al., 1992). Deverall and Deakin (1985) detected an elicitor in IWFs from wheat leaves infected with the leaf rust fungus *Puccinia recondita* f. sp. *tritici*. In the infected tissue, rust hyphae present in the intercellular spaces contributed the elicitor materials to these washing fluids (Holden and Rohringer, 1985). Elicitors have been isolated from intracellular fluids (Rowland et al., 2005) and IFs (Gelli et al., 1997) collected from tomato leaves infected by different races of *Cladosporium fulvum*. These studies suggest that elicitors released from fungal cells are available at the infection site to trigger defense responses in plants.

2.14 RECEPTORS FOR ELICITOR SIGNALS IN PLANT CELL MEMBRANE

2.14.1 Receptor Sites for Binding Oligosaccharide Elicitors

Several studies have indicated that receptors (binding sites) for the fungal oligosaccharide elicitors may exist in host plant cell membranes (Dixon, 1986; Basse et al., 1993; Montesano et al., 2003). Indirect evidences have been presented to show that the fungal elicitors have to be bound with the receptor sites of the host for their action. The activity of oligosaccharide elicitor from *Phytophthora sojae* was shown to be inhibited by certain methyl sugar derivatives, which were presumed to act by competing for elicitor-binding sites (Ayers et al., 1976). Yoshikawa et al. (1983) used ^{14}C-labeled mycolaminarin isolated from *Phytophthora* spp. as elicitor. Mycolaminarin bound with membrane preparations from soybean cotyledons and the binding was inhibited by pretreatment of the membranes with heat or pronase indicating the presence of a proteinaceous binding site. The binding was characterized by a dissociation constant of 11.5 μM with respect to the one class of binding site identified and a total of 16,500 binding sites/cell were calculated. Maximum specific binding per milligram of protein was associated with a fraction containing plasma membranes.

The presence of saturable, high-affinity, and specific binding sites for the *P. sojae* glucan elicitors in a microsomal fraction of soybean roots has been identified (Cosio et al., 1992; Cheong et al., 1993). Schmidt and Ebel (1987) used β-1,3-(^{3}H) glucan elicitor fraction from *P. sojae*, the soybean pathogen, to identify putative receptor sites in soybean tissues. The studies successfully demonstrated that highest binding activity was associated with a plasma membrane-enriched fraction. The binding was abolished by pronase treatment of the microsomal fraction and stabilized in the presence of dithiothreitol indicating proteinaceous nature of the binding site. The maximum number of binding sites was 0.5 pmol/mg of protein. Competition studies with the (^{3}H) glucan elicitor and a number of polysaccharides demonstrated that only polysaccharides of a branched β-glucan type effectively displaced the radiolabeled ligand from membrane binding (Schmidt and Ebel, 1987).

A 75 kDa protein has been identified as the receptor site for the HG elicitor of *P. sojae* in soybean (Cosio et al., 1992). The 75 kDa elicitor-binding protein was identified in the microsomal membrane of the soybean root (Cosio et al., 1992). The binding protein was purified (Mithöfer et al., 1996b) and the cDNA was cloned and sequenced (Umemoto et al., 1997). Expression of the cDNA clone in tobacco suspension-cultured cells conferred the glucan elicitor-binding activity (Umemoto et al., 1997). An antibody against the recombinant protein was found to inhibit the elicitor binding with the soybean cotyledon membrane fractions as well as the resulting accumulation of phytoalexin. Immunolocalization assays

indicated that the elicitor-binding protein is located in the plasma membrane (Umemoto et al., 1997). High-affinity binding sites of similar characteristics have also been detected in the microsomal fractions of alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris*), lupine (*Lupinus albus*), and pea (*Pisum sativum*) (Cosio et al., 1996; Côté et al., 2000; Mithöfer et al., 2000). However, none of these proteins exhibit a signaling domain, suggesting that these β-glucan-binding proteins (GBPs) may interact with other components to transduce the elicitor signal (Mithöfer et al., 2000).

Recent studies have shown that the specific and high-affinity HG elicitor-binding site is contained in the GBP, which in turn is part of a receptor complex in soybean (Fliegmann et al., 2004). The GBP is composed of two different carbohydrate reactive protein domains, one containing the β-glucan-binding site and the other related to glucan endo-glucosidases of fungal origin. The glucan hydrolase displayed an endo-specific mode of action, cleaving only 1,3-β-D-glucosidic linkages of oligoglucosides consisting of at least four moieties. During initial contact with *Phytophthora*, the intrinsic endo-1,3-β-glucanase activity released oligoglucoside fragments enriched in motifs that constituted ligands for the affinity-binding site present in the same protein (Fliegmann et al., 2004). Binding sites for COS molecules have been detected in plasma membrane of tomato (Baureithel et al., 1994), soybean (Day et al., 2001), rice (Shibuya et al., 1993, 1996; Yamaguchi et al., 2000b), wheat, barley, and carrot cells (Okada et al., 2002). Some specific COS-binding proteins have been identified (Ito et al., 1997; Day et al., 2001; Okada et al., 2002). A novel membrane-bound chitinase-related receptor-like protein that contains both chitinase and serine/threonine kinase domains has been identified in tobacco (Kim et al., 2000). A rice 75 kDa COS-binding membrane protein has been identified as a functional receptor for the COS elicitor (Ito et al., 1997; Yamaguchi et al., 2000b). Lectins may be important groups of receptors for oligosaccharide elicitors and their role in binding the elicitor molecules is discussed separately.

2.14.2 Receptor Sites for Binding Proteinaceous Elicitors

The presence of elicitor-binding sites with a high affinity for the protein elicitor cryptogein has been demonstrated in tobacco cells (Blein et al., 1991). Cryptogein high-affinity binding sites with receptor properties occur in tobacco plasma membrane preparations (Wendehenne et al., 1995). The presence of a single family of high-affinity sites for elicitins has been reported (Blein et al., 1991). They are located on the plasma membrane, represent 220 fmol of sites/mg of protein ($K_d = 2$ nM), and exhibit a sharp optimum pH near 7.0 (Wendehenne et al., 1995). These binding sites showed a functional molecular mass of 193 kDa (Bourque et al., 1999). They contained an N-linked carbohydrate moiety determinant in elicitin binding (Bourque et al., 1999). The binding was saturable and susceptible to displacement by unlabelled ligand (Blein et al., 1991).

Class I-A and Class I-B elicitins interact with the same tobacco receptor, with the same affinity (Bourque et al., 1998). Class III elicitin γ-megaspermin may also interact with the same receptor of Class I-A and Class I-B elicitins (Baillieul et al., 2003). Class I-A and Class I-B elicitins function as sterol carriers (Mikes et al., 1997, 1998). It appears that the formation of an elicitin–sterol complex is a prerequisite for binding to the receptor and subsequent elicitor activity (Ponchet et al., 1999; Osman et al., 2001).

A 100 kDa protein has been identified as a receptor site for Pep-13 peptide elicitor of *P. sojae* in the plasma membrane of parsley cells (Baureithel et al., 1994; Nürnberger et al., 1994; Nennstiel et al., 1998). Specific, saturable, and reversible binding of Pep-13 to this receptor protein has been reported in parsley cells (Nürnberger et al., 1994). The binding site for the xylanase elicitor of *Trichoderma viride* in tobacco was identified as a 44 kDa protein (Hanania and Avni, 1997). Binding sites for AVR9 peptide elicitor of *Cladosporium fulvum* in

tomato (Kooman-Gersmann et al., 1997; Nennsteil et al., 1998) and Pgt-glycoprotein elicitor of *Puccinia graminis* in wheat (Wendehenne et al., 1995) have been characterized.

Binding site for EIX elicitor has been characterized. EIX binds to the plasma membrane of some specific tomato- and tobacco-responding cultivars (Hanania and Avni, 1997). The response of EIX in tobacco and tomato cultivars is controlled by a single dominant locus (Bailey et al., 1990; Ron et al., 2000). The EIX-responsive locus (LeEix) was mapped to the short arm of chromosome 7 of the tomato cultivar. A yeast artificial chromosome (YAC) clone carrying a 300 kb DNA segment, derived from the LeEix region (Ron et al., 2000), was isolated. Mapping the ends of this YAC clone showed that it spans the LeEix locus (Ron et al., 2000). A novel gene cluster was isolated from the LeEix locus in tomato. The gene cluster contained two members, *LeEix1* and *LeEix2*. The deduced amino acid sequences encoded by *LeEix1* and *LeEix2* contain a leucine zipper (LZ), an extracellular leucine-rich repeat (LRR) domain with glycosylation signals, a transmembrane (TM) domain, and a C-terminal domain with a mammalian endocytosis signal (Ron and Avni, 2004).

Silencing expression of the *LeEix* genes prevented the binding of EIX to cells of an EIX-responsive plant and thus inhibited the HR. Overexpression of either *LeEix1* or *LeEix2* in EIX-nonresponsive tobacco plants enabled the binding of EIX (Ron and Avni, 2004). Over-expressing *LeEix2* in mammalian COS-7 cells enables binding of EIX, indicating physical interaction between the EIX elicitor and the *LeEix2* gene product (Ron and Avni, 2004). These results suggest the LeEix gene products represent the binding site for EIX elicitor. Structural analysis of the LeEix proteins suggests that they belong to a class of cell-surface glycoproteins with a signal for receptor-mediated endocytosis. Mutating the endocytosis signal in LeEix2 (Tyr to Ala) abolished its ability to induce the HR, suggesting that endocytosis plays a key role in the signal transduction pathway (Ron and Avni, 2004).

2.14.3 Protein Kinases as Receptor Sites

Some of the receptors, which bind with elicitors, have been identified as receptor-like protein kinases (RLKs). These plant RLKs are serine/threonine kinases. RLKs contain an extracellular domain that is probably involved in signal perception, a TM domain and a cytoplasmic kinase domain, which may initiate a signal transduction cascade into the cell (Satterlee and Sussman, 1998; Shiu and Bleecker, 2001). Large number of RLKs have been reported in plants. There are at least 340 genes encoding putative RLKs in the *Arabidopsis* genome (Montesano et al., 2003). The diversity of the large number of RLKs in plants suggests that they may be involved in perception of a wide range of elicitors in plant–pathogen interactions. Plant resistance genes (*R*) have been suggested to be involved in the signal transduction system (see Chapter 3), and several *R* genes encode cytoplasmic proteins related to RLKs (Shiu and Bleecker, 2001). Some *R* gene products are RLKs (Song et al., 1995; Feuillet et al., 1997).

Some RLKs contain extracellular domains with similarity to PR proteins. The RLK from tobacco, CHRK1, shows similarity to chitinase. CHRK1 may be involved in the recognition of pathogen-derived chitin oligosaccharides (Kim et al., 2000). The RLK from *Arabidopsis*, PR5K, contains extracellular domains with similarity to PR-5 protein (Wang et al., 1996). Some RLKs show similarity to lectin proteins, and the lectin-binding motifs of these RLKs may bind the oligosaccharide elicitors of the fungal pathogens (Hervé et al., 1996).

2.14.4 LRR-Type Receptors

LRR-containing TM RLK may also act as receptor for the elicitor. A bacterial elicitor, flagellin, interacts with an LRR-containing TM RLK receptor in *Arabidopsis* (Gómez-Gómez and Boller, 2000, 2002). The LRR regions participate in protein–protein interactions

(Bauer et al., 2001). The *Arabidopsis* receptor shows a structural similarity to *Drosophila* Toll- and mammalian Toll-like receptors, and both of them are LRR-type receptors involved in mediating the innate immune responses in animals (Montesano et al., 2003). The systemin receptor from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family (Scheer and Ryan, 2002).

2.14.5 Lectins as Receptors

Lectins may be the important receptors for oligosaccharide elicitors in plant cells. Garas and Kuc (1981) showed that elicitors from *Phytophthora infestans* were precipitated by potato lectin. The lectin–potato elicitor complexes nonetheless retained most of the elicitor activity when applied to potato discs. These results suggested that lectins in potato may serve as binding or receptor sites for the elicitor. GlcNAc oligomers are most likely recognized by specific lectin-like receptors (Etzler, 1985). Lectins specific for GlcNAc have been implicated in the recognition of chitin oligomers (Etzler, 1985). High-affinity binding sites for GlcNAc oligomers have been detected in suspension-cultured rice and tomato cells that can be elicited by chitin (Shibuya et al., 1993; Baureithel et al., 1994). Lectins specific for GlcN have been shown to be involved in binding of chitosan in plant cells (Lièнart et al., 1991).

2.14.6 Resistance Gene Products as Receptors

Some receptors for the fungal elicitor appear to be similar to some resistance gene products (Ron and Avni, 2004). The *LeEix1* gene encodes receptor site for the xylanase elicitor in tomato. It comprises 3247 nucleotides and has 1931 amino acids (Ron and Avni, 2004) The *LeEix1* cDNA-deduced amino acid sequence shares 48% similarity and 31% identity with the tomato Cf2 resistance protein (Dixon et al., 1996) and 45% similarity and 30% identity with the tomato Ve1 resistance protein (Kawchuck et al., 2001). Tomato *Ve* disease resistance genes encode cell surface-like receptors (Kawchuck et al., 2001). The *Arabidopsis* resistance gene, *NHL3*, encodes a plasma membrane receptor protein (Varet et al., 2003). These studies indicate that the fungal elicitors bind with receptor sites in host-plasma membrane, before initiating the signal transduction process through a series of second messengers.

2.15 CALCIUM ION MAY ACT AS SECOND MESSENGER

2.15.1 Function of Calcium Ion as Second Messenger

Calcium ion acts as an intracellular second messenger, coupling extracellular stimuli to intracellular and whole-plant responses (Sanders et al., 1999). Elicitation of defense response by elicitors was more effective in the presence of Ca^{2+} in plants (Ebel, 1984). Ebel (1984) showed that chalcone synthase activity was induced more in elicitor-treated soybean cells after calcium treatment. Calcium ion has been shown to be required for activation of 1,3-β-glucan synthase in soybean cells by the elicitors (Kauss et al., 1983; Kohle et al., 1985).

Plant responses to pathogens are regulated by changes in cytosolic Ca^{2+} levels (Bush, 1993). Changes in host cytosolic Ca^{2+} concentrations that are due to an increased influx of Ca^{2+} across the plasma membrane in response to pathogens and their elicitors have been widely reported (Dixon et al., 1994; Zimmermann et al., 1997; Blume et al., 2000; Klüsener et al., 2002). Calcium concentration increases in plant cells by two ways: influx of Ca^{2+} across the plasma membrane (Gelli et al., 1997; Zimmermann et al., 1997; Pei et al., 2000) and release of Ca^{2+} from internal stores (Blume et al., 2000). Influx of Ca^{2+} may depend upon Ca^{2+}-channel currents. Ca^{2+}-permeable currents in the plasma membrane are termed I_{Ca} channel (Pei et al., 2000). I_{Ca} channels have been shown to be permeable to several cations including Mg^{2+} (Pei et al., 2000).

Ca^{2+} permeation through the plant plasma membrane may occur due to the activation of Ca^{2+}-permeable channels either at hyperpolarized potentials (Stoeckel and Takeda, 1995; Gelli and Blumwald, 1997) or at depolarized membrane potentials (Huang et al., 1994; Thuleau et al., 1994; Piñeros and Tester, 1995). Hyperpolarization-dependent Ca^{2+}-permeable current in the plasma membrane of plant cells leads to Ca^{2+} influx and an increase in the cytoplasmic free Ca^{2+} concentration (Hamilton et al., 2000; Pei et al., 2000; Klüsener et al., 2002). Klüsener et al. (2002) showed that both chitosan and yeast elicitor activated a hyperpolarization-dependent current in *Arabidopsis* cells. In the absence of elicitors, only a small background current with a mean amplitude of −5.7 pA was observed. The common fungal elicitor chitosan induced a hyperpolarization-activated current with a mean peak current amplitude of −119.6, whereas the yeast elicitor induced a mean peak current amplitude of −5.7 pA (Figure 2.5; Klüsener et al., 2002). Elicitor-induced currents (I_{Ca}) have also been described in parsley (*Petroselinum crispum*) (Zimmermann et al., 1997).

Hyperpolarization of the electrical membrane potential difference resulted in the activation of Ca^{2+}-permeable channels in the plasma membrane of tomato cells (Gelli and Blumwald, 1997). A race-specific elicitor (*avr5* elicitor) from *Cladosporium fulvum* induced activation of the plasma membrane Ca^{2+}-permeable channel in tomato (Gelli et al., 1997). Guanosine-5′-[β-thio]diphosphate, a GDP analog that locks heterotrimeric G-proteins into their inactivated state, abolished the channel activation induced by the fungal elicitor, whereas 5′[γ-thio]triphosphate, a nonhydrolyzable GTP analog that locks heterotrimeric G-proteins into their activated state, produced an effect similar to that observed with the fungal elicitor. The addition of HA1004 (a protein kinase inhibitor) in the presence of the elicitor totally abolished channel activity, whereas okadaic acid (a protein phosphatase inhibitor) enhanced channel activity. These results suggest that the activation of the channel by fungal elicitors is modulated by a heterotrimeric G-protein-dependent phosphorylation of the channel protein (Gelli et al., 1997).

Heterotrimeric G-proteins have been implicated in mediating the defense responses of host cells to fungal elicitors (Sano and Ohashi, 1995). Ion channel coupling appears to involve the activation of intermediate membrane-associated effectors, including protein kinases and phosphatases (Brown, 1991). The function of protein kinases and phosphatases in signal transduction is described later in this chapter.

Ca^{2+} permeation through the plant plasma membrane may also occur due to the activation of Ca^{2+}-permeable channels at depolarized membrane potentials. The voltage-dependent Ca^{2+} transport system mediates Ca^{2+} influx across the membrane of plant cells at depolarized

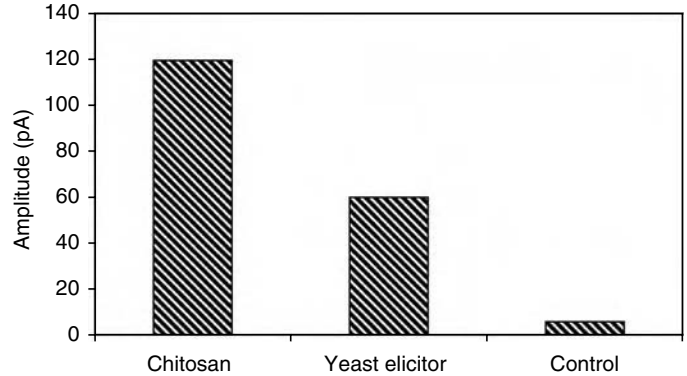

FIGURE 2.5 Activation of Ca^{2+}-channel currents by fungal elicitors. (Adapted from Klüsener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hogouvieux, V., and Schroeder, J.I., *Plant Physiol.*, 130, 2152, 2002.)

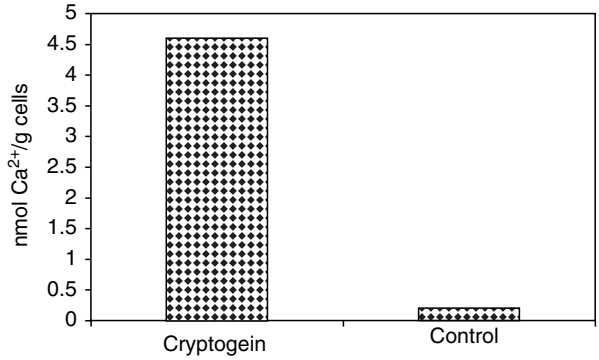

FIGURE 2.6 Induction of calcium ion influx by the elicitor cryptogein. (Adapted from Lecourieux-Ouaked, D., Pugin, A., and Lebrun-Garcia, A., *Mol. Plant Microbe Interact*., 13, 821, 2000.)

membrane potential (Huang et al., 1994). The plasma membrane Ca^{2+} channel is opened upon depolarization of the membrane potential. The voltage-gated Ca^{2+} channels are characterized by bell-shaped current–voltage relationships, with channel opening upon depolarization and subsequent increase in Ca^{2+} current to a maximum upon further successive depolarization. The Ca^{2+} current then decreases upon further depolarization and the electrochemical driving force for Ca^{2+} influx diminishes (Huang et al., 1994). Depolarization was induced by fungal toxins or oligogalacturonide elicitors in carrot protoplasts and the depolarization of the plasma membrane positive to −135 mV activated Ca^{2+}-permeable channels (Thuleau et al., 1994).

Elicitor treatment induces rapid Ca^{2+} influx into cytoplasm of plant cells. Massive influx of Ca^{2+} influx in tobacco-cultured cells was observed within 15–30 min after treatment with cryptogein elicitor (Figure 2.6; Lecourieux-Ouaked et al., 2000).

Elicitor treatment increases the cytoplasmic free calcium $[Ca^{2+}]_{cyt}$ (Klüsener et al., 2002). $[Ca^{2+}]_{cyt}$ oscillations do not occur in the absence of extracellular Ca^{2+} (Klüsener et al., 2002). $[Ca^{2+}]_{cyt}$ elevations accompany membrane hyperpolarization (Grabov and Blatt, 1998). Ca^{2+} influx mediates the initial phase of $[Ca^{2+}]_{cyt}$ transients induced by external Ca^{2+} (McAinsh et al., 1995). Although the presence of external Ca^{2+} may be a prerequisite for the induction of cytoplasmic transients, internal Ca^{2+} release may also play an important role in increase of $[Ca^{2+}]_{cyt}$. An inhibitor of plant phospholipase C (PLC), U-73122, inhibited increases in $[Ca^{2+}]_{cyt}$ in *Commelina communis* (Staxén et al., 1999). The PLC-activated $[Ca^{2+}]_{cyt}$-release mechanisms and Ca^{2+} influx may be interdependent. More than one plasma membrane Ca^{2+} channel is likely to contribute to cytosolic Ca^{2+} elevations in plant cells (Hamilton et al., 2000). A hyperpolarization-independent mechanism for generation of $[Ca^{2+}]_{cyt}$ elevations in *Arabidopsis* cells has been reported (Klüsener et al., 2002).

2.15.2 Upstream Events of Ca^{2+} Signaling

It has been reported that ROS signal transduction activates Ca^{2+} channels (Mori and Schroeder, 2004). Elicitors induce both cytosolic Ca^{2+} increases and ROS generation. In some cases, Ca^{2+} elevations have been reported upstream of ROS production (Kawano and Muto, 2000); in other cases, Ca^{2+} elevations occur downstream of ROS production (Blume et al., 2000; Bowler and Fluhr, 2000), indicating complex spatiotemporal Ca^{2+} elevation mechanisms. Membrane-bound NAD(P)H oxidases may be involved in ROS production (Mori and Schroeder, 2004). Elicitor-induced elevation in cytosolic Ca^{2+} lies upstream of NADPH-oxidase activation (Blume et al., 2000). Protein kinase and phosphatase also modulate I_{Ca} channels (Köhler and Blatt, 2002).

2.15.3 Downstream Events of Ca^{2+} Signaling

Cellular Ca^{2+} levels are tightly regulated, and hence, small changes in intracellular Ca^{2+} can provide information for the modification of enzyme activity and gene expression needed for subsequent responses (Gong et al., 2004). Pathogen signals may trigger an oscillation in the cytosolic free Ca^{2+} concentration, which is then perceived by various intracellular sensors/binding proteins to regulate a series of signaling cascades (Gong et al., 2004). According to Sanders et al. (2002), Ca^{2+} sensors can be classified into sensor responders and sensor relay. On binding with Ca^{2+}, sensor responders change their conformation and modulate their own activity or function through intramolecular interactions (Gong et al., 2004). CDPKs are the sensor responders (Kim et al., 2003b). CDPKs have protein kinase and calmodulin (CaM)-like Ca^{2+}-binding domains in a single protein, which allows direct activation by Ca^{2+} (Horn and Walker, 1994; Cheng et al., 2002; Hrabak et al., 2003). CaM protein acts as sensor relay and communicates the changed conformation to interacting partners such as protein kinases, resulting in a change in kinase activity (Zielinski, 1998; Cheng et al., 2002; Guo et al., 2002).

Oscillations in $[Ca^{2+}]_{cyt}$ are sensed by intracellular Ca^{2+}-binding proteins, one of which is CaM (Chin and Means, 2000; Snedden and Fromm, 2001; Yang and Poovaiah, 2002). CaM is a ubiquitous intracellular mediator of Ca^{2+} signals that have four helix–loop–helix Ca^{2+} motifs referred to as EF-hands. The Ca^{2+}-bound CaM transduces the signals into many cellular processes through modulation of a variety of CaM-binding proteins (Park et al., 2004). The CaM-binding proteins include G-proteins, protein kinases, protein phosphatases, nitric oxide synthase (NOS), receptors, and transcription factors (Liao et al., 1996; Snedden and Fromm, 1998).

Several CaM isoforms have been detected in plant cells. They are encoded by multiple CaM genes. Over 30 genes encoding CaM isoforms are found in the *Arabidopsis* genome (Park et al., 2004). Five CaM isoforms, SCaM1–5, have been identified in soybean (Lee et al., 1999b). The different CaM isoforms may activate different sets of enzymes. For example, SCaM1 activates NAD kinase, whereas SCaM4 activates NOS (Lee et al., 1995, 2000). SCaM1 and SCaM4 also exhibit differences in the Ca^{2+} concentrations required for target enzyme activation (Lee et al., 2000).

Cellular level of CaM protein rapidly rises in response to infection with pathogens. Specific CaM isoforms were activated in soybean by pathogen-derived elicitors. Soybean CaM genes *SCaM4* and *SCaM5*, which encode for divergent CaM isoforms, were induced within 30 min by a fungal elicitor. This elicitor-triggered induction of these genes specifically depended on the increase of intracellular Ca^{2+} level (Heo et al., 1999). Transcription of *SCaM4* is dramatically induced within 30 min of a bacterial pathogen inoculation (Park et al., 2004). Deletion analysis of the promoter of the gene revealed that a 130 bp region located between nucleotide positions −858 and −728 is required for the pathogen to induce expression of SCaM4. GT-1-*cis*-element (GAAAAA) was identified as a core *cis*-acting element for the induction of the *SCaM4* gene. A GT-1-like transcription factor interacts with the GT-*cis*-element. The interaction between a GT-1-*cis*-element and a GT-1-like transcription factor plays a role in pathogen-induced *SCaM4* gene expression in soybean and *Arabidopsis* (Park et al., 2004).

After binding to Ca^{2+}, CaM binds with several proteins. An early ET-responsive gene *NtER1* encodes a CaM-binding protein in tobacco (Yang and Poovaiah, 2000). Six genes related to the tobacco gene encoding CaM-binding protein have been cloned from *Arabidopsis*. All these genes were rapidly induced by signal molecules such as ET, methyl jasmonate (MeJA), abscisic acid (ABA), SA, and H_2O_2, and hence these genes were designated as signal-responsive genes (Yang and Poovaiah, 2002). Ca^{2+}/CaM was found to bind to all these signal-responsive genes, and their CaM-binding regions were located on a conserved

basic amphiphilic α-helical motif in the C terminus. The studies revealed that the signal-responsive gene (six genes) family encoded a family of CaM-binding proteins involved in multiple signal transduction pathways in plants (Yang and Poovaiah, 2002). It suggests that Ca^{2+}/CaM may play an important role in activating several signal transduction pathways. Signaling systems involving H_2O_2, SA, ET, JA, and ABA may function downstream of Ca^{2+}-signaling system.

2.16 PHOSPHORYLATION OF PROTEINS AS A COMPONENT IN SIGNAL TRANSDUCTION SYSTEM

2.16.1 Phosphorylation/Dephosphorylation Events

Posttranslational protein phosphorylation is a general mechanism in the reception/transduction of signals originating from pathogens. There may be continuous phosphorylation/dephosphorylation of proteins in plant cells. Increases in phosphorylation trigger the induction of host plant defense responses. Increases in phosphorylation may occur either by activating phosphorylation or by inhibiting dephosphorylation. Protein kinases activate phosphorylation, whereas protein phosphatases are involved in dephosphorylation of proteins. Activation of protein kinases and inhibition of protein phosphatases may result in increased phosphorylation of proteins. Thus, both plant kinases and protein phosphatases are involved in protein phosphorylation. Although protein kinases may positively activate phosphorylation, inhibition of phosphatases may negatively regulate protein phosphorylation. In tobacco cells, a protein phosphatase could negatively regulate a protein kinase that phosphorylates specific polypeptides involved in cryptogein signal transduction (Bowler and Chua, 1994; Lecourieux-Ouaked et al., 2000). Elicitation of plant defense reactions implicates an increase in the phosphorylation level of polypeptides by activation of protein kinase and inhibition of protein phosphatase activities in tobacco cells (Lecourieux-Ouaked et al., 2000).

Changes in the level of phosphorylation of cellular proteins have been observed upon elicitor treatments of a variety of plant cell cultures (Dietrich et al., 1990; Felix et al., 1991, 1993a,b; Boller, 1995; Chandra and Low, 1995). Enhanced or decreased phosphorylation of different proteins occurred rapidly in spruce cells after elicitor treatment (Salzer et al., 1996). Drastic changes in phosphoprotein labeling have been reported in tomato cells treated with xylanase or chitotetraose (Felix et al., 1991, 1993b). Large differences in the phosphoprotein patterns were observed in tobacco cells after treatment with oligogalacturonide elicitor, reflecting a cascade of phosphorylation/dephosphorylation events from the signal perception to the final output response (Droillard et al., 1997). Cryptogein induced phosphorylation of a number of polypeptides in tobacco cells (Lecourieux-Ouaked et al., 2000).

In many studies, it has been demonstrated that protein kinase inhibitors, such as K-252a and staurosporine, blocked elicitor-induced ion fluxes, ROS production, or activation of defense-related genes, whereas protein phosphatase inhibitors such as calyculin A and okadaic acid stimulated the inducible defenses in the absence of elicitors (Levine et al., 1994; Mathieu et al., 1996; He et al., 1998).

2.16.2 Calcium Ion in Phosphorylation

The phosphorylation of membrane proteins is dependent on Ca^{2+} in many cases. CDPK, Ca^{2+}-/CaM-dependent protein kinase (CaMK), protein kinase C (PKC), or Ca^{2+}-modulated phosphatases play an important role in protein phosphorylation (Lecourieux-Ouaked et al., 2000). CDPKC-type protein kinases have been shown to participate in elicitor-induced phosphorylation in some cases (Subramaniam et al., 1997; Xing et al., 1997). When tobacco cells were treated with cryptogein (elicitor), increased phosphorylation was observed, and out

of about 100 polypeptides, 19 polypeptides were highly phosphorylated (Lecourieux-Ouaked et al., 2000). When tobacco cells were treated with cryptogein and lanthanum (La^{3+}), which are known to block cryptogein-induced calcium influx, the phosphorylation of 12 of the 19 phosphorylated polypeptides (numbers 1, 3, and 6–15) was strongly decreased, suggesting that the process leading to their phosphorylation was Ca^{2+}-dependent. The other polypeptides (numbers 2, 4, 5, and 16–19) showed a level of phosphorylation comparable with that observed in cryptogein-treated cells without La^{3+}, indicating that the phosphorylation of these seven polypeptides occurs upstream or independently of the Ca^{2+} influx (Lecourieux-Ouaked et al., 2000). It also suggests that besides CDPK, other protein kinases may also be involved in elicitor-induced protein phosphorylation. A 40 kDa protein kinase may be positioned downstream of anion channels in the cryptogein-signaling cascade (Wendehenne et al., 2002). The activation of the protein kinase was dependent on NO_3^- efflux induced by the elicitor (Wendehenne et al., 2002). MAPKs have been shown to be involved in plant defense reactions (Jonak et al., 1999).

2.17 MITOGEN-ACTIVATED PROTEIN KINASE CASCADES IN SIGNAL TRANSDUCTION

MAPK cascade forms an important component in the signaling machinery that transduces extracellular signals into a wide range of intracellular responses (Tena et al., 2001; Liu et al., 2003; Menke et al., 2004). Activation of MAPKs by elicitors from different plant pathogens in various plant species has been reported (Zhang and Klessig, 1997, 2001; Lebrun-Garcia et al., 1998; Cardinale et al., 2002; Link et al., 2002).

The MAPK cascade involves three functionally linked protein kinases, such as a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). In response to extracellular signals, MAPKKK activates MAPKK via phosphorylation of serine (S) and serine/threonine (T) residues within the SXXXS/T motif, where X denotes any amino acid. MAPKK, which is a dual-specificity protein kinase, then activates MAPK by phosphorylating specific effector proteins, which leads to activation of cellular responses (Mizoguchi et al., 1993, 1996; Seo et al., 1995; Zhang and Klessig, 1997; Yang et al., 2001; Menke et al., 2004). Several types of MAP kinases have been recognized. SIPK (salicylic acid-induced protein kinase) and WIPK (wounding-induced protein kinase) are activated by fungal elicitors and pathogens (Zhang and Klessig, 1997, 1998a,b; Zhang et al., 1998; Romeis et al., 1999; Kumar and Klessig, 2000). MPK6, which is the ortholog of SIPK, is activated by elicitor treatment (Nuhse et al., 2000; Asai et al., 2002). Two MAP kinases, p48 and p44, were rapidly activated in pea treated with a polysaccharide elicitor (Uppalapati et al., 2004). Two MAP-like kinases (50 and 46 kDa kinases) are activated in tobacco cells treated with cryptogein (Lebrun-Garcia et al., 1998). SIPK and WIPK are activated in Cf9-transgenic tobacco treated with Avr9 elicitor (Romeis et al., 2001).

MAPK cascade regulates defense responses in tobacco (*N. tabacum*), and SIPK and WIPK induced defense gene expression (Yang et al., 2001). Rapid activation of SIPK and transient activation of WIPK by cryptogein elicitor in tobacco have been reported (Zhang and Klessig, 2001). In *N. benthamina*, silencing of MAPKKK, MAPKK, and MAPK components resulted in reduction in a resistance gene (N)-mediated resistance to tobacco mosaic virus (Jin et al., 2002), suggesting the role of MAPKs in triggering defense responses. A bacterial elicitor induced the MAPK cascade consisting of MEKK1, MKK4/MKK5, and MPK3/MPK6 in *Arabidopsis*, and overexpression of these MAPKs conferred resistance against bacterial and fungal pathogens (Asai et al., 2002). The elicitor Pep-13 induced a MAPK in parsley (Ligterink et al., 1997).

The importance of MAPK in triggering defense gene activation has been demonstrated by developing MAPK-silenced plants. *Arabidopsis* MAPK (MPK6)-silenced plants were developed. The wild-type *Arabidopsis* plants responded to ET treatment and showed enhanced

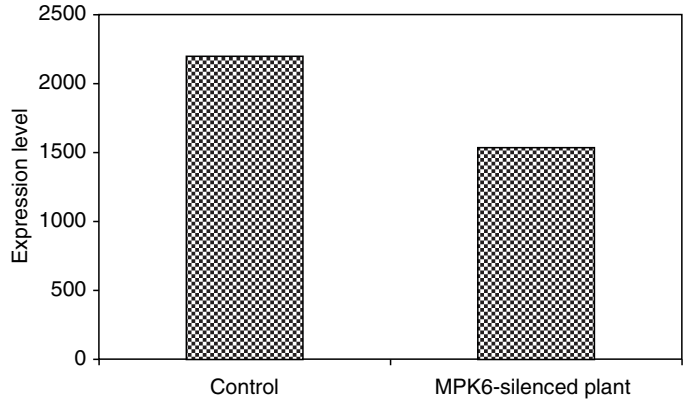

FIGURE 2.7 Role of the MAPK MPK6 in induction of *VSP1* gene in *Arabidopsis*. (Adapted from Menke, F.L.H., van Pelt, J.A., Pieterse, C.M.J., and Klessig, D.F., *Plant Cell*, 16, 897, 2004.)

expression of the pathogen-inducible gene *VSP1* (vegetative storage protein 1), whereas the MAPK-silenced plants showed reduced expression of *VSP1* (Figure 2.7; Menke et al., 2004). *VSP1* gene has been shown to be involved in expression of defense responses (Ellis and Turner, 2001). These observations suggest that the mitogen-activated kinase may play an important role in signaling the defense gene expression.

Some MAP kinases may negatively regulate the defense response. The inactivation of the *MPK4* gene encoding the mitogen-activated kinase MPK4 conferred enhanced resistance and constitutive expression of defense responses in *Arabidopsis* (Petersen et al., 2000).

Both NO and SA activated SIPK (Kumar and Klessig, 2000). Additional analyses with transgenic NahG tobacco revealed that SA is required for the NO-mediated induction of SIPK. Neither JA nor ET activated SIPK (Kumar and Klessig, 2000). Thus, SIPK may function downstream of SA in the NO-signaling pathway for defense responses. NO and SA did not activate WIPK. JA and ET also did not activate WIPK. The signals involved in resistance-associated WIPK activation are not known (Kumar and Klessig, 2000).

The *edr1* (enhanced disease resistance 1) mutation of *Arabidopsis* confers resistance to powdery mildew disease caused by *Erysiphe cichoracearum* (Frye et al., 2001). The *EDR1* gene codes for a MAPKKK. All *edr1*-associated phenotypes are suppressed by mutations that block SA perception (*nim1*) or reduce SA production (*pad4* and *eds1*). The *NahG* transgene, which lowers endogenous SA levels, also suppressed *edr1* (Frye et al., 2001). These results suggest that the MAPKKK may function at the top of a MAPK cascade that negatively regulates SA-inducible defense responses. The *ein2* (ethylene-insensitive 2) mutation did not suppress *edr1*-mediated resistance, indicating that ET and JA-induced responses are not required for the expression of MAPKKK-signaling pathway (Frye et al., 2001). A MAPK mediating ET signaling has also been reported (Ouaked et al., 2003). The *Arabidopsis* gene *CTR1* (constitutive triple-response 1) encodes a protein that belongs to the Raf family of serine/threonine protein kinases that initiate MAPK-signaling cascades. ET signaling operates through this MAPK cascade (Wang et al., 2002; Ouaked et al., 2003).

2.18 PHOSPHOLIPID-SIGNALING SYSTEM

2.18.1 Plant Cell Membrane Phospholipids as Signal Molecules

Several phospholipids commonly found in plant cell membranes play important roles in signal transduction. Phospholipids may contain different fatty acid side chains. PC and

phosphotidylinositol are the major groups of membrane lipids. The inositol headgroup can be reversibly phosphorylated at various positions by the combined action of various kinases and phosphatases, producing different phosphoinositides. Three main forms of phosphoinositides are phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-(4,5)-bisphosphate (PIP_2). Phosphorylation of inositol lipids by phosphoinositide 3-kinase results in the production of PIP and PIP_2 from PI. Activities of PI kinase and PIP kinase were elevated *in vitro* by a fungal elicitor treatment (Toyoda et al., 1992).

Several membrane-associated phospholipases are involved in biosynthesis of various other phospholipids. The important phospholipases detected in plant cell membranes include phospholipase A1 (PLA1), PLA2, PLC, and phospholipase D (PLD). PLA1 and PLA2 catalyze the hydrolysis of the fatty acyl group in glycerol, producing AA and lysophospholipids. The PLA2 activity generates lysophosphatidylcholine (LPC) in California poppy (*Eschscholtzia californica*) cells (Viehweger et al., 2002). PLC hydrolyzes phosphorylated forms of PI (PIP and PIP_2) and produces diacylglycerol (DAG) and IP_3. DAG is rapidly phosphorylated to phosphatidic acid (PA) by DAG kinase (Munnik et al., 1998b; Shigaki and Bhattacharyya, 2000; van der Luit et al., 2000). The rapid synthesis of PA is followed by the formation of diacylglycerol pyrophosphate (DGPP) (van der Luit, 2000). PLD hydrolyzes PC, producing PA and choline. Another phospholipid detected in plants is sphingomyelin (ceramide phosphorylcholine). Sphingomyelinase is an important phospholipid-degrading enzyme and it generates ceramide and sphingosine from sphingomyelin (Lynch and Dunn, 2004).

Various phospholipids generated by the action of various phospholipases have been reported to be involved in signal transduction system (Chapman, 1998; Munnik et al., 1998a; Munnik, 2001; Wang, 2001). IP_3 stimulates calcium efflux (Mithöfer et al., 1999). DAG activates protein phosphorylation and stimulates synthesis of IP_3, which releases Ca^{2+} from internal stores (Berridge and Irvine, 1989). PA triggers production of superoxide anion (O_2^-) (Sang et al., 2001). LPC stimulates the plasma membrane proton pump and causes a transient increase of the vacuolar pH in plant cells (Viehweger et al., 2002). Ceramide activates MAPKs. Ceramide synthesis prevents the action of AAL toxin produced by the tomato pathogen *Alternaria alternata* f. sp. *lycopersici* and triggers HR in tomato (Spassieva et al., 2002; Spassieva and Hille, 2003). Exogenous application of ceramide prevents the action of AAL toxin on tomato leaves and triggers defense reactions (Brandwagt et al., 2000). Sphingosine stimulates the hydrolysis of PI and the production of IP_3 and Ca^{2+}. Sphingosine-1-phosphate has second messenger activity and is involved in the inositol-independent release of Ca^{2+} from intercellular stores (Ng and Hetherington, 2001). It stimulates anion efflux (Coursol et al., 2003). Sphingosine-1-phosphate acts both as a ligand for certain G-protein-coupled receptors (GPCRs) and as an intracellular second messenger (Lynch and Dunn, 2004). Interaction between sphingosine-1-phosphate and the G-protein subunit in signaling has been reported in rice (Suharsono et al., 2002).

2.18.2 Role of Phospholipase A in Phospholipid-Signaling System

The involvement of phospholipase A (PLA) in elicitor-triggered signal cascades has been reported in cultured cells of soybean (Chandra et al., 1996), tobacco (Roy et al., 1995), and potato (Senda et al., 1998) and tomato leaves (Narvaez-Vasquez et al., 1999). Activation of PLA2 in tomato leaves exposed to pathogens has been demonstrated (Lee et al., 1997). The elicitor treatment triggered fluorescence development in California poppy (*Eschscholtzia californica*) cells and this elicitor-induced fluorescence was prevented by an inhibitor of PLA2, 5,8,11,14-eicosatetrynoic acid (ETYA) (Figure 2.8; Viehweger et al., 2002). It suggests that the elicitor-induced fluorescence is due to the activity of PLA2.

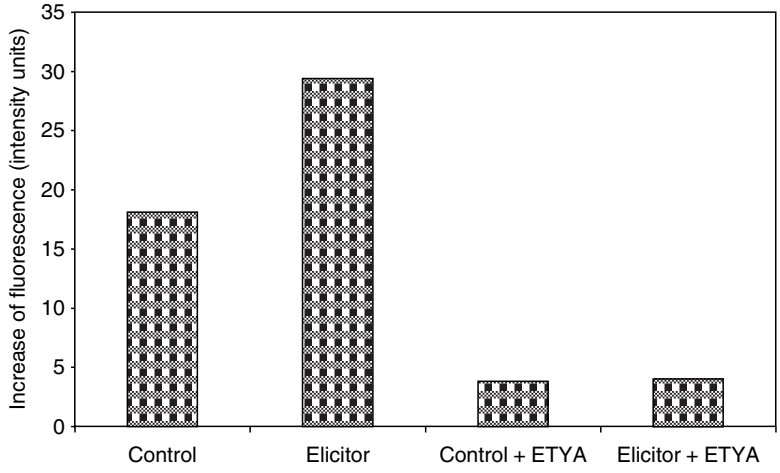

FIGURE 2.8 Induction of phospholipase A2 by fungal elicitor. (Adapted from Viehweger, K., Dordschbal, B., and Roos, W., *Plant Cell*, 14, 1509, 2002.)

In California poppy cells, the stimulation of PLA2 activity by the elicitor was correlated with the elicitation of phytoalexin synthesis (Roos et al., 1999). The PLA2 activity generates LPC in California poppy cells (Viehweger et al., 2002). LPC that is overproduced transiently by elicitor-induced PLA2 stimulates the plasma membrane proton pump (Viehweger et al., 2002). It causes a transient increase of the vacuolar pH by increasing the Na^+ sensitivity of Na^+-dependent proton efflux (Viehweger et al., 2002).

An increase of PLA2 activity has been correlated with the perception of elicitors in several plant–pathogen systems (Roy et al., 1995; Senda et al., 1998; Narvaez-Vasquez et al., 1999) and with early downstream effects such as the production of active oxygen species (Chandra et al., 1996) and the increase of free fatty acids (Müller et al., 1993). The fatty acids released by PLA2 are likely to act as second messengers in the transmission of systemin-triggered signals (Lee et al., 1997; Ryan, 2000). They may also serve as starting material for the octadecanoid-signaling system (Blechert et al., 1995; Narvaez-Vasquez et al., 1999; Ryan, 2000).

2.18.3 Phospholipase C in Phospholipid-Signaling System

PLC plays an important role in signal transduction system. The role of PLC in signal transduction in wheat leaves (Arz and Grambow, 1995) and tomato cells (van der Luit et al., 2000) has been demonstrated. PLC appears to be involved in regulating intracellular Ca^{2+} concentrations, which act as second messenger in signal transduction system (Drobak and Ferguson, 1985; Berridge and Irvine, 1989). It catalyzes hydrolysis of the cell membrane phosphoinositides to release DAG and inositol phosphates, predominantly IP_3 (Berridge and Irvine, 1989). IP_3 increases in pea because of fungal elicitor treatment (Toyoda et al., 1993b). Inositol phosphates may be involved in elicitor signal transduction (Renelt et al., 1993). Elicitor treatment increased inositol trisphosphate (produced by the action of PLC), and this increase preceded phytoalexin accumulation in cultured carrot cells. It suggests the involvement of a PI turnover-mediated signal pathway in carrot (Kurosaki et al., 1987). Inositol phospholipid turnover is involved in several signal transduction pathways (Kamada and Muto, 1994). IP_3 has been reported to stimulate calcium efflux (Drobak and Ferguson, 1985; Rincon and Boss, 1987) and activate defense genes. Elicitor treatments activate PLC

and consequently change IP_3 and polyphosphoinositide (PPI) levels in pea epicotyl tissue (Toyoda et al., 1992, 1993b) and in cell suspensions of tobacco (Kamada and Muto, 1994), soybean (Legendre et al., 1993b), and lucerne (Walton et al., 1993).

Elicitor treatment increased IP_3 in several plants (Mithöfer et al., 1999), and IP_3 has been reported to stimulate calcium efflux (Drobak and Ferguson, 1985; Rincon and Boss, 1987; Berridge and Irvine, 1989; Mithöfer et al., 1999). DAG activates protein phosphorylation and functions as a second messenger. DAG is rapidly phosphorylated to PA by DAG kinase (Munnik et al., 1998b; van der Luit et al., 2000).

PA is a second messenger. PA activates production of O_2^- in plant cells (Sang et al., 2001). PA is the product of two signaling pathways, those of PLC and PLD (Munnik, 2001). In the PLC pathway, inositol lipids are phosphorylated by phosphoinositide 3-kinase (Chapman, 1998; Munnik et al., 1998a). PLC hydrolyzes phosphorylated forms of PI such as PIP and PIP_2 producing DAG and IP_3. DAG activates production of PA by phosphorylation (Figure 2.9; Munnik et al., 1998b; van der Luit et al., 2000).

PA is also produced by the action of PLD. It is produced by the cleavage of PC by PLD1. PLD1 activation is induced by agonist binding to GPCRs or receptor tyrosine kinases (RTKs) and is mediated by PKC as well as by the small GTP-binding proteins Rho (RhoA, Rac1, and Cdc42) and ADP-ribosylation factor 1 (ARF1). PLD hydrolyzes phospholipids into free head groups and PA. Both free head group and PA serve as cellular signal messengers (Sang et al., 2001).

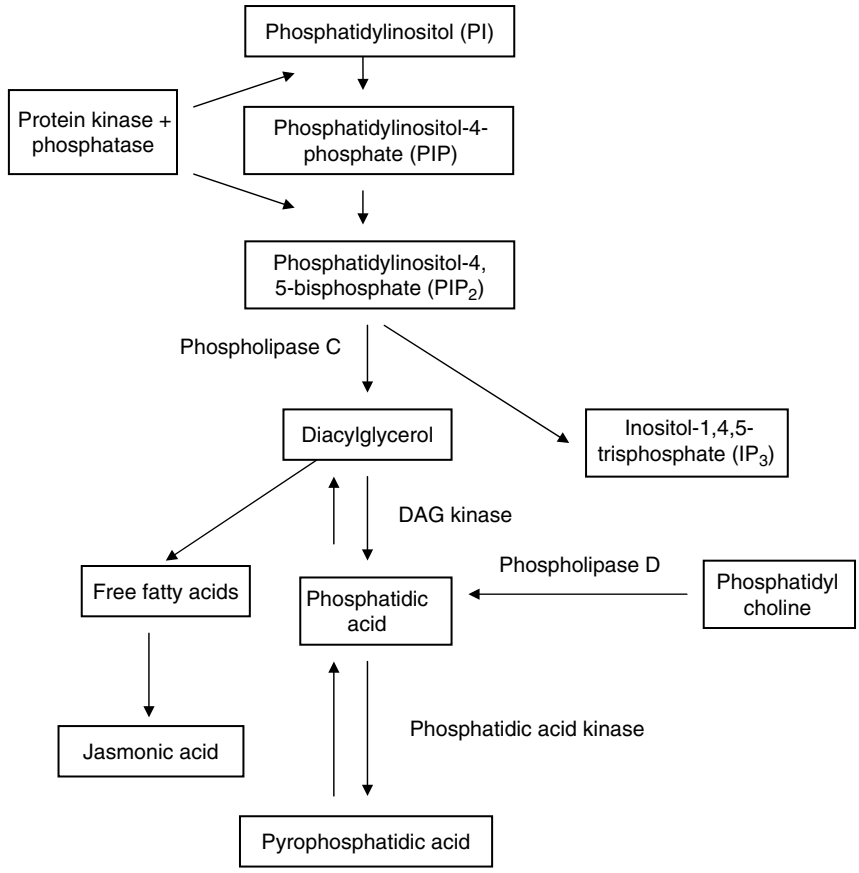

FIGURE 2.9 Pathway of biosynthesis of phospholipids.

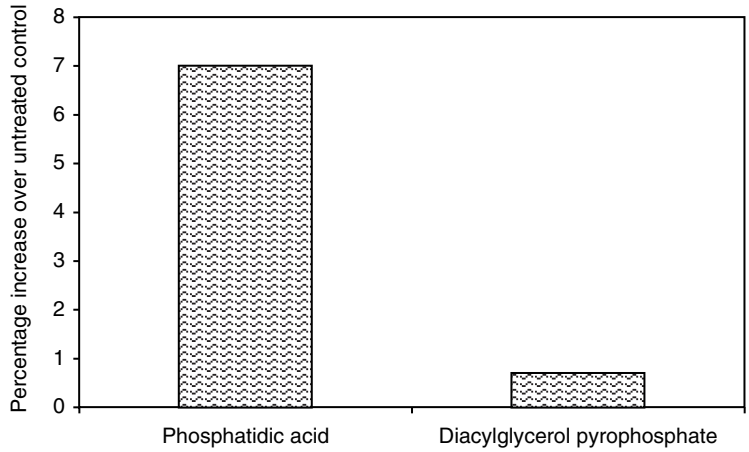

FIGURE 2.10 Increases in lipid levels in tomato cells treated with xylanase elicitor. (Adapted from van der Luit, A.H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T., and Munnik, T., *Plant Physiol.*, 123, 1507, 2000.)

When tomato cells were treated with xylanase elicitor, the level of PA increased (Figure 2.10; van der Luit et al., 2000) and the increase was observed even at 2 min after treatment. The elicitor treatment also increased the level of DGPP (Figure 2.10; van der Luit et al., 2000). The rapid synthesis of PA and the subsequent formation of DGPP are signaling events that take place when tomato cells are treated with elicitors (van der Luit et al., 2000).

2.18.4 Phospholipase D in Phospholipid-Signaling System

PLD regulates cellular processes through the production of lipid and lipid-derived messengers (Wang, 1999; Sang et al., 2001). The important messengers generated by PLD include PA, DAG, free polyunsaturated fatty acids, PIP_2, and JA (Wang et al., 2000). PLD has been proposed to participate in cellular events that lead to ABA responses (Jacob et al., 1999) and the production of ET (Lee et al., 1998). Cellular activity of PLD is regulated by several messengers such as Ca^{2+} (Zheng et al., 2000), PPIs (Qin et al., 1997), G-proteins (Munnik et al., 1995; Ritchie and Gilroy, 2000), H_2O_2 (Yamaguchi et al., 2004), pH changes (Pappan and Wang, 1999), and membrane perturbation (Pappan et al., 1998).

Increased PLD may trigger the hydrolysis of structural phospholipids, resulting in the production of PA (Munnik et al., 1998a,b) and a free head group, such as choline, which serve as second messengers (Dennis et al., 1991). PA enhances production of O_2^- (Sang et al., 2001). PA also can be further metabolized by PA phosphorylase to form DAG. DAG activates PKC. PLD is a critical component in signal transduction (Divecha and Irvine, 1995). PLD, which is often the most abundant phospholipase in plants, plays a role in plant-signaling system (Munnik et al., 1995). Multiple forms of PLD have been identified in plants (Pappan and Wang, 1999). Depletion of PLDα (the most prevalent isoform of PLD in plants) in *Arabidopsis* decreased the levels of PA and superoxide production in *Arabidopsis* (Sang et al., 2001). PLD plays a role in mediating superoxide production in plants through the generation of PA as a lipid messenger.

The role of PLD in tobacco cells in signal transduction has been demonstrated (Laxalt et al., 2001; Laxalt and Munnik, 2002). Fungal elicitors increase PLD activity. Chitotetraose,

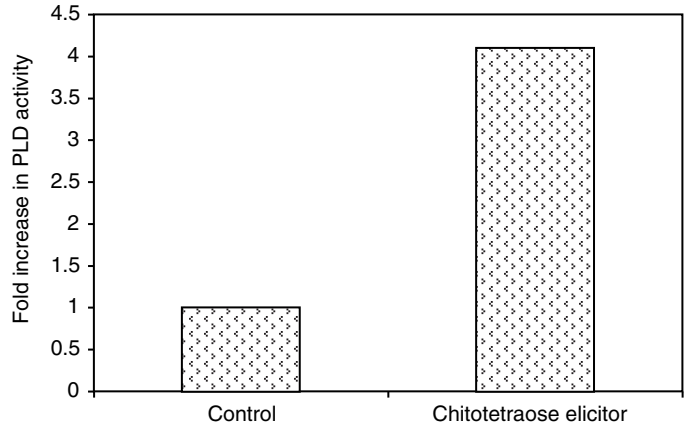

FIGURE 2.11 Increases in phospholipase D (PLD) acivity in tomato cells treated with chitotetraose elicitor. (Adapted from van der Luit, A.H., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T., and Munnik, T., *Plant Physiol*., 123, 1507, 2000.)

a chitin elicitor, treatment increased PLD activity in tomato cells (Figure 2.11; van der Luit et al., 2000).

N-acetylchitooligosaccharide elicitor treatment activated both PLC and PLD in rice cells (Yamaguchi et al., 2005). The activation of both enzymes was observed for the first phase of ROS generation, but only the activation of PLD was evident for the second response. Enzymatic products of these phospholipases, DAG, and PA, could induce ROS generation by themselves. DAG and PA also induced the expression of elicitor-responsive genes in the absence of the elicitor. They could not induce phytoalexin synthesis by themselves but greatly enhanced the elicitor-induced phytoalexin accumulation. The 1-butanol treatment, which inhibited PLD, inhibited the elicitor-induced phytoalexin accumulation in rice cells. It suggests the importance of phospholipid signaling, especially PLD and its product PA in plant defense responses (Yamaguchi et al., 2005).

2.19 ANION CHANNELS IN SIGNAL TRANSDUCTION

2.19.1 Anion Channels in the Signaling System

The plasma membrane anion channels may be essential components of early signal transduction processes in plants. The elicitors activate the anion channels. These channels, which because of the outward-directed anion gradients across the plasma membrane, drive passive effluxes from the cytoplasm into the extracellular space (Wendehenne et al., 2002). Anion channels may mediate Cl^- and NO_3^- efflux, and Cl^- and NO_3^- permeability seems to be a general feature of plant plasma membrane anion channels (Barbier-Brygoo et al., 1999, 2000). Cl^- efflux is one of the earliest events in elicitor-treated tobacco and parsley cells (Nürnberger et al., 1994; Pugin et al., 1997; Zimmermann et al., 1998). Anion channel antagonists have been shown to suppress the early and late elicitor- or pathogen-induced responses (Ebel et al., 1995; Jabs et al., 1997; Zimmermann et al., 1998). The anion channel blockers, such as niflumic acid, glibenclamide, and ethacrynic acid, reduced and delayed the hypersensitive cell death and the induction of several defense-related genes in tobacco plants (Wendehenne et al., 2002). These results suggest that anion channels are involved in signal transduction inducing defense responses in plants.

2.19.2 Upstream Events of Anion Channel-Signaling System

Protein phosphorylation followed by Ca^{2+} influx are key upstream steps of the anion-signaling system (Viard et al., 1994; Tavernier et al., 1995; Lecourieux-Ouaked et al., 2000; Binet et al., 2001). Activation of the NO_3^- efflux depends on protein phosphorylation (Wendehenne et al., 2002). A prolonged activation of protein kinases appears to be required to maintain the NO_3^- efflux because the addition of staurosporine (a general inhibitor of protein kinases) in the middle of the cryptogein elicitor response prevented any further NO_3^- efflux (Wendehenne et al., 2002). Phosphatases negatively controlled the anion channel cascade, whereas protein kinases acted as positive regulators in the chain of events leading to anion channel activity (Wendehenne et al., 2002). Ca^{2+} influx from the extracellular space was found to be required for the initiation and maintenance of anion channel in the cryptogein elicitor-treated cells (Wendehenne et al., 2002). Ca^{2+} influx was a prerequisite for the activation of plasma membrane anion channels by elicitors of the plant defense reaction (Jabs et al., 1997). The link between Ca^{2+} influx and anion efflux may involve a complex network of signals, including nucleotides, phosphorylation/dephosphorylation events, cytoplasmic free Ca^{2+}, voltage, and cytoplasmic pH (Wendehenne et al., 2002).

A link between channel-mediated anion efflux and alkalinization of the extracellular medium has been reported (Long and Iino, 2001). Alkalinization may occur if an efflux of anions resulting from channel activation provided substrate for a H^+/anion symporter at the plasma membrane (Wendehenne et al., 2002).

2.19.3 Downstream of Anion Channel-Signaling System

Anion channels are involved in signal transduction inducing defense responses in plants. The anion channels initiate or amplify plasma membrane depolarization, which in turn may activate K^+ channels and Ca^{2+} voltage-dependent channels (Ward et al., 1995). Oxidative burst and induction of a 40 kDa protein kinase are the downstream events of the anion channel-signaling system (Wendehenne et al., 2002). The elicitor-induced NO_3^- efflux triggers hypersensitive cell death in tobacco plants. It also induced several defense-related genes (Wendehenne et al., 2002).

2.20 EXTRACELLULAR ALKALINIZATION AND CYTOPLASMIC ACIDIFICATION IN SIGNALING SYSTEM

Transport of solutes across the plasma membrane is driven by H^+-ATPase that produces an electric potential and pH gradient. Transient shifts of intracellular and apoplastic pH are essential steps in several signal transduction processes of plant cells (Mathieu et al., 1996; Lapous et al., 1998; Roos et al., 1998; Felle et al., 2004). Cytoplasmic acidification induced by biotic or abiotic stress is considered a plant-specific trigger for the synthesis of phytoalexins and other secondary metabolites (Sakano, 2001). In cultured plant cells, microbial elicitors often trigger rapid alkalinization of the apoplast and the outer medium (Felix et al., 1993; Mathieu et al., 1996). Chitin oligomer ($GlcNAc_8$) induced apoplastic alkalinization in barley leaves within 2 h after treatment (Felle et al., 2004). There was a change of up to two pH units above control in the barley leaves after elicitor treatment, and this change was observed in resistant genotypes and not in susceptible genotype (Felle et al., 2004). This type of extracellular alkalinization is combined with intracellular acidification, loss of K^+, and influx of Ca^{2+}. These early events lead to some late events such as synthesis of PR proteins, activation or expression of antimicrobial enzymes, and hypersensitive cell death (Mathieu et al., 1996; Simon-Plas et al., 1997; Lebrun-Garcia et al., 1999).

Analogous to elicitor treatment, artificial acidification of the cytoplasm caused increased mRNA levels of PAL, the first enzyme of phenylpropanoid pathway, and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the first enzyme of isoprenoid pathway in cultured cells of tobacco (Lapous et al., 1998). Acidification of the cytoplasm induced increased levels of mRNA of PAL in rice (He et al., 1998). Acidification of the cytoplasm that was fed by vacuolar protons has been reported in elicitor-treated cells of California poppy (*Eschscholtzia californica*) (Roos et al., 1998). Approximately 2 min after elicitor contact, a transient decrease of the cytoplasmic pH started simultaneously with a stoichiometrically related loss of vacuolar H^+. Artificial acidification of the cytoplasm via permeant acids triggered phytoalexin accumulation in the absence of elicitor (Roos et al., 1998). These results suggest the role of cytoplasmic acidification in signaling system induced by elicitor. After depletion of the vacuolar proton pool by preincubation with the membrane-permeant base methylamine (i.e., in the absence of a pH gradient across the tonoplast) neither cytoplasmic acidification nor phytoalexin biosynthesis could be elicited. Both reactions were restored by withdrawing methylamine and subsequent recovery of vacuolar acidity to a pH of about 6.0. These results suggest that a transient efflux of vacuolar protons is a necessary and sufficient step in the elicitation of phytoalexin synthesis (Roos et al., 1998).

The increase of external pH originates from an influx of protons into the challenged cells. It may be due to the inhibition of the plasma membrane H^+-ATPase via reversible phosphorylation. The activation of serine/threonine protein kinases is required for intracellular acidification in elicited tobacco cells (Mathieu et al., 1991), and reversible changes of the phosphorylation state of the proton pump have been found to occur after exposure of tomato cells to a fungal pathogen (Xing et al., 1996). The defense pathways appear to depend on changes in the proton electrochemical gradient across the plasma membrane (Schaller and Oecking, 1999).

2.21 REACTIVE OXYGEN SPECIES IN SIGNAL TRANSDUCTION

2.21.1 Oxidative Burst

The oxidative burst is the fastest active defense response induced by pathogens in the resistant interactions. A rapid and transient production of ROS, including O_2^-, H_2O_2, and hydroxyl radical ($^{\bullet}OH$) is called oxidative burst (Legendre et al., 1993b; Levine et al., 1994; Tenhaken et al., 1995; Otte et al., 2001; Ortmann et al., 2004). Singlet oxygen ($^1O_2$) is the physiologically energized form of dioxygen and it is also called ROS.

The accumulation of ROS has been recognized as an early event of the plant defense responses (Alvarez et al., 1998). Production of ROS was initiated in tomato cells within 2 min of treatment with elicitors isolated from *Cladosporium fulvum* (Vera-Estrella et al., 1992). Massive production of H_2O_2 was observed within 10 min in tobacco cells treated with cryptogein elicitor produced by *Phytophthora cryptogea* (Figure 2.12; Lecourieux-Ouaked et al., 2000).

H_2O_2 is produced within 5 min of *Verticillium dahliae* elicitor addition in cultured soybean cells (Apostol et al., 1989). The earliest event, which occurred in bean cells treated with *Colletotrichum lindemuthianum* elicitor, was the production of H_2O_2 (Anderson et al., 1991). When suspension-cultured white clover (*Trifolium repens*) cells were treated with an elicitor, H_2O_2 production was observed within 10 min after treatment (Devlin and Gustine, 1992).

In potato leaves inoculated with *Phytophthora infestans*, O_2^- was generated even before penetration of the leaf tissues by the fungus (Chai and Doke, 1983). A superoxide-generating system was activated almost immediately after penetration of the cells of potato tuber tissues by incompatible races of *P. infestans* (Doke, 1983a,b). Injection of elicitor preparation containing the *Avr9* gene product of *Cladosporium fulvum* race 4 into tomato carrying the

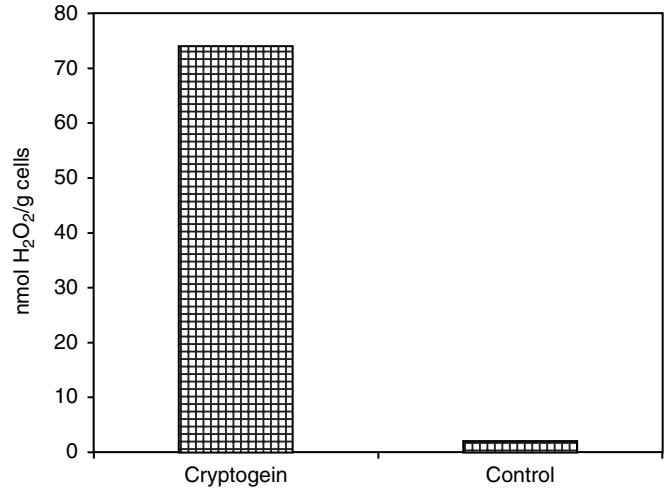

FIGURE 2.12 Induction of H_2O_2 by the elicitor cryptogein. (Adapted from Lecourieux-Ouaked, D., Pugin, A., and Lebrun-Garcia, A., *Mol. Plant Microbe Interact*., 13, 821, 2000.)

Cf9 gene induced production of O_2^- and H_2O_2 (Lu and Higgins, 1998). An elicitor prepared from cell walls of *Phytophthora* sp. induced O_2^- generation and H_2O_2 accumulation in *Rosa damascene* (Arnott and Murphy, 1991; Auh and Murphy, 1995). O_2^- was detected in pear leaves inoculated with *Venturia nashicola* (Faize et al., 2004). Generation of O_2^- in rice leaf protoplasts treated with the *Magnaporthe grisea* proteoglucomannan elicitor has been reported (Haga et al., 1995).

Early production of $^{\bullet}OH$ radical has been reported in *P. infestans*-infected potato leaf tissues (Jordan and DeVay, 1990). Production of $^{\bullet}OH$ in elicitor-treated soybean cotyledons has been reported (Epperlein et al., 1986). $^1O_2$ is generated during bacterial pathogenesis (Keppler et al., 1989; Salzwedel et al., 1989). Production of ROS has been reported in several other plant–pathogen interactions (Keppler and Novacky, 1986; Chai and Doke, 1987; Doke and Ohashi, 1988; Adam et al., 1989; Keppler and Baker, 1989; Moreau and Osman, 1989; Devlin and Gustine, 1992; Lamb and Dixon, 1997; Faize et al., 2004).

2.21.2 Mechanisms of Production of Reactive Oxygen Species

2.21.2.1 Production of O_2^-

The first reaction during the pathogen-induced oxidative burst is the one-electron reduction of molecular oxygen to form O_2^- (Mehdy, 1994; Auh and Murphy, 1995):

$$2O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^+ + \text{H}^-.$$

O_2^- bears an unpaired electron and is routinely generated, in low concentrations, by electron transport system. O_2^- is also produced by the action of a number of enzymes, which participate in oxidation–reduction processes. Enzymes such as xanthine oxidase, aldehyde oxidase, and other flavin dehydrogenases are capable of generating O_2^- as a catalytic product. An NADPH-dependent oxidase catalyzes the single electron reduction of oxygen to form O_2^-, using NADPH as the reductant (Doke, 1985; Sutherland, 1991; Tenhaken et al., 1995; Desikan et al., 1996). The O_2^- production is inhibited by compounds, such as diphenylene iodonium, that inhibit NADPH oxidase (Auh and Murphy, 1995) suggesting the importance of NADPH oxidase in O_2^- production. NADPH oxidase may reside in plasma membrane of

the plant cell (Mehdy, 1994). Cross and Jones (1991) reported that synthesis of O_2^- occurs directly at the extracellular surface of the plasma membrane through a one-electron reduction of molecular oxygen. The trans-plasma membrane redox components transfer electrons from cytosolic NAD(P)H to molecular oxygen.

An NADPH-dependent peroxidase, which is associated with the external surface of the plasma membrane, has also been demonstrated to be involved in generation of O_2^- in plant tissues (Doke, 1985; Vianello and Macri, 1991; Sutherland, 1991; Vera-Estrella et al., 1992). The cell wall peroxidase produces O_2^- from O_2 by a complex pathway involving a cycling of apoplastic NADH, NAD radical, and NAD^+ (Halliwell, 1978). The cell wall peroxidase and the plasma membrane peroxidase may be the same enzyme (Auh and Murphy, 1995). NADH/NADPH is oxidized to NAD/NADP in the presence of peroxidase and Mn^{2+} to reduce molecular oxygen to O_2^- (Halliwell, 1978).

LOX catalyzes the direct oxygenation of polyunsaturated fatty acids (Saniewski, 1979) and LOX activity produces O_2^- (Lynch and Thompson, 1984; Thompson et al., 1987). The oxidative catabolism of purine leads to synthesis of the ureides, allantoin, and allantoic acid, which constitute major organic nitrogen compounds present in a variety of plants (Schubert, 1986). The purine catabolism is catalyzed by xanthine dehydrogenase, leading to uric acid formation (Sandalio et al., 1988). Xanthine oxidase is a reductase supplying electrons to NAD^+ to produce NADH (Halliwell and Gutteridge, 1989) and is also involved in the production of O_2^- (Montalbini, 1992). O_2^- arises as a by-product from many oxidoreductase enzymes when electrons leak from the reaction and reduce molecular oxygen, for example, ferridoxin-$NADP^+$-reductase (Halliwell and Gutteridge, 1989).

2.21.2.2 Production of H_2O_2

H_2O_2 may be produced from the O_2^-, which undergoes spontaneous dismutation to produce H_2O_2 or through the action of superoxide dismutase (SOD) (Auh and Murphy, 1995; Wojtaszek, 1997). The dismutation of two O_2^- produces H_2O_2. SOD activity produces H_2O_2 as a direct consequence of the disproportionation of O_2^- (Apostol et al., 1989):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2.$$

Several enzyme activities can lead to the production of H_2O_2 inside and at the surface of plant cells. Plants may produce H_2O_2 via an NADPH oxidase system (Keller et al., 1998; Sagi and Fluhr, 2001; Simon-Plas et al., 2002). When a NADPH oxidase inhibitor, diphenylene iodinium (DPI), was added to the cultured rice cells before COS elicitor treatment, the oxidative burst induced by the elicitor was inhibited (Ning et al., 2004). DPI treatment prevented the elicitor-induced cell death and it indicates that the elicitor treatment induces oxidative burst via an NADPH oxidase system (Ning et al., 2004). No induction of *Rhoba* gene (rice *gp91* phox subunit gene) encoding an NADPH oxidase responsible for H_2O_2 production was observed in rice suspension cells, suggesting that the COS-stimulated H_2O_2 generation depends on a posttranslational activation of the NADPH oxidase in rice (Ning et al., 2004). Desikan et al. (1996) isolated protein components from *Arabidopsis* extracts that share immunological properties with the mammalian NADPH oxidase complex. Keller et al. (1998) isolated an *Arabidopsis rbohA* (respiratory burst oxidase homolog A) gene that has pronounced similarity to one of the subunits of the neutrophil respiratory burst NADPH oxidase. This gene was shown to be involved in the oxidative burst in *Arabidopsis*. In tobacco, NtrbohD (*N. tabacum* respiratory burst oxidase homolog D) has been shown to be involved in ROS production (Morel et al., 2004). The gene *Ntrac5* was found to negatively regulate NtrbohD. The *Ntrac5* mRNA is repressed when tobacco leaves and cells were treated with the fungal elicitor cryptogein (Morel et al., 2004).

A membrane-associated NADH-dependent redox enzyme might be involved in the reactions, which generate H_2O_2 (Apostol et al., 1989; Desikan et al., 1996; Keller et al., 1998). An NADPH-dependent oxidase in conjunction with SOD may be involved in the H_2O_2 production (Milosevic and Slusarenko, 1996). H_2O_2 can also be produced by peroxidases (Wojtaszek, 1997). The H_2O_2 may result from NAD(P)H oxidation by peroxidase (Mader and Amberg-Fisher, 1982). Peroxidase activity may contribute to the production of H_2O_2 and other ROS (Vianello and Macri, 1991; Vera-Estrella et al., 1992; Mehdy, 1994; Bestwick et al., 1995). Xanthine oxidase activity produces both O_2^- and H_2O_2 (Montalbini, 1992). Oxalate oxidase is also a H_2O_2-generating enzyme (Zhou et al., 1998a). Increased activity of this enzyme has been reported in barley and wheat leaves following inoculation with the powdery mildew fungus *Blumeria graminis* (Dumas et al., 1995; Hurkman and Tanaka, 1996). A germin/oxalate oxidase system is also able to produce H_2O_2 in response to pathogen challenge (Wojtaszek, 1997). Some enzymes, such as urate oxidase and glycollate oxidase, can produce H_2O_2 without going via O_2^- (Halliwell and Gutteridge, 1989).

SA may increase the accumulation of H_2O_2 in plant cells (Delaney et al., 1994). SA inhibits catalase, which can remove H_2O_2 (Milosevic and Slusarenko, 1996). Catalases are present as multiple isoforms in plants, and isolation of cDNA clones from several plant species has shown that catalases exist as small gene families (Scandalios, 1994). Expression analysis of catalases in plants showed that each of the genes is associated with a specific H_2O_2-producing process (Willekens et al., 1994). The COS elicitor treatment reduced the transcript level of catalase gene (Ning et al., 2004), suggesting that catalase could be a negative regulator of the plant defense responses. Inhibition of catalase may lead to accumulation of H_2O_2 (Chen et al., 1993a; Takahashi et al., 1997). Xanthine oxidase and peroxidase also reduce the level of catalase and hence increase the production of H_2O_2 (Milosevic and Slusarenko, 1996).

2.21.2.3 Production of $^{\bullet}OH$ Radical

O_2^- reacts with H_2O_2 to produce $^{\bullet}OH$ radical (Sutherland, 1991):

$$O_2^- + H_2O_2 + H^+ \rightarrow O_2 + H_2O + {}^{\bullet}OH.$$

O_2^- can also act as a reducing agent for transition metals such as Fe^{3+} and Cu^{2+}. These metals may be reduced even if they are complexed with proteins or low molecular weight chelators. The consequence of metal reduction is that it can lead to the H_2O_2-dependent formation of hydroxyl radicals in the Fenton reaction (Mehdy, 1994):

$$O_2^- + Fe^{3+}(Cu^{2+}) \rightarrow Fe^{2+}(Cu^{2+}) + O_2,$$

$$Fe^{2+}(Cu^{+}) + H_2O_2 \rightarrow Fe^{3+}(Cu^{2+}) + OH^- + {}^{\bullet}OH.$$

Superoxide and H_2O_2 can react in a Haber–Weiss reaction to generate the hydroxyl radical (Scandalios, 1993):

$$H_2O_2 + O_2^- \rightarrow {}^{\bullet}OH + OH^- + O_2.$$

2.21.2.4 Production of Singlet Oxygen ($^1O_2$)

$^1O_2$ is an excited state of molecular oxygen that can be generated in a number of ways including the spontaneous dismutation of two O_2^- radicals (Elstner, 1982; Knox and Dodge, 1985). $^1O_2$ production requires energy, and an activation energy of 22 kcal/mol is required to raise molecular O_2 from its ground state to its first singlet state (Scandalios, 1993).

2.21.3 Upstream of ROS Signaling

The oxidative burst reaction begins with the recognition of the elicitor molecule by a corresponding receptor molecule that lies on the plasma membrane. The receptors have been only partially characterized (Wojtaszek, 1997). Components of the signaling pathway downstream of the receptor may include heterotrimeric GTP-binding or G-proteins (Legendre et al., 1993b). Agents known to interact with heterotrimeric G-proteins were shown to promote generation of ROS in soybean cell cultures either in the presence or the absence of elicitor (Legendre et al., 1992). ROS generation in several plant species appears to depend on increased intracellular Ca^{2+} level. A Ca^{2+} channel blocker, La^{3+}, inhibited elicitor-induced ROS production in tobacco (Baker et al., 1993). A calcium ionophore, A23187, induced active oxygen formation in spruce cell suspensions (Schwake and Hager, 1992). The results suggest that Ca^{2+} signaling may act upstream of the ROS signaling. It has been reported that ROS signal transduction activates Ca^{2+} channels (Mori and Schroeder, 2004). ROS induce cytosolic Ca^{2+} increases in plant cells (Kawano and Muto, 2000; Pier et al., 2000).

Phosphorylation activated by protein kinases and phosphatases may trigger the ROS signaling. The protein kinase inhibitors, staurosporine and K-252a, inhibited elicitor-induced increases in active oxygen species in tobacco (Baker et al., 1993) and spruce cell cultures (Schwake and Hager, 1992). Cyclic AMP-signaling system may also be an upstream event in ROS signaling. Finally, NADPH oxidase is activated, O_2^- produced and dismutated to H_2O_2 (Wojtaszek, 1997).

In the model in which oxidative burst is created by pH-dependent cell wall peroxidases, elicitor is recognized by a receptor molecule, which leads to the activation of ion channels (Wojtaszek, 1997). Ion fluxes in turn cause transient alkalinization of the extracellular matrix in the apoplast, leading to the activation of pH-dependent cell wall peroxidases, forming H_2O_2. The ROS-producing system activated in response to cryptogein is dependent on NADPH oxidase and involved in pH alterations (extracellular medium alkalinization and cytosol acidification) (Pugin et al., 1997). Cryptogein induces NADPH oxidase, which may be responsible for the alkalinization of the extracellular medium (Pugin et al., 1997). NO_3^- efflux activation seems to be essential to induce NADPH oxidase (Wendehenne et al., 2002). Hence, NO_3^- efflux may be involved in ROS production. Anion efflux has been shown to be necessary for the induction of the oxidative burst in elicitor-treated parsley or soybean cells (Ebel et al., 1995; Jabs et al., 1997).

2.21.4 Downstream of ROS Signaling

Besides elevation of Ca^{2+} upstream of ROS production, Ca^{2+} elevations also occur downstream of ROS production (Blume et al., 2000; Bowler and Fluhr, 2000). ROS stimulates a rapid Ca^{2+} influx (Levine et al., 1996). The role of Ca^{2+} influx in downstream signal transduction is known. SA and JA are also induced by ROS, and their role as systemic signals is discussed later in this chapter.

ROS induce various defense responses, including strengthening of plant cell walls by a peroxidase-catalyzed cross-linking of cell wall structural proteins (Brisson et al., 1994; Otte and Barz, 1996) and triggering the transcription of defense-related genes (Levine et al., 1994). O_2^- triggered defense gene activation and phytoalexin synthesis in parsley (Jabs et al., 1997). H_2O_2 induced defense gene activation and cell death in *Arabidopsis* (Desikan et al., 1998). ROS induces HR during the barley–powdery mildew interaction (Thordal-Christensen et al., 1997). Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus were associated with H_2O_2 (Hückelhoven et al., 1999). The role of ROS in inducing resistance has been demonstrated by developing transgenic plants. Transgenic tobacco plants with reduced capability to detoxify reactive oxygen intermediates were hyperresponsive to pathogen infection (Mittler et al., 1999).

2.22 NITRIC OXIDE IN SIGNAL TRANSDUCTION

2.22.1 Increases in Nitric Oxide

NO is a gaseous free radical that diffuses readily through biomembranes (Bethke et al., 2004). It is a well-characterized mammalian-signaling molecule and it also acts as a cellular mediator in plant (Wendehenne et al., 2001; Gould et al., 2003). It is now well established that NO is involved in the plant defense signaling (Delledonne et al., 1998, 2001; Durner et al., 1998; Clarke et al., 2000a; Foissner et al., 2000; Klessig et al., 2000; Polverari et al., 2003; Xu et al., 2004). NO content dramatically increased in wheat at 24 h after inoculation with *Puccinia striiformis* race CY22-2 (incompatible interaction) (Xu et al., 2004). An elicitor of *Phytophthora cryptogea*, cryptogein triggers a NO burst within minutes in epidermal sections from tobacco leaves (*N. tabacum* cv. Xanthi) (Foissner et al., 2000). NO production was observed in tobacco cells within 5 min after treatment with the cryptogein elicitor, and maximum increase was observed within 30 min (Figure 2.13; Lamotte et al., 2004).

2.22.2 Biosynthesis of Nitric Oxide

In animal systems, NO is synthesized predominantly by the enzyme NO synthase (NOS). NOS converts L-arginine into L-citrulline in a NADPH-dependent reaction (Figure 2.14). In this process, one molecule of NO is released for each molecule of L-arginine (Bethke et al., 2004). Similar NO synthesis by a NOS-type enzyme also occurs in plants (Durner et al., 1998; Foissner et al., 2000). A mammalian-type NOS has been detected in plants (Wendehenne et al., 2001; Zeidler et al., 2004). This pathogen-inducible enzyme activity has been identified as a variant of the P protein of Gly decarboxylase complex (GDC) and named variant P (Chandok et al., 2003). Despite the lack of sequence homology with animal NOS, variant P exhibits a high level of NOS-like activity and displays biochemical features similar to those of its animal counterparts (Chandok et al., 2003). Elicitor-induced NOS has been shown to induce defense genes in *Arabidopsis* (Zeidler et al., 2004). The cryptogein-elicited burst of NO in tobacco cells was reduced by NOS inhibitors, suggesting the occurrence of a NOS-like enzyme. The elicitor-induced NOS-like enzyme corresponded to variant P (Lamotte et al., 2004).

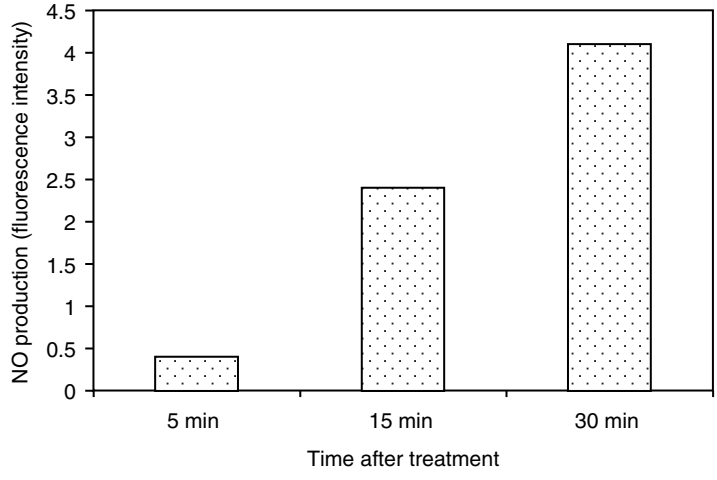

FIGURE 2.13 NO production in tobacco cells treated with cryptogein elicitor. (Adapted from Lamotte, O., Gould, K., Lecourieux, D., Sequeira-Legrand, A., Lebrun-Garcia, A., Durner, J., Pugin, A., and Wendehenne, D., *Plant Physiol.*, 135, 516, 2004.)

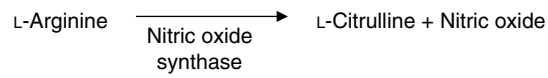

FIGURE 2.14 Role of nitric oxide synthase in nitric oxide synthesis.

Plants also synthesize NO from nitrite. Nitrate reductase has been found to catalyze the NAD(P)H-dependent reduction of nitrite to NO (Yamasaki, 2000; Desikan et al., 2002; Morot-Gaudry-Talarmain et al., 2002). Nitrate reductase reduces nitrate to nitrite and can further reduce nitrite to NO (Bethke et al., 2004). Nitrite-dependent NO production has been observed in soybean (Delledonne et al., 1998) and sunflower (Rockel et al., 2002).

NO may also be synthesized from nitrite in a nonenzymatic manner (Yamasaki, 2000). In this process nitrite is protonated to form nitrous acid (HNO_2) in a freely reversible reaction that is favored under acidic conditions. Two molecules of HNO_2 interact through a series of reactions and give rise to NO and nitrogen dioxide (NO_2), and NO_2 can be converted to NO plus $^1/_2O_2$ as shown in the following equation:

$$2NO_2^- + 2H^+ \leftrightarrow 2HNO_2 \leftrightarrow NO + NO_2 + H_2O \leftrightarrow 2NO + {}^1/_2\,O_2 + H_2O.$$

In this nonenzymatic pathway, NO synthesis may occur in the apoplast rather than in the cytoplasm, because only the apoplast is acidic, which is suitable for the chemical reaction (Yamasaki, 2000).

2.22.3 Upstream Events of Nitric Oxide Signaling

NO synthesis is tightly regulated by a signaling cascade involving Ca^{2+} influx and phosphorylation events. In cryptogein-treated tobacco cell suspensions, NO was produced by variant P and was dependent on upstream protein phosphorylation events and cytosolic free Ca^{2+} elevation (Lamotte et al., 2004). Protein kinase inhibitor staurosporine inhibited cryptogein-induced NO production in tobacco cells (Figure 2.15; Lamotte et al., 2004). Another protein

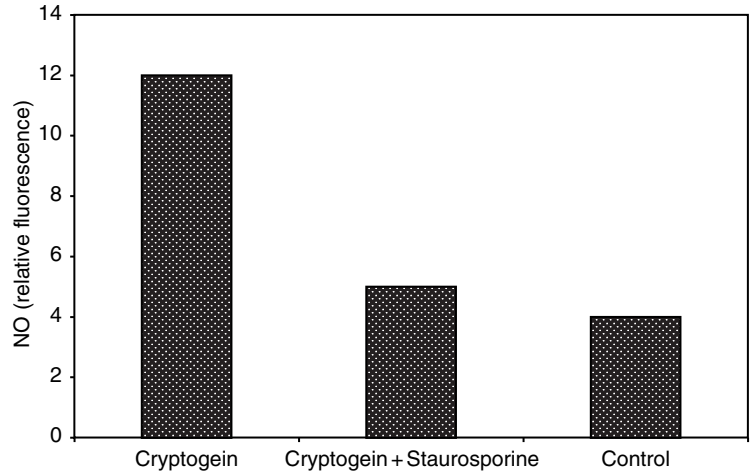

FIGURE 2.15 Effect of the protein kinase inhibitor staurosporine on NO production elicited by cryptogein. (Adapted from Lamotte, O., Gould, K., Lecourieux, D., Sequeira-Legrand, A., Lebrun-Garcia, A., Durner, J., Pugin, A., and Wendehenne, D., *Plant Physiol.*, 135, 516, 2004.)

kinase inhibitor K-252a also completely suppressed the burst of NO mediated by the elicitor. The results suggest that phosphorylation is the upstream event in NO production.

Downstream of protein phosphorylation, the influx of Ca^{2+} constitutes a significant event in NO production. Lanthanum or EGTA are the inhibitors of Ca^{2+} influx in plant cells (Tavernier et al., 1995). Both lanthanum and EGTA prevented cryptogein-elicited NO production in tobacco cell suspension. It suggests that Ca^{2+} influx is required for activation of NO production (Lamotte et al., 2004). NO production in tobacco cells was not triggered by H_2O_2 produced in response to cryptogein treatment, suggesting that the ROS may not be the upstream event in the production of NO (Lamotte et al., 2004).

2.22.4 Downstream Events of Nitric Oxide Signaling

NO acts through a cyclic GMP-dependent pathway (Minorski, 2003). In this pathway, NO posttranslationally activates guanylate cyclase (GC), which leads to a transient increase in the second messenger guanosine-3′,5′-cyclic monophosphate (cGMP) (Klessig et al., 2000). cGMP in turn activates ADP-ribosyl cyclase (ADPRC), through a cGMP-dependent protein kinase. This results in elevated levels of another second messenger, cyclic ADP ribose (cADPR). NO induces defense gene expression via signaling pathways that likely involve cyclic GMP and cADPR (Willmott et al., 1996; Durner et al., 1998; Klessig et al., 2000; Garcia-Mata et al., 2003). NO induced a transient increase in cGMP levels in tobacco (Durner et al., 1998). A membrane-permeable analog of cGMP activated the expression of *PAL* gene encoding PAL, the key enzyme in phenylpropanoid pathway (Klessig et al., 2000). In tobacco, NO induction of *PAL* was suppressed by GC inhibitors, 6-anilino-5,8-quinolinedione (LY83583), and 1*H*-(1,2,4)-oxadiazole[4,3-α]quinoxalin-1-one (ODQ). It suggests that cGMP is involved in NO-induced defense gene expression. cADPR also activated *PR1* and *PAL* expression. cADPR antagonist 8-bromo-cADPR suppressed *PR1* activation in tobacco. It demonstrates the importance of cADPR in the NO-signaling system (Klessig et al., 2000). This activation was suppressed by the Ca^{2+} channel inhibitor ruthenium red, suggesting that Ca^{2+} participates downstream of cADPR in the signal transduction pathway (Klessig et al., 2000).

cADPR releases Ca^{2+} and participates in the increase of free cytosolic Ca^{2+} concentration (Allen et al., 1995). It suggests that NO could mobilize intracellular Ca^{2+} in plants (Xu et al., 1994a). NO participates in the cryptogein-mediated elevation of cytosolic free Ca^{2+} through the mobilization of Ca^{2+} from intracellular stores (Lamotte et al., 2004). NO triggered an increase of $[Ca^{2+}]_{cyt}$ in *Vicia* guard cells by promoting Ca^{2+} release from intracellular stores (Garcia-Mata et al., 2003). Lecourieux et al. (2002) reported that first $[Ca^{2+}]_{cyt}$ increase in elicitor-treated *N. plumbaginifolia* cells results from a Ca^{2+} influx, which in turn leads to Ca^{2+} release from internal stores. In cryptogein-treated cells, the NO scavenger cPTIO, the NOS inhibitor PBITU, and the variant P inhibitor carboxymethoxylamine did not modify Ca^{2+} influx but clearly reduced the amplitude of the first surge of $[Ca^{2+}]_{cyt}$ (Lamotte et al., 2004). The second sustained increase, which was reported to originate mainly from Ca^{2+} influx, was not strongly affected by these inhibitors. Ruthenium red, which is a well-known ryanodine receptor (RYR) antagonist, showed similar inhibition profile. These observations suggest that NO might specifically mobilize $[Ca^{2+}]_{cyt}$ from internal stores through the activation of RYR (Lamotte et al., 2004).

NO inhibits cytosolic and mitochondrial aconitase activities in tobacco (Navarre et al., 2000). NO has been shown to convert the cytosolic aconitase into an mRNA-binding protein known as iron regulatory protein-1 (IRP-1) in mammals (Klessig et al., 2000). IRP-1 binds specific sites, termed iron-responsive elements, on the transcripts of genes involved in iron metabolism and energy metabolism, such as the transferring receptor. IRP-1 regulates free iron concentrations and through this mechanism NO stimulates increased levels of

intracellular free iron. In the presence of ROS, free iron promotes oxidative damage via the Fenton reaction. Thus, NO-mediated increases in iron may contribute to induction of HR in plants (Navarre et al., 2000).

NO was shown to activate a MAPK, SIPK in tobacco (Kumar and Klessig, 2000). This activation appears to be mediated via a SA-dependent pathway because SIPK enzymatic activity was not induced in NOS-treated transgenic tobacco plants expressing the *nahG* gene (Kumar and Klessig, 2000). NO cooperates with ROS in the activity of inducing HR (Delledonne et al., 2001; Polverari et al., 2003). NO accumulation coincides with that of H_2O_2 in soybean suspension cells resisting infection by a bacterial pathogen (Delledonne et al., 1998). NO production precedes the accumulation of H_2O_2 in elicitor-induced NO burst in tobacco (Foissner et al., 2000). NO inhibited the H_2O_2-scavenging enzymes catalase and ascorbate peroxidase activities in tobacco (Clark et al., 2000). It suggests that NO may participate in redox signaling during the activation of defense responses following pathogen attack (Van Camp et al., 1998). The resultant increased H_2O_2 accumulation may trigger the H_2O_2-induced defense mechanisms. NO plays a role in regulating H_2O_2 levels during the resistance response by reversibly inhibiting catalase and ascorbic acid peroxidase.

NO increases SA levels in elicitor-treated cells (Durner et al., 1998). The role of SA in triggering defense genes has been discussed in the later part of this chapter. SA was critical for the NO^-- and cADPR-mediated activation of *PR1* expression. Neither NO nor cADPR could induce induction of *PR1* gene in SA-deficient transgenic tobacco expressing the bacterial *nahG* gene, suggesting the function of NO-signaling system. However, the NO- and cADPR-mediated induction of another defense gene *PAL* was unaffected in these transgenic tobacco plants (Klessig et al., 2000). Thus, NO and cADPR appear to regulate the expression of various defense genes through either SA-dependent or -independent pathway.

NO appears to be involved in the pathway leading to the accumulation of transcripts encoding the ET-forming enzyme and cell death (Lamotte et al., 2004). NO induces several defense genes including many genes encoding PR proteins, glutathione-*S*-transferases and cytochrome P450 in *Arabidopsis thaliana* (Polverari et al., 2003; Zeidler et al., 2004). NO has been shown to induce expression of the *PR1* gene through cGMP, a well-known mediator of NO effects in various mammalian processes (Durner et al., 1998).

2.23 SALICYLIC ACID-SIGNALING SYSTEM

2.23.1 Salicylic Acid in Signaling Defense Response in Plants

SA has been reported as one of the important signal molecules, which act locally in intracellular signal transduction and systemically in intercellular signal transduction (Raskin, 1992). SA accumulates in plants inoculated with pathogens (Meuwly et al., 1995; Shah et al., 1997). SA levels increase both in tissue proximal and distal to the infection (Malamy et al., 1990; Rasmussen et al., 1991). The increased levels of SA resulted in induction of various defense-related genes (Dorey et al., 1997). The importance of SA-signaling system in induction of host defenses was studied by developing transgenic plants expressing the bacterial gene *NahG*. This gene encodes the enzyme salicylate hydroxylase, which inactivates SA by converting it to catechol. Some of the *NahG* transgenic plants are unable to accumulate SA and are incapable of developing HR, indicating that SA accumulation is required for HR to occur (Delaney et al., 1994).

Mutants or transgenic plants with impaired SA signaling show increased susceptibility to pathogens (Cao et al., 1998; Jirage et al., 1999). Mutants with high constitutive levels of SA show enhanced resistance (Bowling et al., 1994; Cao et al., 1998; Clarke et al., 2000b). The *Arabidopsis* mutants, such as accelerated cell death mutant *acd2* (Mach et al., 2001), lesion-simulating disease mutants *lsd1*, *lsd2*, and *lsd7* (Dietrich et al., 1997), and constitutive

expression of PR-1 mutant *cep* (Silva et al., 1999), possessed much higher levels of SA and also showed increased disease resistance to virulent pathogens. Benzothiadiazole (BTH) induces SA synthesis in plants and BTH treatment rendered wild-type plants more resistant than control plants (Audenaert et al., 2002). Zimmerli et al. (2001) showed that *NahG Arabidopsis* plants were more susceptible to *Botrytis cinerea* than wild-type *Arabidopsis* Columbia-O plants and that a soil drench application of BTH drastically slowed down the *B. cinerea* infection on *Arabidopsis*.

Disease resistance is also induced in plants by spray treatments with SA (Murphy et al., 2000; Navarre and Mayo, 2004) or more potent synthetic mimics such as isonicotinic acid (INA) (Durner et al., 1997). These results suggest that SA accumulation contributes for disease resistance. Systemic acquired resistance (SAR) in plants has been widely reported and SA has been implicated as a signal in triggering SAR (Vidhyasekaran, 2004). SA-mediated signaling may operate differentially in different plants. It has been well characterized in the *Arabidopsis*, tobacco, and cucumber systems, but less is known in plants with high basal levels of SA such as potato and rice (Raskin et al., 1990; Yalpani et al., 1991; Coquoz et al., 1995; Dempsey et al., 1999; Vleeshouwers et al., 2000). High basal SA levels may make cells less competent to perceive or transduce the SA signal (Yu et al., 1997). SA-signaling pathway may mediate the resistance to biotrophic pathogens, such as *Erysiphe orontii* and *Peronospora parasitica* in *Arabidopsis* (Thomma et al., 2001b; Rojo et al., 2003).

2.23.2 Biosynthesis of Salicylic Acid

SA is a phenolic compound commonly present in the plant kingdom. It was found in all the 34 plant species tested for its content (Raskin et al., 1990). Plants synthesize SA (*O*-hydroxybenzoic acid) by the action of PAL. Accumulation of SA in cucumber during SAR is preceded by a transient increase in PAL in stem and petioles (Smith-Becker et al., 1998). PAL is a key regulator of the phenylpropanoid pathway, which yields a variety of phenolics with structural and defense-related functions and is involved in the synthesis of *trans*-cinnamic acid from phenylalanine (Yalpani and Raskin, 1993). Two pathways for the formation of SA from phenylalanine by the action of PAL have been suggested (Figure 2.16). In the first pathway,

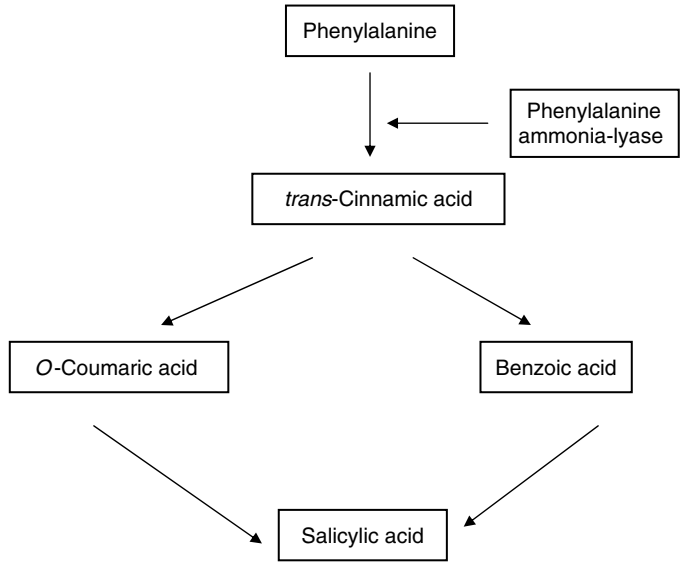

FIGURE 2.16 Biosynthetic pathway of salicylic acid.

trans-cinnamic acid (the product of PAL action) is converted first to 2-hydroxy-cinnamic acid and then through oxidation to SA. In the alternative pathway, *trans*-cinnamic acid is β-oxidized to benzoic acid and then ortho-hydroxylated to SA (Ward et al., 1991).

Radioactive SA was formed via *O*-coumaric acid by leaf segments of *Primula acaulis* and *Gaultheria procumbens* after they were fed ^{14}C-labeled phenylalanine or cinnamic acid (El-Basyouni et al., 1964). In the same species, labeled SA was also formed after treatment with (^{14}C) benzoic acid (El-Basyouni et al., 1964). Similarly, in tomato also SA has been reported to be formed through both the pathways (Chadha and Brown, 1974). However, benzoic acid alone serves as a precursor for the formation of SA in potato, pea, and sunflower (*Helianthus annuus*) (Klambt, 1962). In tobacco, cucumber, and rice, SA is formed from cinnamic acid via benzoic acid (Leon et al., 1993; Yalpani and Raskin, 1993; Meuwly et al., 1995; Silverman et al., 1995). H_2O_2 and other ROS induce benzoic acid 2-hydroxylase activity, which catalyzes the synthesis of SA from benzoic acid (Leon et al., 1995).

Induction of SA has been shown to require Ca^{2+}. A specific calcium chelator (EGTA) and a calcium blocker (verapamil) inhibited the production of SA (Schneider-Muller et al., 1994). The results suggest that SA may play a key role in transferring intracellular signal transmitted by calcium ion. SA interacts with Fe^{2+} in heme and nonheme containing proteins, either through chelation or as an electron donor to generate a highly reactive salicylate radical (Ghosh and Kopp, 1995; Durner et al., 1997). SA accumulates both locally and systemically. However, it is not known whether SA moves symplastically via plasmodesmata. It is still debated whether SA is a phloem mobile signal (Cameron, 2000).

2.23.3 Signal Perception

Several proteins, including catalase and ascorbate oxidase through which the SA signal acts, have been identified (Du and Klessig, 1997). A soluble binding site for SA that appears as an aggregate of approximately 650 kDa has been reported in tobacco (Chen and Klessig, 1991; Chen et al., 1993b). The SA-binding protein was originally termed SABP (SA-binding protein) and it was identified as catalase (Chen et al., 1993b; Conrath et al., 1995). Another soluble SABP2 in tobacco has been described. It has an apparent molecular weight of approximately 25,000. It reversibly binds SA with an apparent dissociation constant of 90 nm, an affinity that is 150-fold higher than that between SA and catalase (Du and Klessig, 1997). Sequence analysis predicted that SABP2 is a lipase belonging to the α/β-fold hydrolase superfamily (Kumar and Klessig, 2003). The lipase activity of SABP2 was stimulated by SA binding and may generate a lipid-derived signal (Kumar and Klessig, 2003). SA binds with SABP2 and induces *PR* genes (Gorlach et al., 1996; Du and Klessig, 1997). Silencing of *SABP2* gene expression suppressed induction of *PR1* gene expression by SA (Kumar and Klessig, 2003).

2.23.4 Upstream Signals for Induction of Synthesis of Salicylic Acid

It is well established that infection by different pathogens leads to synthesis of SA. H_2O_2 appears to be important for SA accumulation. High levels of H_2O_2 stimulate SA biosynthesis (Leon et al., 1995; Neuenschwander et al., 1995; Summermatter et al., 1995). When catalase expression leading to accumulation of H_2O_2 was suppressed in leaves of transgenic tobacco plants through sense cosuppression or antisense suppression, most plants failed to show constitutive *PR* gene expression (Chamnongpol et al., 1996; Takahashi et al., 1997). H_2O_2 and H_2O_2-inducing chemicals were unable to induce *PR* gene expression in transgenic plants expressing NahG (salicylate hydroxylase from *Pseudomonas putida* which degrades SA) although they could activate *PR-1* genes in wild-type tobacco (Bi et al., 1995; Neuenschwander et al., 1995). Although exogenous application of SA can trigger accumulation of ROS,

elicitation by incompatible pathogens trigger elevated ROS before SA becomes elevated (Dangl and Jones, 2001). These results suggest that SA may act downstream of H_2O_2 in inducing *PR* genes.

G-proteins, which modulate phosphorylation, also induce SA accumulation. Transgenic tobacco plants expressing the cholera toxin gene, which modulates signaling system induced by G-proteins, constitutively accumulate high levels of SA (Beffa et al., 1995). Transgenic tobacco plants expressing *Halobacterium* opsin gene with proton pump function show elevated levels of SA (Dangl et al., 1996). These results suggest that G-proteins and ion channels may act upstream of SA-signaling system.

2.23.5 Downstream of Salicylic Acid Signaling

NPR1 (nonexpressor of *PR1*) gene also known as *NIM1* (nonimmunity1) is an important regulator of responses downstream of SA in *Arabidopsis* (Shah et al., 1997; Friedrich et al., 2001; Yu et al., 2001; Mou et al., 2003; Zhang et al., 2003). The *NPR1* gene encodes a novel protein with ankyrin repeats (Cao et al., 1997). The NPR1 protein is stimulated by SA to translocate to the nucleus where it interacts with TGA transcription factors that bind the TGACG motif leading to the expression of various defense-related genes (Kinkema et al., 2000; Fan and Dong, 2002; Mou et al., 2003). Nuclear localization of NPR1 has been shown to be essential for its function in inducing *PR* gene expression (Kinkema et al., 2000). NPR1 interacts with the *Arabidopsis* TGA family members of basic-region leucine zipper (bZIP) transcription factors (Zhang et al., 1999; Després et al., 2000, 2003; Zhou et al., 2000; Fan and Dong, 2002). TGA2 has been demonstrated to be a SA-responsive and NPR1-dependent transcription activator (Fan and Dong, 2002).

Interaction between NPR1 and TGA proteins facilitates binding of the TGA proteins to the SA-responsive LS5 and LS7 elements of the PR1 promoter (Després et al., 2000). The TGA proteins may directly connect NPR1 with *PR* gene induction in the SA signal transduction pathway (Zhang et al., 1999). The initial increase in oxidative state, induced by the release of ROS by the elicitors, is followed by establishment of a reducing state, which is essential for the movement of NPR1 protein into the nucleus (Mou et al., 2003) and the modification of some TGA transcription factors to induce binding to NPR1 (Després et al., 2003). The reducing state may be induced by SA-mediated induction of antioxidant encoding genes including glutathione-*S*-transferase and glucosyltransferase (Uquillas et al., 2004).

SA may enhance release of H_2O_2 and H_2O_2-derived active oxygen species and induce activities of defense-related genes (Shirasu et al., 1997). SA binds with catalase (Conrath et al., 1995) and catalase suppresses H_2O_2 activity. SA suppresses the H_2O_2-degrading activity of catalase, both *in vivo* and *in vitro* (Chen et al., 1993a; Conrath et al., 1995). The other major H_2O_2-scavenging enzyme, ascorbate peroxidase, is also inhibited by SA (Durner and Klessig, 1995). Elevated levels of H_2O_2 resulting from the inhibition of catalase and ascorbate peroxidase might be directly or indirectly involved in the activation of defense responses (Chen et al., 1993a; Conrath et al., 1995; Dempsey and Klessig, 1995; Durner and Klessig, 1995). H_2O_2 and H_2O_2-derived active oxygen species are known to function in the signal transduction pathway (Du and Klessig, 1997).

Another mechanism through which SA-mediated inhibition of catalase and ascorbate peroxidase might activate defenses is via the generation of SA free radicals. SA has been shown to inhibit catalase by serving as a one-electron donating substrate. In this process, SA is converted into a free radical, which could then initiate lipid peroxidation. Lipid peroxides are potent signaling molecules in animals (Durner et al., 1997). SA induces lipid peroxidation in tobacco suspension cells, and exogenously applied lipid peroxides induce *PR-1* genes in these cells (Klessig et al., 2000). It has also been demonstrated that elevated SA stimulates the

lipase activity of the SA-binding protein SABP2 in tobacco and generates a lipid-derived signal (Kumar and Klessig, 2003).

SA-induced defense responses are mediated in part by a MAPK cascade and MAP plays an important role in SA signal transduction (Menke et al., 2004). SA regulates the expression of several *PR* genes encoding antimicrobial proteins, including PR1, PR2, and PR5, which results in an efficient protection against pathogens (Malamy et al., 1990; Metraux et al., 1990; Gaffney et al., 1993; Pervieux et al., 2004). Increased levels of SA induced *PR* genes in various plants. Several *Arabidopsis* mutants like *acd2*, *lsd1*, *lsd2*, *lsd3*, *lsd4*, *lsd5*, *lsd6*, *lsd7*, *cep1*, *cpr1*, and *cim3* constitutively express elevated levels of SA and all of them show constitutively high *PR* gene expression (Dangl et al., 1996; Ryals et al., 1996). The SA-insensitive (*sai1*) *Arabidopsis* mutant plants do not induce *PR* genes even after treatment with SA (Cao et al., 1997). When tobacco plants, which are deficient in catalase, are transformed with tobacco catalase gene, elevated levels of SA were observed in those transgenic plants. These transgenic plants showed constitutively high expression of *PR* genes (Chamnongpol et al., 1996; Takahashi et al., 1997).

Exogenous application of SA also induces *PR* genes in different plants (Uknes et al., 1992). SA induced acidic PR-1a, PR-1b, and PR-1c protein genes in tobacco (Eyal et al., 1992). Treatment of tobacco plants with SA strongly induces accumulation of mRNAs of Class II and Class III β-1,3-glucanases and certain other PR proteins (Ward et al., 1991; Niki et al., 1998). Promoter activity of the Class II PR-2b and Class PR-2d genes is induced in tobacco in response to SA (Eyal et al., 1992; Van de Rhee et al., 1993). *TIMP* genes encoding inhibitors of microbial proteinases (PR-6 proteins) accumulated in tobacco plants treated with SA (Heitz et al., 1999). The SA-dependent pathways activate expression of Class II and Class III β-1,3-glucanase genes in *Arabidopsis* (Silverman et al., 1993; Lawton et al., 1994; Ryals et al., 1996).

Exogenous application of SA normally induces acidic/intercellular PR proteins in many plants. Excised healthy tobacco leaves were fed SA for 72 h through the cut petiole and SA and PR-1 proteins were analyzed in opposite half-leaves (Yalpani et al., 1991). The level of SA in a leaf was proportional to the concentration of SA in the solution in which the petiole was immersed. Induction of acidic PR-1 proteins was positively correlated with leaf SA (Yalpani et al., 1991). SA induced several acidic PR proteins in tobacco (Van de Rhee et al., 1993). Spraying of the leaves of young potato plants with SA induces the appearance of eight intercellular acidic PR proteins including β-1,3-glucanase, chitinase, and PR-5 (Pierpoint et al., 1990).

Many basic PR proteins like Class I PR-2 proteins Ggl50 and Glb are not induced by SA in tobacco (Castresana et al., 1990). Transcripts of tobacco Class I β-1,3-glucanase and chitinases are not induced in response to SA (Linthorst et al., 1990; Ohme-Takagi and Shinshi, 1990; Beffa et al., 1995; Niki et al., 1998). SA induced only very small amounts of PR-S (a basic PR-5 protein) in tobacco (Koiwa et al., 1994). However, some basic PR proteins are also induced by SA (Eyal et al., 1992). The promoter of *N. plumbaginifolia* Class I PR-2 protein Gn1 is strongly induced (about 14-fold) in transgenic tobacco plants treated with SA (Castresana et al., 1990). In sunflower, multiple PR-5 isomers of similar molecular weight but of different isoelectric points were excreted from the plant cells in response to the SA treatment (Jung et al., 1993). SA also induced some intracellular PR proteins (PR-10 proteins) such as SAM22 (soybean PR protein) and AoPR1 (asparagus PR protein) (Crowell et al., 1992; Warner et al., 1994). However, another PR-10 protein, PBZ1, is not induced by SA in rice (Midoh and Iwata, 1996).

2.23.6 Methyl Salicylate

Besides SA, methyl salicylate is naturally produced by a number of plants (Loughrin et al., 1993). Bacterial pathogens induced accumulation of methyl salicylate in plants (Seskar

et al., 1998). SA accumulation seems to be required for methyl salicylate production. Transgenic tobacco plants expressing the salicylate hydroxylase gene (*NahG*) from *Pseudomonas putida*, which converts SA to catechol, were unable to produce SA as well as methyl salicylate (Seskar et al., 1998). Methyl salicylate induced PR-1 transcripts in tobacco. Methyl salicylate treatment did not induce PR-1 transcripts in NahG plants, in contrast to its dramatic effect in wild-type plants (Seskar et al., 1998). The results suggest that methyl salicylate is synthesized from SA and methyl salicylate induces PR proteins.

Methyl salicylate has been shown as a translocatable form of SA (Shulaev et al., 1997). Methyl salicylate accumulation was detected in healthy tobacco leaves located above the inoculated leaf. It has been detected in phloem exudates of TMV-inoculated leaves (Shulaev et al., 1997). Besides phloem translocation of nongaseous methyl salicylate, the gaseous form of it may be the signal inducing *PR* genes in an adjacent plant (Seskar et al., 1998).

2.23.7 Salicylate-Independent Signaling Systems

Although SA-signaling system is the major signaling system in plants, SA has been shown not to be involved in inducing host defense responses in many plant–pathogen interactions (Hückelhoven et al., 1999). SA application had no effect on the induction of the defensin, PGD1 (*Picea glauca* defensin 1)-type protein, in white spruce (*P. glauca*) (Pervieux et al., 2004). Exogenous application of methyl salicylate did not enhance phenolic synthesis in the conifer *Pseudotsuga menziesii* (Hudgins and Franceschi, 2004). The race-specific *Cladosporium fulvum* resistance in tomato is SA-independent (Brading et al., 2000). The downy mildew (*Hyaloperonospora parasitica*) resistance in *Arabidopsis* (McDowell et al., 2000) is also reported to be SA-independent. Resistance to *Botrytis cinerea* (Govrin and Levine, 2002), *Phytophthora porri* (Roetschi et al., 2001), and *Alternaria alternata* (Ryals et al., 1996) in *Arabidopsis* and *Phytophthora infestans* in tomato has been observed to be SA-independent. Several defense responses can be activated without increases in the levels of SA. It suggests that besides SA, some other signaling systems may operate in plants in triggering defense responses.

2.24 JASMONATE-SIGNALING PATHWAY

2.24.1 Jasmonate Signaling in Induction of Defense Responses

JAs, which were first detected in essential oils of *Jasminum grandiflorum* (Demole et al., 1962), occur ubiquitously in all plant tissues, and they are a major group of signaling compounds in inducing host defense (Creelman and Mullet, 1997). JA and its cyclic precursors and derivatives are collectively referred to as JAs (Li et al., 2005). The JAs, derived from peroxidized linolenic acid, are members of a large class of oxygenated lipids called oxylipins (Hamberg and Gardner, 1992). Oxylipins are acyclic or cyclic oxidation products derived from the catabolism of fatty acids (Creelman and Mulpuri, 2002). JA, MeJA, 12-oxo-phytodienoic acid (OPDA), and other oxylipins act as signals for defense against pathogens (Krumm et al., 1995; Bate and Rothstein, 1998). The accumulation of JAs is followed by the activation of JA-mediated defense responses (Wasternack and Hause, 2002).

The importance of JA in signaling induction of defense genes has been demonstrated by using plant mutants deficient in JA synthesis and perception, or by applying the JA on plants to trigger the defense genes inducing disease resistance, or by developing transgenic plants overproducing JAs. Constitutive production of JA in an *Arabidopsis* mutant was accompanied by constitutive expression of defensin *PDF1.2*, thionin *Thi2.1*, and chitinase *CHI* genes (Ellis and Turner, 2001), and this mutant showed enhanced resistance against *E. cichoracearum* and a bacterial pathogen *Pseudomonas syringae* (Ellis et al., 2002b). *Arabidopsis*

(Feys et al., 1994; Staswick et al., 1998; Thomma et al., 1998; Vijayan et al., 1998) and tomato (Li et al., 2002) mutants defective in JA biosynthesis or perception are deficient in defense responses. JA synthesis and perception mutants in *Arabidopsis* were highly susceptible to *Pythium mastophorum*, and exogenous application of MeJA protected the mutants to a level close to that of wild-type controls (Vijayan et al., 1998). The application of JA to tomato and potato induces local and systemic protection against *Phytophthora infestans* (Cohen et al., 1993). JA treatment of barley plants provides some protection against *B. graminis* f. sp. *hordei* (Schweizer et al., 1993; Mitchell and Walters, 1995). Local treatment of rice leaves with JA induced partial resistance against *Magnaporthe grisea* (Schweizer et al., 1998). Transgenic *Arabidopsis* overexpressing JA carboxyl methyl transferase (JMT) accumulated MeJA, expressed the JA-responsive gene *PDF1.2* constitutively, and displayed enhanced resistance to *Botrytis cinerea* (Seo et al., 2001).

JA induces a number of proteins (called JA-induced proteins, JIPS), most of which are of unknown function, but some may have antimicrobial activity (Reinbothe et al., 1994). JA and MeJA induce resistance against various pathogens (Dong, 1998; Penninckx et al., 1998; Thomma et al., 1998; Vijayan et al., 1998; Kozlowski et al., 1999). JA signaling is needed for inducing resistance against *Alternaria brassicicola* in *Arabidopsis* (Kariola et al., 2005).

2.24.2 Biosynthesis of Jasmonates

Exogenous application of JAs does not induce the biosynthesis of JA in plants (Kozlowski et al., 1999). JA is produced rapidly (within hours of inoculation) in plant–pathogen interactions (Kenton et al., 1999). Elicitors trigger JA/MeJA accumulation in plants (Creelman and Mulpuri, 2002). Doares et al. (1995b) reported that oligogalacturonide and chitosan elicitors activate JA-signaling system.

Ellis et al. (2002b) identified the early signals involved in inducing JA-signaling system employing different *Arabidopsis* mutants. *Cev1* (constitutive expression of *VSP1*) mutant showed constitutive expression of JA-inducible genes such as *PDF1.2*, *Thi 2.1*, and the chitinase *CHI* (Ellis and Turner, 2001). The *cev1* mutant phenotype was partially suppressed in the *coi1* (coronatine sensitive 1; the JA-responsive gene) and *etr1* (ethylene resistant 1; ethylene-responsive gene) backgrounds and the triple mutant, *cev1*; *coi1*; *etr1*, was wild type except for slightly shorter roots (Ellis et al., 2002b). This indicated that *cev1* induces biosynthesis of JA and also ET, and its mutant phenotype is largely determined by responses to these signaling molecules. *Cev1* may act as a first step in the JA biosynthesis. *Cev1* has been cloned and it was identified as cellulose synthetase gene *CESA3*. Accordingly, *cev1* had reduced cellulose content, and wild-type plants treated with cellulose synthetase inhibitors had enhanced JA responses and exhibited a near-phenocopy of the *cev1* mutant (Ellis et al., 2002b). It suggests that alterations in the cell wall can initiate JA signaling.

The biosynthesis of JAs begins with the production of linolenic acid (Creelman and Mulpuri, 2002). Plant membranes are a rich source of linolenic acid, and the activation of phospholipases release linolenic acid from membranes (Farmer and Ryan, 1992). JAs are synthesized by the octadecanoid pathway that involves enzymes located in two different subcellular compartments such as chloroplast and peroxisome (Schaller, 2001; Wasternack and Hause, 2002; Li et al., 2005; Theodoulou et al., 2005). JA biosynthesis is initiated in the chloroplast and terminated in the peroxisome. In the first part of the pathway occurring in chloroplasts, following phospholipase-dependent release of linolenic acid (18:3) from chloroplast membrane lipids, molecular oxygen is introduced by 13-LOX activity to generate 13-hydroperoxy octadecatrienoic acid. Allene oxide synthase (AOS) catalyzes the formation of an unstable intermediate, 12,13-epoxy-octadecatrienoic acid, which is converted to (9*S*,13*S*) OPDA by allene oxide cyclase (AOC) (Laudert and Weiler, 1998; Li et al., 2005; Theodoulou et al., 2005).

The second part of the pathway takes place in peroxisomes. The OPDA produced in the chloroplast is transported to the peroxisome by the peroxisomal ATP-binding cassette (ABC) transporter COMATOSE (CTS) in *Arabidopsis* (Theodoulou et al., 2005). The OPDA is reduced by OPDA reductase (OPR3), which is located in the peroxisome, to produce 3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoic acid (OPC8) (Figure 2.17; Mussig et al., 2000; Schaller et al., 2000; Strassner et al., 2002; Li et al., 2005). Removal of six carbons from the octanoate side chain of OPC8 yields JA. The conversion of OPC8 to JA involves three cycles of β-oxidation (Hause et al., 2000; Creelman and Mulpuri, 2002). The final stages of JA synthesis may be catalyzed by the three core enzymes of the β-oxidation cycle, namely acyl-CoA oxidase (ACX), the multifunctional protein (containing 2-*trans*-enoyl-CoA

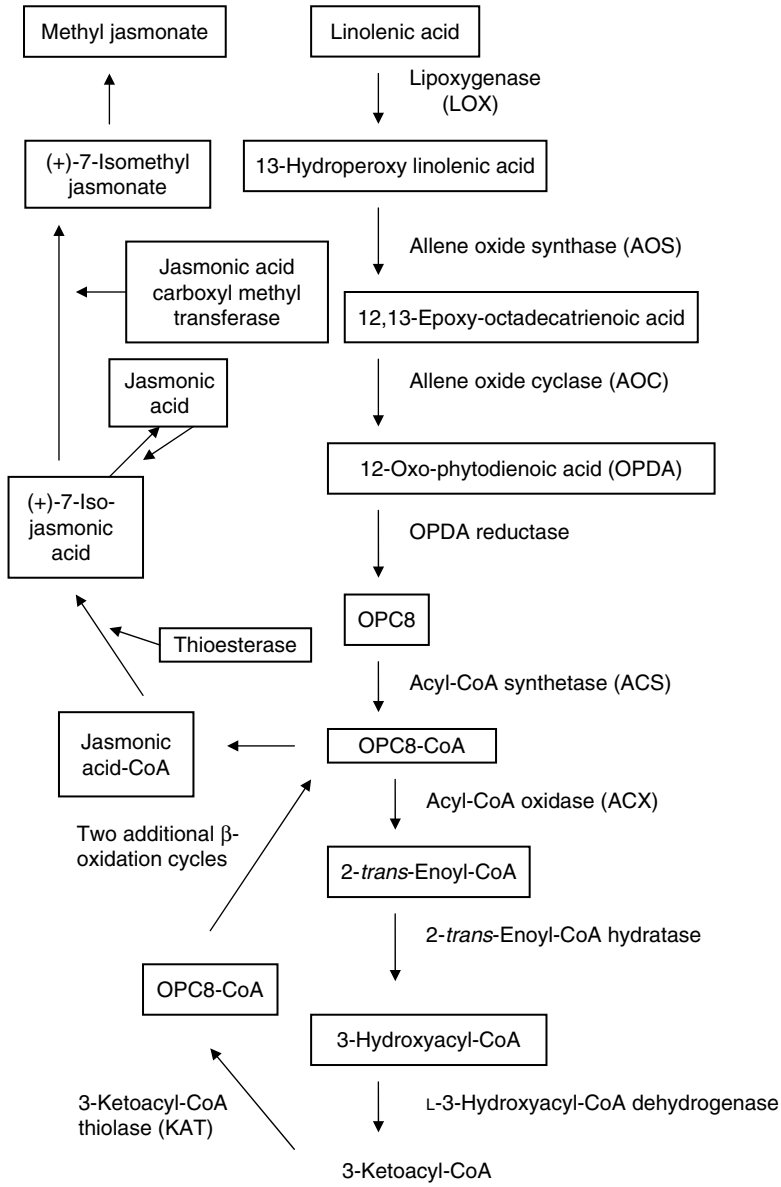

FIGURE 2.17 Biosynthetic pathway of jasmonic acid/methyl jasmonate.

hydratase and L-3-hydroxyacyl-CoA dehydrogenase activities), and 3-ketoacyl-CoA thiolase (KAT). An additional thioesterase activity is also involved in the release of JA from JA-CoA, the product of the final round of β-oxidation (Li et al., 2005). JA is catabolized to form MeJA by JMT (Figure 2.17; Hamberg and Gardner, 1992; Seo et al., 2001; Creelman and Mulpuri, 2002).

The JA signal can be amplified by JA itself. It is shown that JA activates JA biosynthetic genes such as *LOX*, *AOS*, *OPR3*, and *JMT* (Heitz et al., 1997; Laudert and Weiler, 1998; Mussig et al., 2000; Ishiguro et al., 2001; Seo et al., 2001). JAs appear to be synthesized locally in response to stress cues, and products of this pathway provide a feedback loop for amplification of the signal (Turner et al., 2002).

2.24.3 Perception of Jasmonate Signals

The JA signal is probably transduced by the activation of receptors that bind the JA molecules; however, no receptors have been so far identified (Turner et al., 2002). *COI1* (coronatine-insensitive 1) and *JAR1* (JA-resistant 1) are the genes in *Arabidopsis* involved in perception of JA signals. COI1 is an F-box protein and JAR1 has similarity to the auxin-induced GH3 gene product from soybean, and both of them show no homology to previously described plant receptor proteins (Gilroy and Trewavas, 2001). *COI1* gene encodes a 66 kDa protein containing an N-terminal F-box motif and a LRR domain (Xie et al., 1998). F-box proteins occur in the eukaryotic kingdom and function as receptors. F-box proteins associate with cullin and Skp1 proteins to form an E3 ubiquitin ligase known as the SCF complex. COI1 forms an SCF^{COI1} complex *in vivo*. SCF^{COI1} regulates JA responses (Turner et al., 2002). *JAR1* also has been found as a requirement for JA-dependent defenses. Thus, both *COI1* and *JAR1* genes may regulate the defense responses induced by JA (Turner et al., 2002).

2.24.4 Jasmonate-Signaling System May Behave Differently in Protecting Plants against Various Pathogens

It is known that JA induces a specific set of defense genes. It is also established that JA induces resistance only against specific pathogens in the particular host. JA deficiency in mutant tomato plants increased the susceptibility of the plants to *Fusarium oxysporum* f. sp. *lycopersici*, *Verticillium dahliae*, and *Phytophthora infestans* (Thaler et al., 2004). In contrast, susceptibility to three other fungi, *Septoria lycopersici*, *C. fulvum*, and *Oidium neolycopersici* was not affected in the JA-deficient mutant plants (Thaler et al., 2004). JA-deficient tomato plants were more susceptible to *Botrytis cinerea* than wild-type plants (Díaz et al., 2002).

Resistance of wild-type and JA-deficient *Arabidopsis* plants varied widely against 10 pathogens. A reduced JA response in *Arabidopsis* resulted in increased susceptibility to *Botrytis cinerea* (Thomma et al., 1998), *Pythium mastophorum* (Vijayan et al., 1998), *Pythium irregulare* (Staswick et al., 1998), and *Alternaria brassicicola* (Penninckx et al., 1996; Thomma et al., 1998). All of them are necrotrophic pathogens. JA deficiency in *Arabidopsis* did not affect the susceptibility of two biotrophic pathogens, *Erysiphe orontii* (Reuber et al., 1998), and *Peronospora parasitica* (Thomma et al., 1998) and a hemibiotrophic pathogen *Phytophthora porri* (Roetschi et al., 2001). One biotroph, *Erysiphe cichoracearum*, was negatively affected by the JA response (Ellis et al., 2002a). JA response to two bacterial pathogens in *Arabidopsis* also differed. JA deficiency resulted in increased susceptibility to *Erwinia carotovora*, whereas *Pseudomonas syringae* was negatively affected by the JA response (Pieterse et al., 1998; Ellis and Turner, 2001). These results suggest that JA signaling may affect different pathogens in varied manner (Thomma et al., 2001a,b).

2.24.5 Induction of Intercellular and Interplant Systemic Transduction of Jasmonate Signals

Application of JA/MeJA to one leaf induces expression of proteinase inhibitors in distal untreated leaves (Farmer and Ryan, 1992), suggesting that JA and MeJA may be involved in intercellular signaling. The volatile methyl JA also induced synthesis of proteinase inhibitors in the nearby untreated control plants. When tomato plants were placed in airtight chambers together with cotton-tipped wooden dowels on to which various dilutions of methyl JA in ethanol have been applied to cotton, synthesis and accumulation of proteinase inhibitors I and II in a dose-dependent manner were observed in tomato leaves (Farmer and Ryan, 1990). When tomato plants were incubated in airtight chambers along with leafy branches of *Artemisia tridentata*, the leaves of tomato plants exhibited elevated levels of proteinase inhibitors I and II. *A. tridentata* contains methyl JA in its leaves (Farmer and Ryan, 1990). These results strongly suggest that methyl JA may be involved in interplant and systemic signal transduction system.

Systemic movement of JA signals has been reported by several researchers (Li et al., 2002, 2003; Strassner et al., 2002). JA-signaling pathway appears to be required for production of the systemic signal (Li et al., 2002, 2003, 2004). Acyl-CoA oxidase1 (ACX1) is a key enzyme involved in β-oxidation of OPDA to JA. *Arabidopsis acx1* mutant plants lacked the ability to produce the systemic signal for defense gene expression (Li et al., 2005). Thus, the active compound involved in production of the systemic signal may be a product of β-oxidation (Li et al., 2005).

2.24.6 Upstream of Jasmonate Signaling

The components of JA-signaling pathway include phosphorylation and calcium ion influx (Leon et al., 1998; Rojo et al., 1998; Jensen et al., 2002). The protein phosphatase 2A activates JA-responsive gene expression, whereas a protein kinase was shown to negatively regulate the JA-dependent pathway (Rojo et al., 1998). Mobilization of intracellular calcium pools blocked induction of JA-responsive genes by JA (Leon et al., 1998). Antagonists of CaM blocked JA-responsive gene induction in *Arabidopsis*, suggesting the role of CaM in the signal transduction pathway (Leon et al., 1998). The action of calcium and CaM appeared to be downstream of the reversible phosphorylation events (Rojo et al., 1998).

2.24.7 Downstream of Jasmonate Signaling

MeJA and JA induce many defense genes (Schenk et al., 2000; Sasaki et al., 2001). The activated defense genes include the genes encoding PAL (Gundlach et al., 1992), proline-rich cell wall protein (Creelman et al., 1992), thionin (Andresen et al., 1992; Epple et al., 1995; Vignutelli et al., 1998), plant defensin (Terras et al., 1992; Penninckx et al., 1996, 1998; Manners et al., 1998), ribosome-inactivating protein (Chaudhry et al., 1994), and proteinase inhibitors (Farmer and Ryan, 1990; Miersch and Wasternack, 2000), chalcone synthase (Creelman and Mullet, 1995), and several secondary metabolites (Gundlach et al., 1992). In *Arabidopsis*, JA-induced defense genes include *CHI* (basic chitinase), *HEL* (hevein-like protein or PR4), and *LEC* (lectin-like protein) (Schenk et al., 2000). JA induced terpenoid biosynthetic enzymes and their associated genes in Norway spruce (Fäldt et al., 2003; Martin et al., 2003) and chalcone synthase in white spruce (*Picea glauca*) (Richard et al., 2000).

2.24.8 Transcriptional Regulation of JA-Responsive Genes

Transcription factor involved in transcriptional regulation of JA-responsive genes has been identified in *Catharanthus roseus* (van der Fits and Memelink, 2001) and *Arabidopsis*

(Fujimoto et al., 2000). ORAC3 is a JA-responsive APETALA′2 (AP2)-domain transcription factor from *C. roseus*. Its overexpression results in enhanced expression of several genes for metabolic biosynthesis and in increased accumulation of terpenoid indole alkaloids. ORCA3 specifically binds to and activates gene expression via a JA- and elicitor-responsive element (JERE) in the promoters of JA-response genes. Transcription of ORCA3 is rapidly induced by MeJA (van der Fits and Memelink, 2001). The transcription factor ORCA3 has similarity to the ethylene response binding factors (ERFs).

JA responses in *Arabidopsis* are regulated by ERF-like transcription factors (Berrocol-Lobo et al., 2002; Turner et al., 2002; Lorenzo et al., 2003). The gene *JIN1* (jasmonate-insensitive 1) encoding a transcription factor, AtMYC2, has been cloned from *Arabidopsis* (Lorenzo et al., 2004). AtMYC2 is a nuclear-localized basic helix–loop–helix–LZ transcription factor, whose expression is rapidly upregulated by JA. AtMYC2 represses the expression of genes involved in defense responses such as *PR4*, *PR1*, and *PDF1.2*. Conversely, ethylene-response factor 1 (ERF1) positively regulates the expression of group of genes involved in defense mechanisms (Lorenzo et al., 2004). ERF1 plays a key role in the integration of JA and ET signals (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003) and in the expression of defense genes. The interaction between AtMYC2 and ERF1 determines the type of expressed defense genes (Lorenzo et al., 2004).

2.24.9 Jasmonic Acid, Methyl Jasmonate, and Cyclic Precursors and Derivatives of Jasmonic Acid as Signal Molecules

In many plants, the terminal products of the octadecanoid pathway, JA and MeJA, are the active signals for expression of defense-related genes (Farmer and Ryan, 1992). OPDA may also serve as a signal in some other plants (Blechert et al., 1995; Koch et al., 1999; Li et al., 2005). Biosynthesis of alkaloids in *Eschscholtzia californica* cell cultures (Haider et al., 2000) and the expression of proteinase inhibitor genes in tomato are mediated by JA rather than OPDA (Miersch and Wasternack, 2000).

In some plants, MeJA may be a more important signal molecule than JA. Transgenic *Arabidopsis* plants overexpressing JMT contained elevated levels of MeJA, yet their JA was unaltered. In these transgenic plants, expression of various JA-responsive genes was elevated in the absence of JA treatment. Transgenic plants also showed enhanced level of resistance against *Botrytis cinerea*, suggesting that MeJA induced pathogen defense responses rather than JA (Seo et al., 2001). Transgenic potato plants carrying flax AOS cDNA contained higher levels of JA; however, these transgenic plants did not show overexpression of JA-responsive genes (Harms et al., 1995). It suggests that JA may not be an important signal, and other JAs such as MeJA may be involved in signaling system in potato. MeJA has been shown to be the primary signal in alkaloid production in *Eschcholtzia californica* cell cultures (Byun, 2000). MeJA induced accumulation of proteinase inhibitors in tomato (Farmer and Ryan, 1990) and potato (Kim et al., 1992). When MeJA was sprayed on leaves of tomato plants, it powerfully induced the synthesis and accumulation of proteinase inhibitor I protein (Farmer and Ryan, 1990). When tobacco and alfalfa plants were exposed to MeJA, trypsin inhibitor content increased severalfold (Farmer and Ryan, 1990; Farmer et al., 1992).

The importance of OPDA in signaling has been demonstrated in *Arabidopsis* and soybean. *Arabidopsis opr3* mutant plants fail to accumulate biologically active JA; however, this mutant retained JA-mediated resistance to fungal attack (Stintzi et al., 2001). The *opr3* plants expressed defense-related genes in response to exogenous OPDA. These results suggest that JA and OPDA may work together in wild-type *Arabidopsis* plants to coordinate the expression of appropriate sets of target genes (Stintzi et al., 2001; Weber, 2002). OPDA has been found to be more effective than JA in eliciting accumulation of glyceollin phytoalexin in soybean (Fliegmann et al., 2003). In these plants, metabolism of OPDA to JA may not be

required for induction of defense responses. OPDA was more effective than JA in eliciting the synthesis of diterpenoids in lima bean (*Phaseolus lunatus*) (Koch et al., 1999). Stintzi et al. (2001) showed that OPDA is a signal that induces broad-spectrum resistance in the absence of JA. These results also suggest that the plant cells may contain receptors that are selective for OPDA and JA (Blechert et al., 1999; Koch et al., 1999).

Some other oxylipins have also been reported to serve as signals in triggering activation of defense-related genes. C6-volatiles derived from the LOX pathway induced a subset of defense-related genes (Bate and Rothstein, 1998). The oxylipins 9-hydroperoxy polyunsaturated fatty acids (Rustérucci et al., 1999) and 13-hydroperoxy-9,11(*Z*,*E*)-octadecadienoic acid induced defense reactions, suggesting that these LOX-mediated intermediates also may function as signal molecules (Creelman and Mulpuri, 2002). Dinor-oxo-phytodienoic acid is another hexadecanoid signal in the JA-signaling system (Weber et al., 1997).

2.25 ROLE OF SYSTEMIN IN SIGNAL TRANSDUCTION SYSTEM

Systemin, an 18 amino acid polypeptide, has been identified as a primary signal for the systemic activation of defense genes in leaves of tomato plants. Systemin moves systemically in plant tissues. When ^{14}C-labeled systemin was synthesized and placed on fresh wounds of tomato plants, the radioactivity had moved throughout the leaf within 30 min, and within 1–2 h it was identified in the phloem exudates (Pearce et al., 1991). Systemin induced synthesis of proteinase inhibitors I and II (Pearce et al., 1991). Cloning and characterization of the cDNA and gene encoding systemin have also been carried out (McGurl et al., 1992). The systemin cDNA was isolated by screening a primary cDNA library synthesized from tomato leaf mRNA with an oligonucleotide corresponding to amino acids 12–18 of systemin, and a second oligonucleotide corresponding to amino acids 1–6 of systemin. One cDNA clone encoding the systemin polypeptide within a larger protein, prosystemin, was obtained. The complete prosystemin mRNA sequence was determined by sequencing the prosystemin gene. The open reading frame was 600 bp encoding a 200 amino acid prosystemin protein. Of the 200 amino acid prosystemin, amino acids 179–196 encode systemin. Prosystemin is encoded by a single gene that consists of 11 exons and 10 introns (McGurl and Ryan, 1992; McGurl et al., 1992).

Prosystemin gene homologs were found in potato also, but not in tobacco and alfalfa. An mRNA species that hybridized to the prosystemin mRNA was identified in potato. Prosystemin gene itself was wound-inducible. Prosystemin mRNA accumulated in the upper, unwounded leaves of young tomato plants that had been wounded on the lower leaves, demonstrating that prosystemin mRNA, like proteinase inhibitor I mRNA, is systemically wound-inducible. Although inhibitor I mRNA was absent from the leaves of unwounded tomato plants, a small amount of prosystemin mRNA was detected there, which may provide a continuous supply of systemin and allow the plant to respond immediately to wounding (McGurl et al., 1992).

To determine if the prosystemin gene product is important in the signal transduction pathway leading to the expression of proteinase inhibitor genes in tomato leaves, tomato plants were transformed with a prosystemin antisense gene. In transformed plants with prosystemin antisense gene, a complete suppression of the systemic, wound induction of proteinase inhibitors was observed. It suggests that systemin is an integral component of the signal transduction system that regulates the synthesis of proteinase inhibitor proteins in tomato (McGurl et al., 1992). Systemin activity has been detected only in tomato plants and it is not known if systemin-like molecules with similar function are present in other plant species (Bostock, 1999).

Systemin induces a cascade of intracellular signaling events leading to the release of linolenic acid from membrane lipids (Schaller and Ryan, 1995; Bergey et al., 1996). The linolenic acid is converted to various oxylipin molecules that signal the expression of defense genes (Ryan, 2000; Turner et al., 2002; Devoto and Turner, 2003; Li et al., 2005). Systemin induces the production of H_2O_2 and the subsequent synthesis of JA (Orozco-Cardenas et al., 2001). Systemin functions amplify the JA-signaling pathway (Li et al., 2002; Lee and Howe, 2003; Stratmann, 2003).

A systemin receptor, SR 160, has been identified from *Lycopersicon peruvianum* (Scheer and Ryan, 2002). The receptor belongs to the LRR receptor kinase family (Scheer and Ryan, 2002). The release of systemin into the vascular system of tomato activates JA biosynthesis in the surrounding vascular tissues in which JA biosynthetic enzymes are located (Ryan, 2000).

2.26 ETHYLENE-DEPENDENT SIGNALING PATHWAY

2.26.1 Ethylene-Signaling System Inducing Disease Resistance or Susceptibility

The increased production of ET is one of the earliest chemically detectable events in pathogen-infected plants or in plants treated with elicitors (Toppan and Esquerré-Tugayé, 1984). ET production was greatly enhanced in melon seedlings infected with *Colletotrichum lagenarium* (Toppan et al., 1982). Treatment of melon petioles with an elicitor from *Colletotrichum lindemuthianum* induced ET within 6 h after treatment (Roby et al., 1985).

The role of ET in plant–pathogen interaction is complex (Geraats et al., 2003). ET stimulates defense mechanisms against several pathogens, and it also induces susceptibility to several other pathogens (Boller, 1991). It is suggested that ET perception is often required for basal resistance, but the production of ET on infection can aggravate symptom development (Geraats et al., 2003). ET applied as pretreatment induces resistance against *Botrytis cinerea* in tomato (Díaz et al., 2002), whereas exogenous application of ET enhances *B. cinerea* (gray mold) incidence in tomato, pepper, cucumber, bean, rose, and carnation (Boller, 1991). The ET-insensitive mutant of tomato showed enhanced resistance to *Fusarium oxysporum* (Lund et al., 1998), and soybean mutants with reduced sensitivity to ET were less susceptible to *Phytophthora sojae* (Hoffman et al., 1999). By contrast, ET insensitivity enhanced susceptibility to various pathogens in different plants. *Arabidopsis* mutant *ein2-1* (for *ethylene-insensitive 2-1*) showed enhanced susceptibility to *B. cinerea* (Thomma et al., 1999) and *Pythium* spp. (Geraats et al., 2002). Soybean mutants with reduced sensitivity to ET showed enhanced susceptibility to the fungal pathogens *Septoria glycinea* and *Rhizoctonia solani* (Hoffman et al., 1999).

Transgenic tobacco plants expressing the mutant *etr1-1* gene from *Arabidopsis thaliana* were insensitive to ET. These transformed plants were susceptible to *Fusarium oxysporum*, *Thielaviopsis basicola*, and *Pythium* spp., whereas nontransformed plants were resistant (Geraats et al., 2003). The ET-insensitive tobacco plants showed enhanced resistance against *Peronospora tabaci* and enhanced susceptibility to *B. cinerea* and *Cercospora nicotianae* (Geraats et al., 2003). The results suggest ET may modulate the disease reaction depending on the pathogen. ET-signaling system may play important roles in various plant disease-resistant pathways. Depending on the type of pathogen and plant species, ET may induce susceptibility or disease resistance.

2.26.2 Biosynthesis of Ethylene

Biosynthetic pathway of ET has been studied in detail (Figure 2.18; Kende, 1993; Wang et al., 2002). *S*-adenosylmethionine (*S*-AdoMet) and ACC are the precursors of ET. The formation of *S*-AdoMet is catalyzed by *S*-AdoMet synthetase. *S*-AdoMet is converted to ACC by ACS.

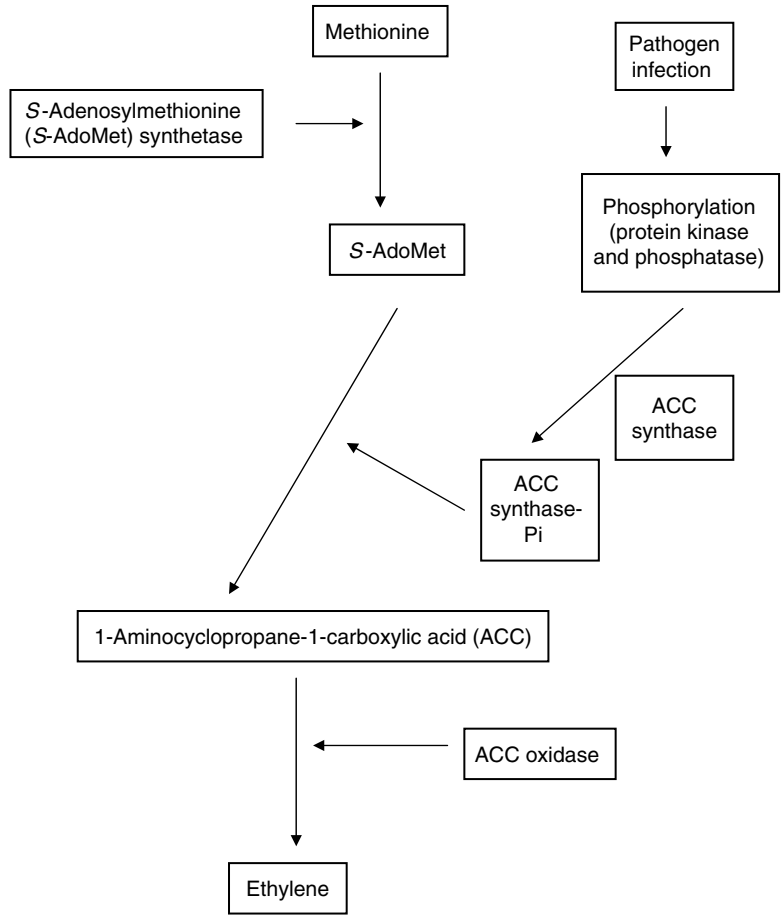

FIGURE 2.18 Biosynthetic pathway of ethylene.

Addition of fungal elicitors to tomato cell culture induces a rapid increase in ACS activity (Spanu et al., 1991). Treatment with the protein kinase inhibitors K-252 or staurosporine inactivated the ACS activity, whereas protein phosphatase inhibitor calyculin A stimulated ACS activity of the tomato cell culture even without elicitors. Besides, calyculin A enhanced the effect of elicitor treatment in increasing the ACS activity (Spanu et al., 1991). These results suggest that phosphorylation of ACS is involved in increased ACS activity. It is suggested that ACS is unstable *in vivo* and present at low abundance, and phosphorylation of ACS may increase its stability to sustain the elevated activity (Wang et al., 2002). It is also possible that an unknown repressor of ACS activity may be inactivated by phosphorylation (Wang et al., 2002). At the final stages, ACC is oxidized by ACO to form ET (Wang et al., 2002).

2.26.3 Upstream Signals in Induction of Synthesis of Ethylene

Protein phosphorylation and dephosphorylation are implicated in the process of ET induction in tobacco (Kim et al., 2003a). The activation of a mitogen-activated kinase, SIPK, resulted in a dramatic increase in ET production in tobacco. The increase in ET after the activation of SIPK coincided with a dramatic increase in ACS activity (Kim et al., 2003b). The elicitor isolated from cell walls of *Phytophthora megasperma* induced ACS activity about 10-fold within 1 h after treatment in parsley cell cultures (Chappell et al., 1984). The increase

in ACS activity activated ACO genes (Kim et al., 2003a). The activated ACO genes promote the production of ET (Wang et al., 2002).

2.26.4 Ethylene Signal Perception

After its synthesis, ET is perceived and its signal is transduced through transduction machinery to trigger specific biological responses. The signaling system consists of two proteins, a histidine kinase and a response regulator. The histidine kinase acts as the sensor that autophosphorylates an internal histidine residue in response to signals, and the response regulator activates the downstream components upon receiving a phosphate from the histidine residue of the sensor on its aspartate residue (Pirrung, 1999). ET is perceived by a family of five membrane-localized receptors (ETR1 [for ethylene response 1], ETR2, EIN4 [for ethylene-insensitive 4], ERS1 [for ethylene response sensor], and ERS2) in *Arabidopsis* (Wang et al., 2002, 2003a; Klee, 2004). In tomato, there are six ET receptors (LeETR1-6) (Klee, 2004). The *Arabidopsis* receptors ETR1 and ERS1 contain three TM domains and a conserved histidine kinase domain, and have been shown to function as homodimers. ETR2, EIN4, and ERS2 have four membrane-spanning regions and a degenerate histidine kinase domain. Only ETR1, ETR2, and EIN4 have reciver domains at their C termini. ET binding occurs at the N-terminal TM domain of the receptors. A copper cofactor is required for binding and RAN1 (RESPONSE-TO-ANTAGONIST 1); the copper transporter is involved in delivery of copper to the ET receptor. This copper-delivery pathway appears to be required to create functional receptors in plants (Wang et al., 2002). The predicted structures of the tomato receptors are very similar to those in *Arabidopsis* (Klee, 2004). One of the receptors, LeETR4, is induced by a bacterial pathogen and is associated with increased ET synthesis following infection (Ciardi et al., 2000).

2.26.5 Downstream Events in Ethylene Signaling

Downstream of ET receptors, the *Arabidopsis* gene *CTR1* may operate. The *CTR1* gene encodes a protein that belongs to the Raf family of serine/threonine protein kinases that initiate MAPK-signaling cascades. It suggests that the ET signaling may operate through an MAPK cascade (Wang et al., 2002; Ouaked et al., 2003). Another gene in *Arabidopsis EIN2* (ET-insensitive 2) acts downstream of CTR1 and upstream of *EIN3*. *EIN2* encodes a novel integral membrane protein (Alonso et al., 1999). N-terminal portion of EIN2 is necessary for sensing the ET signal from upstream components in the pathway, whereas the C-terminal portion of the EIN2 protein is required for transducing the signal to the downstream components. EIN3 encodes a novel nuclear-localized protein, which is involved in the early signal transduction pathway in *Arabidopsis* (Chao et al., 1997). EIN3-like transcription factors have been identified in other plant species, such as tomato (Tieman et al., 2001) and tobacco (Kosugi and Ohashi, 2000). The EIN3 proteins were able to bind primary ET response elements in the promoters of ERF1 (Solano and Ecker, 1998). After ET production, there was strong activation of *ERF* genes (Kim et al., 2003a).

ERF1 belongs to large family of plant-specific transcription factors referred to as ET-response-element binding proteins (EREBPs) (Berrocal-Lobo et al., 2002). EIN3 stimulates ERF1 expression. EREBPs bind to the GCC box, a DNA motif associated with ET- and pathogen-induced gene expression in plants. GCC box is a general transcriptional regulatory element (Wang et al., 2002). In tomato, a transcription factor, Pti4, has been identified. Pti4 protein shares extensive similarity with the amino acid sequences of EREBPs and specifically binds the GCC-box *cis*-element present in the promoter of pathogen-related (*PR*) genes. ET rapidly induces the expression of *Pti4* in tomato leaves, and *Pti4* induces expression of several *PR* genes (Gu et al., 2000). ET induces PR-1 proteins, β-1,3-glucanase, chitinase, PAL,

HRGPs, and osmotin-like proteins (Boller, 1991; Knoester et al., 1995; Deikman, 1997). ET pretreatment induced expression of several *PR* genes (Díaz et al., 2002).

2.27 ABSCISIC ACID SIGNALING

During fungal infection, ABA accumulates in the infected tissues (Bothe et al., 1994; Kettner and Dörffling, 1995). ABA may also be a key factor in systemic induction of proteinase inhibitor genes (Peña-Cortés et al., 1989). Spraying ABA on potato plants induces proteinase inhibitor mRNA synthesis in leaves (Peña-Cortés et al., 1991). ABA-deficient plants do not accumulate proteinase inhibitor II following systemin treatment (Peña-Cortés et al., 1996). ABA induces *PR-1* in rice plants (Agrawal et al., 2001). These observations suggest that ABA is involved in the induction of defense genes. ABA is believed to operate upstream of the octadecanoid pathway, possibly through an effect on release of the JA precursor linolenic acid (Bostock, 1999).

ABA has been implicated in enhancing disease susceptibility in various plant species (McDonald and Cahill, 1996; Audenaert et al., 2002; Mohr and Cahill, 2003; Anderson et al., 2004; Koga et al., 2004). ABA increases susceptibility of soybean (*Glycine max*) plants to *Phytophthora sojae* (McDonald and Cahill, 1996). ABA determines basal susceptibility of tomato to *Botrytis cinerea* (Audenaert et al., 2002). Tomato mutants with reduced ABA levels (*sitiens* plants) are much more resistant to *B. cinerea* than wild-type plants. Exogenous application of ABA restored susceptibility to *B. cinerea* in *sitiens* mutant plants and increased susceptibility in wild-type plants (Audenaert et al., 2002). ABA induced susceptibility in rice plants to the pathogen *Magnaporthe grisea* (Koga et al., 2004). ABA influences the susceptibility of *Arabidopsis thaliana* to *Peronospora parasitica* (Mohr and Cahill, 2003).

ABA may suppress the defense signaling systems. ABA appears to modulate the SA-dependent defense signaling system in tomato, and by suppressing the SA defense signaling system ABA may induce susceptibility to *B. cinerea* (Audenaert et al., 2002). SA signals induce increase in PAL mRNA and PAL activity in plants (Audenaert et al., 2002), and a correlation between PAL and disease resistance has been widely reported (De Meyer et al., 1999). PAL activity is repressed by ABA in tomato. The tomato mutants with reduced ABA levels (*sitiens* plants) showed a clear increase in PAL activity after infection with *B. cinerea*, which was not observed in wild-type plants (Audenaert et al., 2002). In soybean, exogenously applied ABA suppressed PAL activity and synthesis of PAL mRNA in the incompatible interaction of soybean with *Phytophthora sojae* (Ward et al., 1989).

ABA may suppress the expression of the *PR1a* gene promoter. This promoter contains a negative-acting ABA-responsive element TAACAAA (Giraudat et al., 1994). This could lead to a transcriptional downregulation of PR1a (Audenaert et al., 2002). ABA downregulates transcription of *PR2* genes encoding β-1,3-glucanase and not PR3 (chitinase) genes in tobacco cell cultures (Rezzonico et al., 1998). ABA-responsive element was absent in the chitinase gene, whereas the ABA-responsive element TAACAAA box was present in the promoter of β-1,3-glucanase gene (Rezzonico et al., 1998). It is also reported that ABA suppresses NPR1 activity. NPR1 is the protein encoded by *NPR1* gene. It functions in the downstream of SA in induction of defense genes (Cao et al., 1994). Thus, ABA functions as a negative modulator of SA-dependent defense responses and confers susceptibility to diseases.

ABA also inhibited ET- and JA-signaling pathways (Anderson et al., 2004). ABA treatment significantly reduced expression of the defensin gene *PDF1.2* induced by treatments with MeJA and ET in *Arabidopsis* (Anderson et al., 2004). AtMYC2, a transcriptional activator, is a positive regulator of ABA signaling in *Arabidopsis* (Abe et al., 2003). When *Arabidopsis* plants were inoculated with *Fusarium oxysporum*, transcripts of AtMYC2 accumulated (Anderson et al., 2004). Overexpression of *AtMYC2* in a transformed plant resulted in an eightfold increase in the *AtMYC2* transcripts and a fourfold reduction in the

transcript levels of *PDF1.2*. It suggests that this positive regulator of ABA signaling negatively regulates JA/ET-responsive defense gene expression (Anderson et al., 2004). ABA-signaling pathway negatively regulated resistance to *F. oxysporum* in *Arabidopsis* (Anderson et al., 2004).

2.28 FATTY ACIDS AS SYSTEMIC SIGNAL MOLECULES

Plant LOXs play an important role in defense mechanisms against pathogens (Preisig and Kuc, 1987; Hildebrand et al., 1988; Bostock and Stermer, 1989; Siedow, 1991). Normal substrates for plant LOXs are linoleic and linolenic acids. LOX products of these fatty acids are metabolized to compounds that may function as signal molecules. MeJA, the important systemic signal molecule is formed because of the action of LOX (Farmer and Ryan, 1992). Another signal molecule, traumatin, is also synthesized by action of LOX (Vick and Zimmerman, 1987).

AA and EPA are the important elicitors of *Phytophthora infestans* and they are also substrates for LOX (Bostock et al., 1981; Preisig and Kuc, 1985). LOX has been suggested to be an intermediate enzyme in the pathway between treatment with AA and induced defense mechanism. Inhibitors of LOX abolished the elicitor activity of AA (Preisig and Kuc, 1987).

Cohen et al. (1991) demonstrated that both AA and EPA induced systemic resistance to *P. infestans* in foliage. Ricker and Bostock (1992) showed that AA diffuses within developing lesions and may therefore provide a signal directly to the surrounding cells.

2.29 OTHER SIGNALING SYSTEMS

Several transcription factors that are not responsive to JA/ET and SA have been identified in *Arabidopsis* (Chen et al., 2002), suggesting that some other signaling systems may also operate. Smart et al. (2003) reported that the partial resistance of tomato plants to *P. infestans* was not dependent upon ET, JA, or SA pathways, suggesting some other signaling systems may exist or cross talk between different signals may be important in inducing resistance against *P. infestans*. Resistance in *Arabidopsis* against *Phytophthora porri* has been shown to be independent of SA-, ET-, and JA-signaling systems (Roetschi et al., 2001). Unknown defense systems independent of SA- and JA-mediated systemic resistance mechanisms have been reported to confer resistance against *Ascochyta rabiei* and *Fusarium oxysporum* f. sp. *ciceri* in chickpea (*Cicer arietinum* L.) (Cho and Muehlbauer, 2004).

2.30 NETWORK AND INTERPLAY OF SIGNALING PATHWAYS

2.30.1 Regulatory Interaction and Coordination among Salicylate-, Jasmonate-, and Ethylene-Signaling Pathways

The SA-, JA-, and ET-dependent signaling pathways may not act independently and the plants may be equipped with regulatory components to control the magnitude of each of these pathways and to mediate the interaction between these diverse signaling pathways (Genoud and Metraux, 1999). An intricate signaling network involving SA, ET, and JA fine-tunes plant defense responses (Nandi et al., 2003). Changes in the expression levels of 2375 selected genes upon pathogen infection or SA, JA, and ET treatment were assessed in *Arabidopsis*. The results revealed that although some genes were affected by one signal or another, many responded to two or more defense signals (Schenk et al., 2000). All the three signaling systems (SA, JA, and ET signals) have been shown to be required for expression of the defensin gene *PDF1.2* and the *PR* genes in *Arabidopsis* (Nandi et al., 2003).

Analysis of some *Arabidopsis* mutants revealed a pathway in which JA and ET signaling are required for SA responses. The *Arabidopsis* mutant *npr1* is insensitive to SA and fails to express SA-induced *PR* genes. A screen for suppressor mutations of *npr1* yielded a dominant mutation named *ssi1* (suppressor of SA insensitivity 1), which had constitutive expression of *PR* genes. *ssi* plants accumulated elevated levels of SA and had constitutive expression of *PDF1.2* also, which is mainly induced by JA and ET (Shah et al., 1999). When SA accumulation in *ssi npr1-5* plants was prevented by expressing *nahG* gene, all of the *ssi* phenotypes were also suppressed, including the expression of *PDF1.2*. Treatment of these plants with BTH, which mimics the action of SA but is not degraded by salicylic hydroxylase, induced SA responses and induced *PDF1.2* expression also (Shah et al., 1999). The results show that the defense gene activation in *ssi* mutants involves the SA-, JA-, and ET-signaling pathways. The *Arabidopsis* mutant *cpr5* (constitutive expression of PR5) gene also requires SA-, JA-, and ET-signaling pathways to induce defense genes (Clarke et al., 2001). The cell death induced by the toxin fumonosin B1 produced by the maize pathogen *F. verticillioides* has been shown to be interdependent of JA-, SA-, and ET-signaling pathways in *Arabidopsis* (Asai et al., 2000).

2.30.2 Coordinated Regulation of Ethylene- and Jasmonate-Signaling Pathways

In most cases, JA- and ET-dependent pathways appear to operate concomitantly (Penninckx et al., 1998; Anderson et al., 2004). ET and JA pathways interact with each other, coregulating expression of some genes involved in plant defense (Wang et al., 2002). JA induced proteinase inhibitor gene expression in tomato. However, the JA required ET for its action in triggering the defense response (O'Donnell et al., 1996). PR proteins induced by ET were also induced by JA in potato (Xu et al., 1994b). A defensin gene in *Arabidopsis* was shown to be induced by a JA- and ET-dependent signaling pathway (Penninckx et al., 1996, 1998). MeJA treatments induced a gene encoding dioxygenase-like protein, which is homologous to E8 protein in tomato (Sasaki et al., 2001). The E8 protein has been shown to regulate ET biosynthesis during fruit ripening (Kneissl and Deikman, 1996). It suggests that an intricate mechanism of interaction exists between JA and ET signal transduction.

Hudgins and Franceschi (2004) showed that MeJA-induced defense responses in the conifer *Pseudotsuga menziesii* are mediated by ET. Pretreatment of *P. menziesii* stems from the ET response inhibitor 1-methylcyclopropene-inhibited MeJA responses, and MeJA treatment produced a higher and more rapid increase in ACO, resulting in increased synthesis of ET. The results suggest that ET may act downstream of JA signaling.

In *Arabidopsis*, responses to different pathogens have been shown to include a synergistic effect of JA and ET for induction of defense-related genes (Xu et al., 1994b; Penninckx et al., 1998). ET has been shown to positively regulate the induction of AOS, which catalyzes the first step in the biosynthesis of JA in tomato and *Arabidopsis* (O'Donnell et al., 1996; Laudert and Weiler, 1998; Sivasankar et al., 2000). Brown et al. (2003) have shown that the transcription factor ERF, which regulates ET-mediated gene expression, also regulates JA-responsive gene expression in *Arabidopsis*.

JA activates a plant defensin gene *PDF1.2* in *Arabidopsis* (Brown et al., 2003). The extended promoter region that positively regulates basal expression from the *PDF1.2* promoter contained a GCC-box region. When this region was deleted, the response to JA was reduced. Point mutations introduced into the core GCC-box sequence substantially reduced JA responsiveness, whereas addition of a 20 nucleotide long promoter element carrying the core GCC-box and flanking nucleotides provided JA responsiveness to a 35S minimal promoter. These results suggested that the GCC-box plays a key role in conferring JA responsiveness to the *PDF1.2* promoter (Brown et al., 2003). The *Arabidopsis* ERF, *AtERF2*, activated transcription from the *PDF1.2*, *Thi2.1*, and *PR4* (basic chitinase) genes,

all of which contain a GCC-box sequence in their promoters. These observations suggest that in addition to their roles in regulating ET-mediated gene expression, ET-responsive factors also play important roles in regulating JA-responsive gene expression, possibly via interaction with the GCC-box.

ET and JA pathways may converge in the transcriptional activation of *ERF1*, which encodes a transcription factor, which regulates the expression of pathogen response genes that prevent disease progression. The expression of *ERF1* can be activated rapidly by ET or JA and can be activated synergistically by both ET and JA (Lorenzo et al., 2003). It has also been shown that both signaling pathways are required simultaneously to activate *ERF1*, because mutations that block any of them prevent *ERF1* induction by any of these hormones either alone or in combination (Lorenzo et al., 2003). *ERF1* regulates *in vivo* expression of a large number of genes responsive to both ET and JA (Lorenzo et al., 2003). Overexpression of *ERF1* rescued the defense-response defects of both *coi1* (defective in JA-dependent pathway) and *ein2* (ethylene-insensitive 2; defective in ethylene-dependent pathway) *Arabidopsis* mutants by restoring *PR* gene expression (Lorenzo et al., 2003). These results suggest that the *ERF1* acts downstream of the intersection between ET and JA pathways. Probably, *ERF1* is a key element in the integration of both ET and JA signals for the regulation of defense response genes.

2.30.3 Interplay between Salicylate- and Jasmonate-Signaling Pathways

In some instances, SA and JA may act synergistically in triggering defense responses (Xu et al., 1994b; Niki et al., 1998). In rice, JA and 2,6-dichloroisonicotinic acid (INA, a SA signal inducer) appear to be synergistic signaling compounds (Schweizer et al., 1997a). Both compounds induce LOX activity in rice (Schweizer et al., 1997a), and both of them induce resistance against pathogens in barley (Schweizer et al., 1993; Kogel et al., 1994) and rice (Schweizer et al., 1997a). In rice, JA enhanced the induction of *PR* gene expression induced by the salicylate-mimic INA (Schweizer et al., 1997b, 1998). SA substantially induced the accumulation of PR-1b protein in Wisconsin 38 tobacco. The combination of SA and MeJA induced the accumulation of PR-1b protein severalfold more (Xu et al., 1994b). In *Arabidopsis*, SA modulates AOS activity, an important step in JA biosynthesis (Laudert and Weiler, 1998). Transgenic tobacco plants expressing *Cf9* gene from tomato produced both JA- and SA-activated MAPK in response to elicitor treatment, suggesting some convergence of JA and SA pathways in signaling (Romeis et al., 1999). In tomato, simultaneous action of both JA- and SA-signaling pathways inducing JA-inducible proteinase inhibitor protein and SA-inducible PR proteins has been reported (Fidantsef et al., 1999). During HR development in tobacco, JA accumulation preceded the accumulation of SA (Kenton et al., 1999). MeJA induces the accumulation of SA in Norway spruce (*Picea abies*) seedlings, establishing a link between the JA and SA pathways in conifers (Kozlowski et al., 1999).

2.30.4 Interplay between Salicylate and Ethylene Pathways

Interplay between ET and SA-dependent pathways has been reported in some instances. A null mutation in the *EDR1* gene has been shown to enhance resistance to *Erysiphe cichoracearum* and induce rapid activation of defense-related genes such as *PR1* in *Arabidopsis* (Frye et al., 2001). The *PR1* gene expression, which is SA-dependent, is highly expressed in *edr1* mutants, whereas it is almost undetectable in wild-type *Arabidopsis* plants. ET may potentiate SA-mediated *PR1* gene expression, and EDR1 may negatively regulate this process. Removal of EDR1 produces a dramatic effect of ET on SA-dependent defense responses, resulting in enhanced disease resistance in *edr1* mutant plants (Frye et al., 2001). Both ET and SA signal transduction pathways have been shown to be necessary to induce effective defense response against *Plectosphaerella cucumerina* in *Arabidopsis* (Berrocal-Lobo et al., 2002).

2.30.5 Cross Talk between Salicylate and Jasmonate/Ethylene Pathways

Cross talk between JA, SA, and ET pathways has been widely reported (Xu et al., 1994b; Niki et al., 1998; Chen et al., 2002; Devoto and Turner, 2003). The word cross talk between different signal transduction pathways indicates the communication between two separate, linear signal transduction pathways that are simultaneously activated in the same cell (Noselli and Perrimon, 2000). Positive and negative interactions between JA- and SA-signaling pathways have been broadly documented (Turner et al., 2002; Farmer et al., 2003; Kachroo et al., 2003a; Rojo et al., 2003). JA-mediated responses are strongly influenced by the type and the nature of interaction with ET and SA (Dong, 1998; Reymond and Farmer, 1998; Genoud and Metraux, 1999). Perturbations in SA-dependent signaling system affect JA/ET-dependent signaling system (Penninckx et al., 1996, 1998; Clarke et al., 1998; Dewdney et al., 2000; Gupta et al., 2000; Rao et al., 2000).

In many plant–pathogen interactions, JA and SA antagonize each other (Felton et al., 1999; Takahashi et al., 2004). Activation of SA pathway suppresses JA pathway and vice versa (Fidantsef et al., 1999). Chen et al. (2002) observed that a group of transcription factors that were repressed in SA-signaling mutants of *Arabidopsis* were induced in JA/ET mutants, and the transcription factors that were repressed in JA/ET-signaling mutants were induced in SA-signaling mutants.

High SA levels may antagonize JA/ET signaling (Wang et al., 2002). Accumulation of high levels of SA in the *Arabidopsis* mutants *ssi* and *fab2* resulted in repression of JA-mediated induction of the defensin *PDF1.2* gene (Kachroo et al., 2003a). *Arabidopsis mpk4* (MAPK) mutant, which has elevated SA levels and constitutive activation of SA-dependent signaling system, failed to induce the expression of *PDF1.2* gene upon JA treatment (Petersen et al., 2000). SA has been shown to inhibit JA biosynthesis (Peňa-Cortés et al., 1993) and JA-dependent induction of host defense response (Doares et al., 1995a). SA inhibits the synthesis of proteinase inhibitors in tomato leaves induced by JA (Doares et al., 1995a) Accumulation of PR protein P4 and proteinase inhibitor II in tomato induced by JA was diminished by treatment with BTH (a SA analog) (Fidantsef et al., 1999). SA applied exogenously to tomato plants inhibited proteinase inhibitor biosynthesis, possibly through the inhibition of JA biosynthesis and action (Doherty et al., 1988; Doares et al., 1995a; Sano and Ohashi, 1995).

Mutants that disrupt SA-mediated responses become sensitized to activation of the JA/ET pathway (Clarke et al., 1998, 2000b; Gupta et al., 2000). A decrease in SA levels resulted in increased *PDF1.2* gene expression (a marker gene in JA/ET-dependent signaling pathway) in *Arabidopsis* (Jirage et al., 2001). *PDF1.2* mRNA accumulated at higher levels in mutants defective in SA signaling compared with levels in the wild type after *Botrytis cinerea* infections (Clarke et al., 2001). It is not known how SA suppresses JA signaling. However, it has been reported that salicylates can interfere with the activity of heme-containing oxidoreductases, such as the AOS involved in JA biosynthesis (Peňa-Cortés et al., 1993; Durner et al., 1997).

As SA inhibits JA/ET-signaling system, JA and ET may inhibit biosynthesis and action of SA. Treatment of tomato plants with JA reduced the PR protein gene expression induced by BTH, which acts by inducing synthesis of SA (Bostock, 1999). Schenk et al. (2000) reported that eight genes that were induced by SA were repressed by MeJA in *Arabidopsis*. Activation of ET responses by the *ERF1* overexpression in *Arabidopsis* plants reduced the SA-mediated tolerance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Berrocal-Lobo et al., 2002).

Synergism between ET and JA also has been reported. ET caused osmotin (PR5 protein) mRNA accumulation in tobacco leaf tissues. After 24 h of ET treatment, 5 day old seedlings containing an osmotin promoter-β-glucuronidase (GUS) fusion gene exhibited a five-fold increase in GUS activity. MeJA treatment did not increase the osmotin promoter activity. But when MeJA was applied in combination with ET, the activity of the osmotin

promoter was induced dramatically beyond that seen with either ET or MeJA in the seedlings (Xu et al., 1994b).

The *Arabidopsis* mutant *cev1* (constitutive expression of vegetative storage protein) was used to study the cross talk between JA, ET, and SA signal pathways (Ellis et al., 2002b). This mutant had constitutive production of JA and ET and constitutive expression of the defensin gene *PDF1.2* and the thionin gene *Thi2.1* (Ellis and Turner, 2001). Treatment of *cev1* with SA suppressed expression of *PDF1.2* and enhanced expression of the pathogenesis-related *PR1* gene (Ellis et al., 2002b). It is known that JA and ET activate the defensin gene *PDF1.2* (Ellis and Turner, 2001), whereas SA activates *PR1* gene. Another *Arabidopsis* mutant *coi1* is deficient in JA perception/response and it showed significant *PR1* expression. The *COI1*-dependent signal normally suppresses *PR1* gene expression. The double-mutant *cev1*; *coi1* expressed neither *PDF1.2* nor *Thi2.1* and it confirmed that expression of these genes required the JA perception/response pathway regulated by *COI1*. The *Arabidopsis* mutant *etr1* was used to make the double-mutant *cev1*; *etr1*. In this mutant, *PDF1.2* expression was absent (Ellis et al., 2002b), suggesting that ET signal is required for *PDF1.2* transcription (Ellis and Turner, 2001). The thionin gene *Thi2.1* was constitutively expressed in this double mutant indicating that ET-signaling system suppresses the transcription of *Thi2.1* (Ellis et al., 2002b). These results suggest the positive and negative cross talk between the SA, JA, and ET signal pathways.

2.30.6 Cross Talk between Abscisic Acid-, Jasmonate-, and Ethylene-Dependent Signaling Pathways

A complex interplay between ABA- and JA-dependent signaling pathways has been reported. Exogenous ABA suppressed both basal and JA-activated transcription from defense genes (Anderson et al., 2004). ABA suppressed expression of the defensin gene *PDF1.2*, induced by JA signal in *Arabidopsis* (Figure 2.19; Anderson et al., 2004).

ABA may also suppress ET-signaling system in plants. It has been demonstrated that the ET-insensitive *Arabidopsis* mutants *etr1-1*, *ein2-1/era3*, and *ein3* show increased sensitivity to ABA, suggesting that ET signaling antagonizes ABA-responsive gene expression (Anderson et al., 2004).

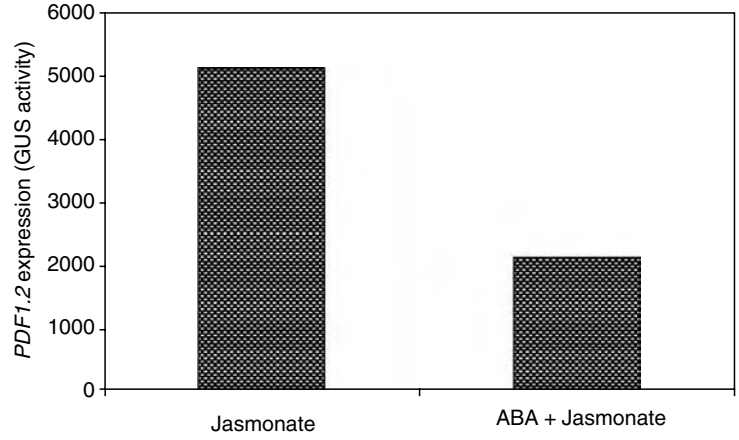

FIGURE 2.19 Suppression of jasmonate-induced expression of defensin (*PDF1.2*) by abscisic acid (ABA) in *Arabidopsis*. (Adapted from Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J., Ebert, P.R., and Kazan, K., *Plant Cell*, 16, 3460, 2004.)

2.30.7 Regulatory Switches to Fine-Tune Signaling Pathways

Several signaling pathways may be involved in induction of host defense responses. SA, JA, ET, and ABA take part in distinctly different pathways. It suggests that plant defense signaling pathways are complex and may involve interaction between various signaling systems. There may be substantial communication between the different signaling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Glazebrook et al., 2003; Devoto and Turner, 2003; Spoel et al., 2003).

Cross talk between JA, ET, and SA plays an important role in fine-tuning defense responses (Creelman and Mulpuri, 2002). Plants may contain regulatory switches to control the temporal expression and the amplitude of multiple pathways. Expression of the defensin *PDF1.2* in *Arabidopsis* is normally JA/ET-dependent (Penninckx et al., 1996). In an *Arabidopsis* mutant, *ssi1*, the expression of *PDF1.2* was responsive to SA (Shah et al., 1999). It suggests that SSI1 functions as a molecular switch to modulate the expression of both SA- and JA-dependent pathways (Shah et al., 1999).

MAPKs may also fine-tune the expression of JA/ET- and SA-signaling pathways (Seo et al., 1995; Petersen et al., 2000). A MAPK gene *MKP4* exerts negative regulation of SA-mediated defense responses and positive regulation of JA-mediated defense responses (Petersen et al., 2000). *Arabidopsis* mutant *mpk1* showed elevated SA levels and constitutive expression of the defense-related PR gene, whereas the expression of the defensin gene *PDF1.2* and the thionin gene *THI1.2* by JA was suppressed in this mutant (Petersen et al., 2000).

A complex interplay between ABA and JA/ET-signaling pathways regulates plant defense gene expression. Exogenous application of ABA suppressed both basal and JA/ET-activated transcription from defense genes. ABA deficiency was conditioned by the mutations in the *ABA1* and *ABA2* genes, which encode enzymes involved in ABA biosynthesis, resulting in upregulation of basal and induced transcription from JA/ET-responsive defense genes. The *Arabidopsis* gene *AtMYC2* (*A. thaliana* myelo blastosis virus c2) (allelic to *JIN1*), which encodes a basic helix–loop–helix–LZ transcription factor, is a positive regulator of ABA signaling. Disruption of this gene resulted in elevated levels of basal and activated transcription from JA/ET-responsive defense genes. The *jin1/myc/aba2-1* mutants showed increased resistance to *Fusarium oxysporum* (Anderson et al., 2004). These results suggest that the antagonistic interactions between multiple components of ABA and JA/ET-signaling pathways modulate defense-responsive gene expression.

A fatty acid involved in the induction of host plant defense responses has been shown to modulate SA- and JA-mediated defense pathways in *Arabidopsis*. The stearoyl-acyl carrier protein desaturase (S-ACP-DES)-derived fatty acid 18:1 activated JA-mediated responses, whereas it repressed the SA-signaling pathway (Kachroo et al., 2003b).

2.31 INDUCTION OF DEFENSE GENES MAY REQUIRE DIFFERENT SIGNAL TRANSDUCTION SYSTEMS

Several defense genes are involved in conferring resistance against diseases. Induction of these defense genes may require different signaling systems. ET induces PR proteins, PR-1 and PR-5, which belong to two different families of PR proteins in tobacco (Raz and Fluhr, 1993; Xu et al., 1994b). However, they may be induced through different signal transduction systems. The protein kinase C inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) could inhibit the induction of PR-1 transcription by ET (Raz and Fluhr, 1993). But, H7 was unable to inhibit the induction of PR-5 transcript accumulation by ET (Xu et al., 1994b). It suggests that ET may act through two different signal transduction systems, one involving PKC and another without participation of this enzyme.

TABLE 2.3
Efficacy of Jasmonate and Salicylate Signals in Induction of Proteinase Inhibitor II and PR Protein 4 Transcripts in Tomato Leaves

Treatment	Proteinase Inhibitor II	PR Protein P4
Jasmonic acid (JA signal)	+++[a]	0[b]
Benzothiadiazole (SA signal inducer)	0[b]	+++[a]

Source: Adapted from Fidantsef, A.L., Stout, M.J., Thaler, J.S., Duffey, S.S., and Bostock, R.M., *Physiol. Mol. Plant Pathol.*, 54, 97, 1999.

[a] +++ indicates strong induction.
[b] 0 indicates no effect.

SA induced accumulation of PR-1b protein in tobacco. However, it could not induce accumulation of PR-5 protein, whereas ET induced the PR-5 protein (Xu et al., 1994b). JA induced nsLTPs in grapevine, whereas SA had no effect (Gomés et al., 2003). In maize embryos infected with *Fusarium moniliforme*, a proteinase inhibitor protein (MPI) and another PR-protein (PRms) mRNAs accumulate (Cordero et al., 1992). Treatment with ABA or methyl JA did not induce PRm mRNA synthesis, whereas these compounds were effective in induction of the accumulation of the MPI mRNA (Cordero et al., 1992). The results suggest that two distinct signal transduction pathways may exist in triggering induction of PR proteins in maize (Cordero et al., 1992) and tobacco (Xu et al., 1994b). JA treatment induced proteinase inhibitor II, but did not induce the PR protein P4 in tomato. By contrast, BTH (a SA signal inducer) induced the PR protein P4 and did not induce proteinase inhibitor II (Table 2.3; Fidantsef et al., 1999). JA treatments induced strong increase of the defensin, PGD1-type transcript level within 8 h after application in white spruce (*Picea glauca*), whereas salicylate treatment could not induce it (Table 2.4; Pervieux et al., 2004).

Endogenous SA and JA may activate separate sets of genes encoding antimicrobial proteins in *A. thaliana*. The PR protein genes *PR-1*, *PR-2*, and *PR-5* require SA signaling for activation, whereas the plant defensin gene *PDF1.2* and PR protein genes *PR-3* and *PR-4* are induced by a SA-independent and JA-dependent pathway (Thomma et al., 1998; Kunkel and Brooks, 2002). In some cases, a single defense gene is induced by different signaling systems. A thionin

TABLE 2.4
Intensity of PgD1 Expression in *Picea glauca* after Jasmonate (JA) or Salicylate (SA) Treatment

	Intensity of PgD1 Expression at Different Hours after Treatment		
Treatment (h)	8	24	48
Control	+[a]	+[a]	+[a]
JA	++[b]	+++[b]	+++[b]
SA	+[a]	+[a]	+[a]

Source: Adapted from Pervieux, I., Bourassa, M., Laurans, F., Hamelin, R., and Séguin, A., *Physiol. Mol. Plant Pathol.*, 64, 331, 2004.

[a] + indicates trace.
[b] ++ and +++ indicate increasing intensity of defensin transcript accumulation.

gene *CATHION1* was induced by treatment with ET, MeJA, and SA in pepper, suggesting involvement of JA/ET- and SA-signal transduction systems in pepper (Lee et al., 2000).

2.32 PERCEPTION AND TRANSDUCTION OF PATHOGEN SIGNALS IN PLANTS LEADING TO SUSCEPTIBILITY

2.32.1 Differential Expression of Signaling System Leading to Susceptibility or Resistance

We have seen so far how plants perceive pathogen signals, and how the signals are transduced through well-orchestrated signaling pathways to induce a plethora of defense genes leading to resistance. Similar sequence of perception and signal transduction processes may also be operative in susceptible interactions leading to disease development. It is now well established that elicitor molecules are present in fungal cell surface. Virulent pathogens, avirulent mutants, or even saprophytes do have elicitors in their cell wall, and these elicitors induce defense mechanisms in all kinds of plants whether they are susceptible or resistant to the pathogen (Veit et al., 2001; Grant et al., 2003; Wang et al., 2003c; Ron and Avni, 2004). Even the amount of elicitors present in the fungal cell wall does not differ much between pathogens and nonpathogens. The amounts of the two elicitors, AA and EPA, present in cell walls of both compatible and incompatible races of the oomycete pathogen *Phytophthora infestans* do not differ significantly (Creamer and Bostock, 1986, 1988). Activation of various signaling systems has been observed in both resistant and susceptible interactions (Rojas et al., 1993; Audenaert et al., 2002; Faize et al., 2004). Some plant defense response genes were also activated in both susceptible and resistant interactions. Defense response marker genes were expressed in *Arabidopsis* plants expressing both susceptible and resistant reactions (Table 2.5). However, the pattern of signal perception and transduction may vary between susceptible and resistant interactions.

When the pathogen invades plants, various signaling systems are activated or suppressed leading to resistance or susceptibility. When rust-susceptible and resistant cowpea plants were inoculated with the pathogen *Uromyces vignae*, five genes were downregulated specifically in susceptible cells, and five were specifically upregulated in inoculated, resistant cells (Mould et al., 2003). The changes in gene expression predicting the subsequent expression of

TABLE 2.5
Expression of Defense Response Marker Genes in Resistant and Susceptible *Arabidopsis* Plants Infected by *Botrytis cinerea*

	Intensity of Expression of Defense Response Genes at Different Hours after Inoculation with *Botrytis cinerea*					
	Wild-Type Plants (with *BOS1* Gene Showing Resistance)			*bos1* Mutant (with Increased Susceptibility)		
Defense Gene	0 h	36 h	60 h	0 h	36 h	60 h
PR1	−[a]	+[b]	+++[b]	−[a]	++[b]	++++[b]
PDF1.2	−[a]	+++[b]	++++[b]	−[a]	+++[b]	++++[b]

Source: Adapted from Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R., *Plant Cell*, 15, 2551, 2003.

[a] − indicates no expression.
[b] +, ++, +++, and ++++ show increased intensity of expression.

susceptibility or resistance to fungal infection occur before the fungus enters the plant cell lumen (Mould et al., 2003). The pathogen signals may trigger or suppress the expression of genes in plants. Both elicitors and suppressors have been identified in pathogen cell walls. The speed and differential action of elicitors and suppressors may result in susceptibility or resistance.

2.32.2 Slower Accumulation of Elicitor-Releasing Enzymes in Susceptible Interactions

Several elicitor molecules are present in fungal cell surface. These elicitor molecules are released from the fungal cell wall because of the action of host enzymes (Lawrence et al., 2000). Release of elicitors appears to be an important phenomenon during pathogenesis. It is now well established that a high amount of elicitor-releasing enzymes in the infection site is needed to release elicitors from the fungal pathogen cell wall to trigger defense responses. In the susceptible interactions, significant amount of accumulation of β-1,3-glucanase (the elicitor-releasing enzyme) was observed in tomato breeding lines only at 8 days after inoculation with *Alternaria solani*. By contrast, the enzyme accumulated even at 2 days after inoculation in the resistant tomato lines (Lawrence et al., 2000). Similar delayed induction of another elicitor-releasing enzyme, chitinase, in the susceptible tomato line infected with *A. solani* has been reported (Lawrence et al., 2000).

Delayed induction of lipase enzymes in susceptible potato–*Phytophthora infestans* interactions has been reported (Bostock, 1989). Cell walls of *P. infestans* contain the elicitors AA and EPA in their lipid fraction (Bostock, 1989), and lipase enzymes release these elicitors from the fungal cell wall. Potato discs were inoculated with compatible or incompatible isolates of *P. infestans*. During the first 12 h after inoculation, lipase activity was suppressed in the compatible interaction but not in the incompatible interaction (Bostock, 1989). Subsequently, the lipase activity was similar in both the compatible and incompatible interactions. The delayed activation of lipase activity in the susceptible interaction resulting in delayed release of elicitors would have favored the development of the pathogen because of reduction in the availability of the elicitors in the initial period of infection (Bostock, 1989; Ricker and Bostock, 1992). These results suggest that the delayed release of elicitor in the susceptible plants compared with that in the resistant plants would have delayed the defense response in susceptible interactions.

2.32.3 Susceptible Varieties May Release Less Amount of Elicitors from Fungal Pathogen Cell Walls

The release of elicitors from fungal cell walls in the infection site is critical in determining susceptibility or resistance. It has been demonstrated that less amount of elicitors are released in susceptible interactions compared with that in resistant ones. Fungal cell walls contain chitin and β-1,3-glucan, and both of them are important elicitors. Chitinase and β-1,3-glucanase are constitutively present in several plants, and these enzymes have been demonstrated to release these elicitors from fungal cell walls (Lawrence et al., 2000). The tomato breeding lines that are susceptible to the early blight pathogen *Alternaria solani* possessed less constitutive levels of chitinase (Figure 2.20; Lawrence et al., 2000) and β-1,3-glucanase (Figure 2.21; Lawrence et al., 2000) than that in resistant lines. Enzyme preparations from resistant and susceptible genotypes differed in their ability to release the elicitors from cell walls of *A. solani*. The enzyme preparations of resistant breeding lines released the elicitors, whereas enzyme preparations from susceptible lines did not (Lawrence et al., 2000). These results suggest that the amount of elicitor-releasing enzymes is the critical factor in deciding

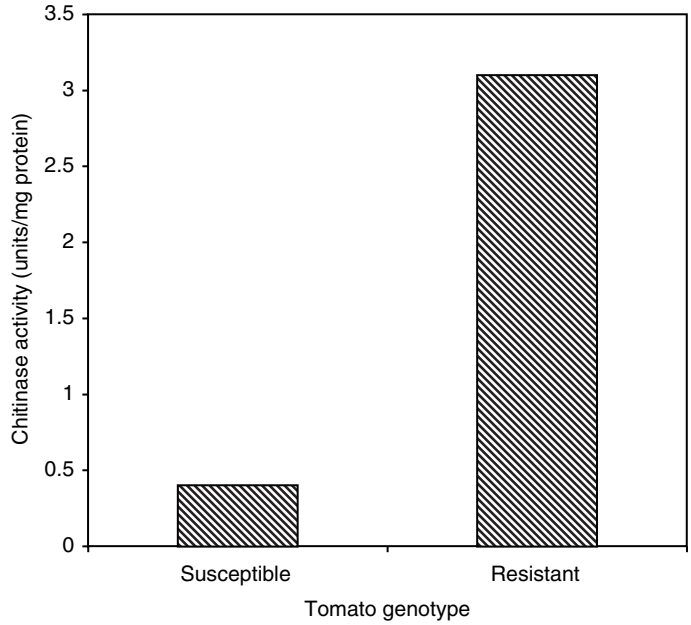

FIGURE 2.20 Constitutive chitinase activity in susceptible and resistant tomato genotypes. (Adapted from Lawrence, C.B., Singh, N.P., Qiu, J., Gardner, R.G., and Tuzun, S., *Physiol. Mol. Plant Pathol.*, 57, 211, 2000.)

the type of disease reaction. There was positive correlation between the amount of β-1,3-glucanase applied to the fungal mycelium of *Phytophthora sojae* and the amount of elicitor released from it (Figure 2.22; Ham et al., 1991).

Exogenous application of β-1,3-glucanase on soybean plants induced the phytoalexin glyceollin synthesis (Yoshikawa et al., 1990). The amount of β-1,3-glucanase applied to

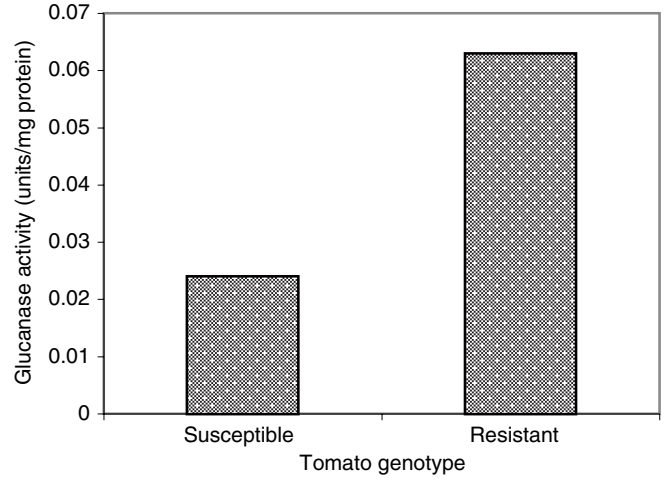

FIGURE 2.21 Constitutive β-1,3-glucanase activity in susceptible and resistant tomato genotypes. (Adapted from Lawrence, C.B., Singh, N.P., Qiu, J., Gardner, R.G., and Tuzun, S., *Physiol. Mol. Plant Pathol.*, 57, 211, 2000.)

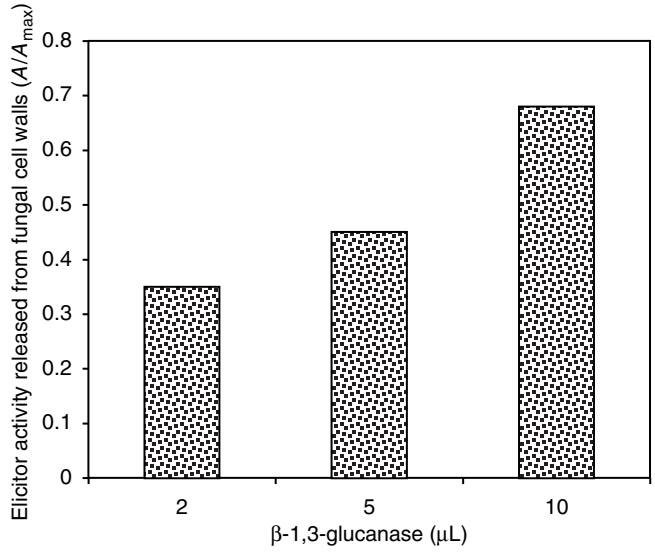

FIGURE 2.22 Efficacy of increased amount of β-1,3-glucanase in releasing increased amount of elicitor from fungal mycelial cell walls. (Adapted from Ham, K.S., Kauffmann, S., Albersheim, P., and Darvill, A.G., *Mol. Plant Microbe Interact*., 4, 545, 1991.)

inoculation sites determined the amount of phytoalexins accumulated in soybean (Yoshikawa et al., 1990). ET increased 50-fold to 100-fold the level of β-1,3-glucanase mRNA (Takeuchi et al., 1990). Exogenous ET increased the level of β-1,3-glucanase and the accumulation of the phytoalexin glyceollin (Yoshikawa et al., 1990). All these studies suggest that the process of elicitor release mediated by host β-1,3-glucanase is an important process leading to defense response. In susceptible interactions, enough quantity of β-1,3-glucanase may not be available to release the elicitors from the pathogen, and hence it results in susceptibility.

The quantity of elicitors available for induction of defense mechanisms may also determine susceptibility or resistance. When large amount of extracellular matrix (ECM) was released from the conidia of the nonpathogens *Blumeria graminis* f. sp. *tritici* and *Erysiphe pisi*, the nonpathogens induced more resistance against the pathogen *B. graminis* f. sp. *hordei* than when a small amount of ECM was released from their conidia in barley leaves (Fujita et al., 2004).

Higher concentration of the elicitor isolated from *Pyricularia oryzae* induces higher chitinase activity in rice leaves (Schaffrath et al., 1995). Peroxidase and cinnamyl-alcohol dehydrogenase activities in rice leaves were also increased with increase in concentration of the elicitor (Schaffrath et al., 1995). If additional elicitor is infiltrated into rice leaves, even the susceptible rice variety becomes resistant to *P. oryzae*, probably due to higher amounts of elicitor present in the intercellular spaces of the host tissue during penetration of the pathogen (Schaffrath et al., 1995).

Activity of glucomannan elicitor isolated from *Phytophthora sojae* increased with increase in its concentration (Keen et al., 1983). Increased induction of coumarin phytoalexins in parsley cells with increased concentration of glucan elicitor from *P. sojae* has been reported (Davis and Hahlbrock, 1987). Similar concentration-dependent induction of phytoalexins in bean has been reported with the elicitor isolated from *Colletotrichum lindemuthianum* (Tepper and Anderson, 1990). These studies suggest that in susceptible interactions, quantity of elicitors released may not be sufficient to induce host defense responses.

2.32.4 Delayed Release of Elicitors in Susceptible Interactions

During successful pathogenesis, the elicitor may not be released or the release may be delayed. A rise in LOX activity was observed during the incompatible interaction in cotyledonary leaves of coffee (*Coffea arabica*) infected with the rust fungus *Hemileia vastatrix* (Rojas et al., 1993). Similar increase was not observed during the compatible interaction. However, an elicitor preparation from germ tubes of the rust fungus stimulated a rise in LOX activity in both resistant and susceptible cultivars (Rojas et al., 1993). The results suggest that the elicitor can induce defense activity in both the susceptible and the resistant varieties, but the fungus could induce it only in the resistant variety. Hence, the release of elicitor from the fungal cell wall may be important in determining the susceptibility or resistance of a host.

The release of elicitors from fungal wall into the host tissues was well documented in potato infected with *Phytophthora infestans* (Ricker and Bostock, 1992). The fungal sporangia were radiolabeled with ^{14}C-arachidonic acid to trace the release of the elicitor into potato leaf tissues. Microautoradiography of these sporangia revealed that radioactivity was distributed throughout fungal cytoplasm and cell walls, and was often associated with lipid bodies. Potato leaves were inoculated with the radiolabeled sporangia of both compatible and incompatible races. At 3 and 6 h after inoculation, radioactivity was not observed within the leaf tissue but was restricted to sporangia. By 9–10 h after inoculation, most sporangia of both races had germinated and had formed appressoria, but there was little or no ramification of infection hyphae into leaf tissue. In the compatible interaction, the label was detected only by 12 h after inoculation, whereas in the incompatible interaction the label was found consistently in plant tissue at 9 h after inoculation itself. By 12 and 14 h after inoculation, the radioactivity was present throughout the tissue sections in both interactions and no difference between compatible and incompatible interactions could be seen (Ricker and Bostock, 1992).

Hence, the only difference between susceptible and resistant reactions was a 3 h delay in release of elicitor in the former interaction. If this delay is compensated by providing the elicitor at an early period of infection process, even the susceptible interaction can become a resistant one. It has been demonstrated that pretreatment of potato tuber slices with AA or EPA suppressed colonization of the tubers by compatible races of *P. infestans* (Bostock et al., 1986).

Similar observations have been reported in rice–*M. grisea* interactions (Schaffrath et al., 1995). The elicitor isolated from *M. grisea*, the blast pathogen, was infiltrated into the intercellular spaces of leaves of a susceptible rice cultivar. After 4 h, the leaves were inoculated with a virulent race of *M. grisea*. There was a significant reduction of blast symptoms on elicitor-treated leaves to water-infiltrated leaves (Schaffrath et al., 1995). The growth of the pathogen was stopped by early induction of defense mechanisms by infiltrating the elicitor in the genetically susceptible rice cultivar, and this response was similar to that observed in the pathogen-inoculated genetically resistant cultivar without any previous treatment with the elicitor (Schaffrath et al., 1995). The results suggest that only due to nonrelease of elicitor in the initial stages of infection, the susceptible cultivar is invaded by the pathogen.

2.32.5 Elicitor of Compatible Pathogens Induces Less Defense-Related Actions than That of Incompatible Pathogens

Elicitors are produced by all pathogens, irrespective of whether they are virulent or avirulent. Three powdery mildew fungi, *Blumeria graminis* f. sp. *hordei* (the barley pathogen), *B. graminis* f. sp. *tritici* (the wheat pathogen), and *Erysiphe pisi* (the pea pathogen) release extracellullar matrix (ECM), which is known to contain elicitors of defense response (Fujita et al., 2004). The elicitor released by each pathogen differed. The ECM released by *B. graminis*

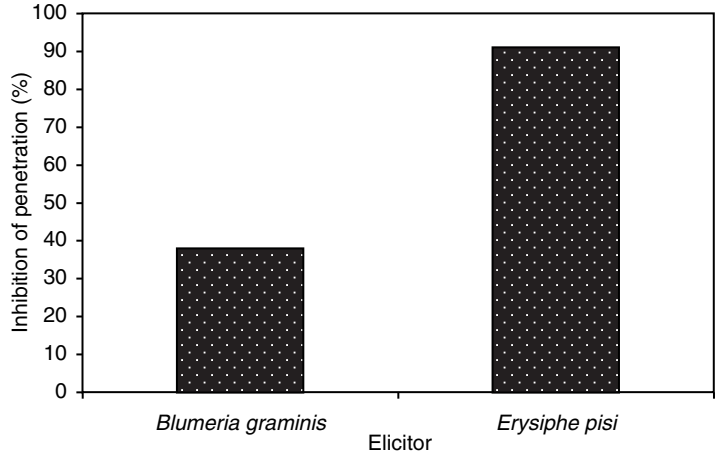

FIGURE 2.23 Effect of elicitors of the nonpathogen *Erysiphe pisi* and the pathogen *Blumeria graminis* f. sp. *hordei* on inducing resistance against *B. graminis* f. sp. *hordei* in barley. (Adapted from Fujita, K., Suzuki, T., Kunoh, H., Carver, T.L.W., Thomas, B.J., Gurr, S., and Shiraishi, T., *Physiol. Mol. Plant Pathol.*, 64, 169, 2004.)

f. sp. *hordei* induced less defense reactions compared with that released by the incompatible pathogen *E. pisi*. Only negligible percentage of inhibition of penetration of the barley coleoptiles by the pathogen was observed, whereas most of the germlings of *E. pisi* failed to penetrate the barley coleoptile cells (Figure 2.23; Fujita et al., 2004). It suggests that the potential of elicitors from compatible and incompatible pathogens in triggering defense responses may vary.

2.32.6 Degradation of Fungal Elicitors by Plant Enzymes in Plant Tissues May Lead to Susceptibility

The fungal (exogenous) elicitors are released and they are found to accumulate in the apoplast tissues during pathogenesis. However, these fungal elicitors are degraded by host enzymes forming inactive derivatives in the plant tissues. These degradation processes may result in susceptibility. When the yeast extract-elicitor was incubated in the medium containing rice cells, the elicitor activity disappeared in time-dependent manner (Felix et al., 1991). Degradation of the elicitor resulted in suppression of the induced defense mechanisms in rice cells (Felix et al., 1991).

Constitutive wheat leaf endochitinases capable of releasing elicitor-active oligosaccharides from chitin hydrolyse elicitor-active chitinoligomers to inactive smaller oligomers. When chitin was administered to wounded wheat leaves, *N*-acetyl glucosamine (GlcNAc, chitin monomer) started accumulating. The monomer became the predominant product at 12 h after treatment (Table 2.6; Barber and Ride, 1994). At the end of the 12 h period, approximately 2% by weight of the applied chitin had been solubilized in wheat leaves. Of this amount, the elicitor-inactive oligomers chitin monomer (GlcNAc), dimer [$(GlcNAc)_2$], and trimer [$(GlcNAc)_3$] accounted for 84% on a molar basis. Extremely little of the applied chitin polymer, only 0.03% by weight, was actually present within the leaf in the form of the known elicitor-active tetramer, pentamer, and hexamer [$(GlcNAc)_{4-6}$] by the end of the 12 h period (Barber and Ride, 1994).

The degradation of the fungal elicitor may be due to host enzymes. It has been demonstrated that elicitor activity of the chitin tetramer can be totally abolished by prolonged

TABLE 2.6
Degradation of Chitin into Smaller Fragment Oligomers in Wheat Leaves Treated with Chitin

	Oligosaccharide Level (pmol/Wound)					
Time (h) after Chitin Treatment	Chitin Monomer (GlcNAc)	Chitin Dimer $(GlcNAc)_2$	Chitin Trimer $(GlcNAc)_3$	Chitin Tetramer $(GlcNAc)_4$	Chitin Pentamer $(GlcNAc)_5$	Chitin Hexamer $(GlcNAc)_6$
0	0	0	0	0	0	0
8	640	219	200	169	85	38
12	1109	323	315	249	107	44

Source: Adapted from Barber, M.S. and Ride, J.P., *Physiol. Mol. Plant Pathol.*, 45, 37, 1994.

exposure to wheat leaf chitinases (Barber et al., 1989). The degradation of elicitor may be high in later stages of the infection process. For example, it was observed that chitinase, the fungal chitin-degrading enzyme, was highly expressed in the susceptible interaction, compared with that in the resistant interaction, when analyses were made at 6 days after inoculation with *Peronospora tabacina* in tobacco (Figure 2.24; Tuzun et al., 1989). Probably, the degraded inactive elicitor fragments may nullify the induction of defense responses; this probability has not been experimentally demonstrated.

2.32.7 Fungal Pathogens May Degrade Host Elicitors during Susceptible Interactions

Endogenous elicitors of host origin are oligomers of pectic fragments. Polygalaturonides with a DP of about 20 (Bishop et al., 1984), 9–12 galacturonosyl units (Robertsen, 1986), or

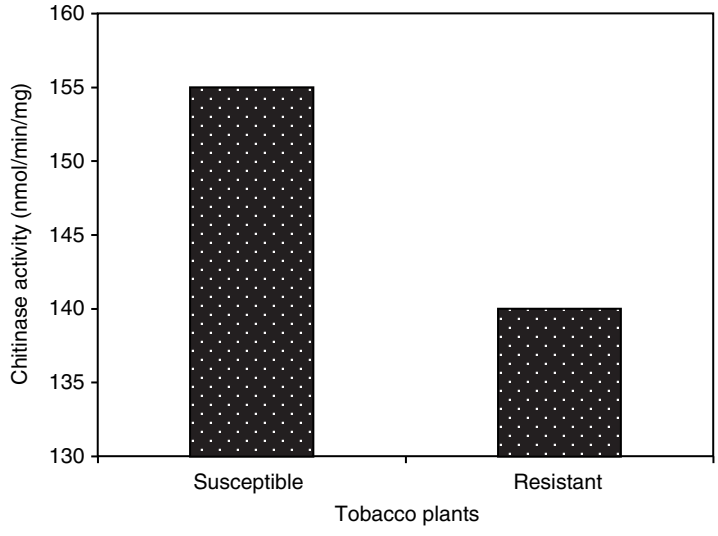

FIGURE 2.24 Chitinase activity in susceptible and resistant tobacco plants after inoculation with *Peronospora tabacina*. (Adapted from Tuzun, S., Rao, M.N., Vogeli, U., Schardl, C.I., and Kuc, J., *Phytopathology*, 79, 979, 1989.)

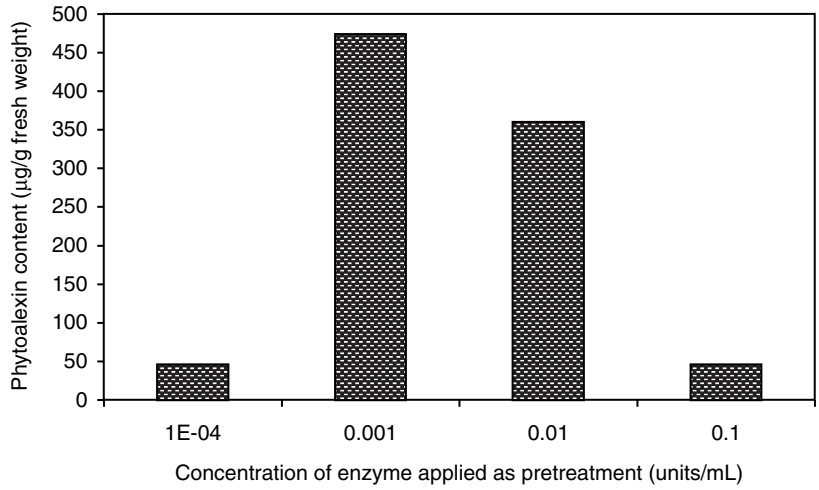

FIGURE 2.25 Role of different concentrations of *Botrytis cinerea* enzyme in induction of phytoalexin in carrot. (Adapted from Movahedi, S. and Heale, J.B., *Physiol. Mol. Plant Pathol.*, 36, 303, 1990.)

nonamers through pentadecamers (Jin and West, 1984) are considered as elicitors, and oligomers of galacturonic acid with a DP of less than 9 had little or no elicitor activity (Hahn et al., 1981; Lorenzo et al., 1990). The elicitor-active pectic fragments are produced because of partial digestion of the host polygalacturonic acid by fungal enzymes. In susceptible tissues, pathogens would have produced more of these pectic enzymes and higher concentration of these enzymes would have degraded the endogenous elicitors into inactive galacturonic acids with low DP. Induction of pectic enzymes in susceptible reactions is much higher than that in resistant reactions (Vidhyasekaran, 1972, 1974a,b).

Endo-PL produced by *Botrytis cinerea*, a pathogen of carrot, induced phytoalexin synthesis in carrot. Lower concentrations of 0.001 and 0.1 units/mL of the pectic enzyme induced high amounts of phytoalexin content, whereas high amounts of the enzyme (0.1 units/mL and still higher concentrations of 1.0 and 10.0 units/mL) did not induce any significant accumulation of the phytoalexin (Figure 2.25; Movahedi and Heale, 1990). These results suggest that the pathogen by overproducing the pectic enzyme in the susceptible interaction may generate highly degraded pectic fraction, which would be inefficient in induction of host defense responses.

2.32.8 Elicitors May Be Released during Pathogenesis but May Not Be Active or Less Active in Susceptible Plants

Release of fungal elicitors in the infection court is very important in inducing defense responses in plants. In some host–pathogen interactions, elicitors may be released in the susceptible plant tissue in abundance. In fact, many studies have revealed that it is difficult to detect the elicitors in plant tissues in the resistant interactions. However, the elicitors may not be active in the susceptible plants. A glycoprotein elicitor has been isolated from germ tube walls of wheat stem rust (*Puccinia graminis* f. sp. *tritici*) uredosporelings (Kogel et al., 1988; Beissmann and Reisener, 1990). When the pathogen was inoculated on primary leaves of a susceptible wheat cultivar, elicitor activity was detected in IWF (Beissmann et al., 1992). Elicitor activity was detected in IWF from the compatible interaction of various wheat cultivars such as Prelude, Kanzler, Little Club, and Ares with *P. graminis* race 32 at 6 days after inoculation. However, no activity was found in IWF from incompatible

interactions of race 32 and the resistant cultivars Feldkrone and Prelude. The results suggest that detectable amount of elicitors are released specifically in the susceptible interactions. Probably enough quantity of elicitor for extraction is produced only when large amount of mycelium accumulates, which can occur only in susceptible interactions. In the resistant reactions, reduced fungal growth occurs and hence only trace amount of elicitor may be present in the IF, which the authors could not have extracted. Although the elicitor was not detected in the infected resistant tissues, the elicitor induced more defense-related PAL activity in resistant variety compared with the susceptible variety (Beissmann et al., 1992).

Elicitors of resistance responses have been isolated from basidiospore-derived infection structures of *Uromyces vignae*, the cowpea (*Vigna unguiculata*) rust pathogen (Chen and Heath, 1990). IWF from teliospore-inoculated leaves of a susceptible variety showed appreciable elicitor activity when tested on a resistant variety. However, no appreciable elicitor activity was observed when it was tested on a susceptible variety (Figure 2.26; Chen and Heath, 1992). The results show that the elicitor may be released in the susceptible variety during pathogenesis; but it may not be active on the susceptible cultivar (Chen and Heath, 1992). The elicitor is active only in the incompatible plant–fungus interaction.

An elicitor from *Cladosporium fulvum* accumulated only in tomato leaves of susceptible cultivars infected with the pathogen. The elicitor was detected in apoplastic fluids from susceptible cultivars infected with a compatible race of the pathogen (DeWit and Spikman, 1982). The elicitor was detected 8–10 days after inoculation of the tomato plants. Mannitol is a specific reserve sugar of *C. fulvum* and is used as a marker of fungal biomass. The appearance of the elicitor coincided with the time when mannitol started to accumulate (Figure 2.27; Schottens-Toma and DeWit, 1988). The results indicate that the elicitor could be detected only when the amount of accumulated fungal mycelium in the plant tissues is sufficient to secrete enough elicitors that can be experimentally detected. The elicitor was not detectable in apoplastic fluids from resistant interactions, probably because of only a trace amount of mycelium, which can be detected in any resistant interaction. Although the elicitor accumulated in compatible *C. fulvum* interactions, the elicitor isolated from the infected susceptible plants did not induce defense responses in susceptible interactions

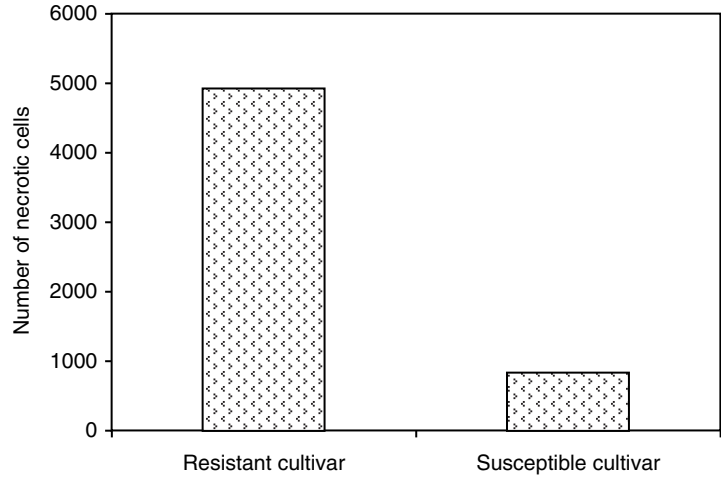

FIGURE 2.26 Specificity of *Uromyces vignae* elicitor inducing necrosis in cowpea cultivars. (Adapted from Chen, C.Y. and Heath, M.C., *Physiol. Mol. Plant Pathol.*, 40, 23, 1992.)

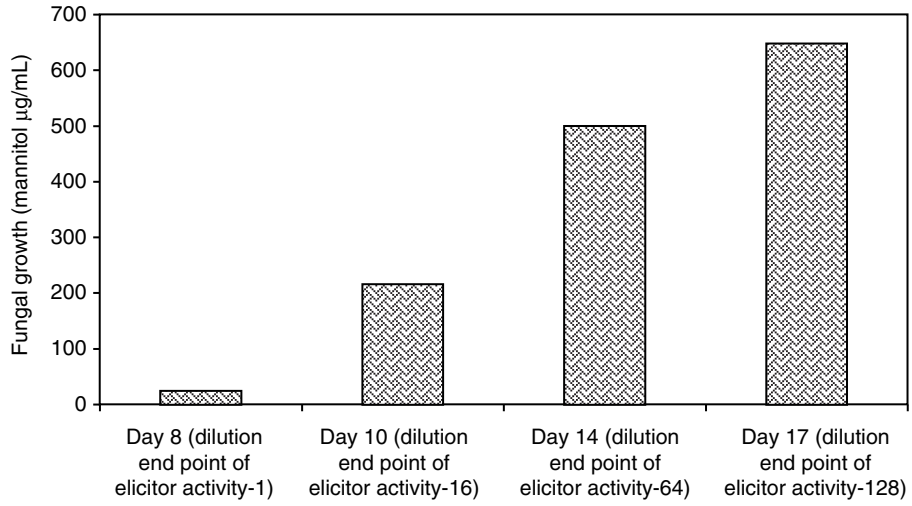

FIGURE 2.27 Relationship between elicitor production and fungal growth in tomato leaves inoculated with *Cladosporium fulvum*. (Adapted from Schottens-Toma, I.M.J. and DeWit, P.J.G.M., *Physiol. Mol. Plant Pathol.*, 33, 59, 1988.)

(Schottens-Toma and DeWit, 1988). These studies suggest that the elicitors are released during the compatible interaction without eliciting defense mechanisms.

2.32.9 Some Elicitors Do Not Act or Show Little Activity on Susceptible Cultivars

In the susceptible interaction, some elicitors may not be active in inducing host defense response, although they may trigger defense-related genes rapidly in the resistant plants. The galactoglucomannan elicitor isolated from *Colletotrichum lindemuthianum* induced accumulation of phytoalexins in bean cultivar resistant to the pathogen and it did not induce any phytoalexin in the susceptible cultivar (Table 2.7; Tepper et al., 1989).

An elicitor from *Phytophthora sojae* was less active on susceptible soybean variety (Keen and Legrand, 1980). Keen et al. (1983) purified the elicitor and found it to be glucomannan. The glucomannan induced less phytolaexin in the susceptible variety, whereas it induced substantial amount of phytolaexin in the incompatible variety.

Race-specific elicitors of resistance have been isolated from appressorium-bearing basidiospore germlings of different races of the cowpea rust fungus (*Uromyces vignae*) (Chen and Heath, 1990). The cowpea cultivar Dixie Cream was susceptible to race 2 of the fungus,

TABLE 2.7
Inability of Elicitor of *Colletotrichum lindemuthianum* to Induce Phytoalexin Accumulation in Bean Cultivars Susceptible to *C. lindemuthianum*

	Phytoalexins (µg/Cotyledon)		
Bean Cultivar	**Phaseollin**	**Phaseollin-Isoflavan**	**Kievitone**
Susceptible	0	0	0
Resistant	4.64	4.30	16.8

Source: Adapted from Tepper, C.S., Albert, F.G., and Anderson, A.J., *Physiol. Mol. Plant Pathol.*, 34, 85, 1989.

whereas it was resistant to race 1. When the elicitor isolated from race 2 was injected into the cultivar Dixie Cream, the defense-related necrosis of mesophyll cells was not significantly induced. However, when the elicitor from race 1 was injected into the same cultivar, the defense-related cell necrosis response was rapidly expressed (Chen and Heath, 1990). The results suggest that some elicitors may not be active in susceptible plants because of their race-specificity (Figure 2.28; Chen and Heath, 1990).

Some of the elicitors act selectively on some varieties, and in other varieties, these elicitors did not induce any defense response. Elicitors isolated from the wheat leaf rust fungus, *Puccinia recondita* f. sp. *tritici*, and the wheat stem rust fungus, *P. graminis* f. sp. *tritici*, induced HR in a group of wheat cultivars although this group of cultivars was variable in disease reaction (Deverall and Deakin, 1987; Sutherland et al., 1989).

Colletotrichum graminicola is a pathogen of maize. It is unable to infect sorghum. The extracts of conidia and the conidial mucilage of *C. graminicola* contained materials that elicited the accumulation of deoxyxanthocyanidin phytoalexins in sorghum mesocotyls and juvenile sorghum leaves. The elicitor preparations did not elicit any defense response in maize (Yamaoka et al., 1990), suggesting that the elicitor may not have any function in susceptible plants. A glycoprotein elicitor of *Phytophthora sojae* elicited phytoalexin only in parsley (nonhost) cells and not in soybean (host) cells (Parker et al., 1988).

The tomato pathogen *Cladosporium fulvum* races 4 and 5 (Gelli et al., 1997), the soybean pathogen *Phytophthora sojae* (Shan et al., 2004), the barley pathogen *Rhynchosporium secalis* (Schürch et al., 2004), the cowpea pathogen *Uromyces vignae* (Chen and Heath, 1993), and the wheat pathogen *Puccinia recondita* f. sp. *tritici* (Sutherland et al., 1989; Saverimuttu and Deverall, 1998) produce elicitors, which elicit defense responses only in resistant cultivars.

Specificity of some general elicitors in eliciting defense responses in some specific cultivars has also been reported. The protein elicitor elicitin from *Phytophthora parasitica* induced defense responses only in specific *Raphanus sativus* and *Brassica campestris* cultivars (Kamoun et al., 1993b). The elicitor xylanase has been reported to be produced by *Fusarium oxysporum* f. sp. *pisi* and *Macrophomina phaseolina* (Dean et al., 1989). It elicits plant defense responses only in specific cultivars of tomato and tobacco (Ron et al., 2000; Ron and Avni, 2004).

The absence or reduced action of some elicitors in the susceptible cultivars may be due to absence or reduced presence of receptor molecules for binding the available elicitor molecules

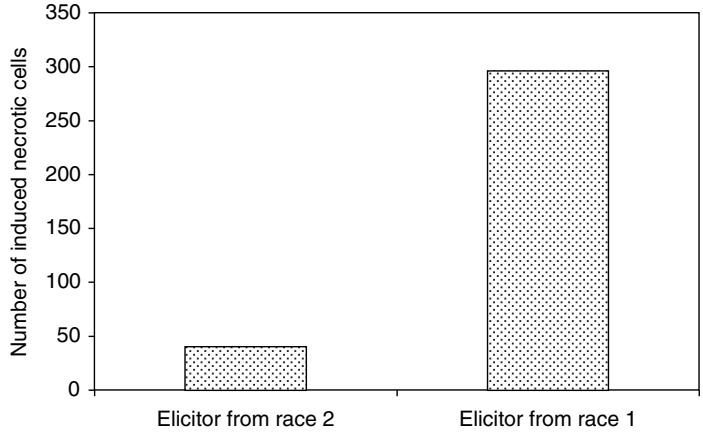

FIGURE 2.28 Role of race-specific elicitors from *Uromyces vignae* races in induction of defense response in cowpea cultivar Dixie Cream. (Adapted from Chen, C.Y. and Heath, M.C., *Physiol. Mol. Plant Pathol.*, 37, 169, 1990.)

in those susceptible cultivars. Several studies have indicated that receptors (binding sites) for the fungal elicitors may exist in host plant cell membranes (Montesano et al., 2003). Binding sites for specific elicitors have been identified in many host plants. The presence of elicitor-binding sites with a high affinity for the protein elicitor cryptogein in tobacco cells (Blein et al., 1991), and for chitin oligomers in rice, wheat, and tomato cells (Shibuya et al., 1993; Baureithel et al., 1994; Ito et al., 1997; Day et al., 2001; Okada et al., 2002) has been reported.

Lectins specific for GlcN have been shown to be involved in binding of chitosan in plant cells (Lièнart et al., 1991). The presence of specific binding sites for glucan elicitors in soybean (Cheong et al., 1993; Umemoto et al., 1997; Fliegmann et al., 2004) and for elicitin elicitors in tobacco cells has been reported (Baillieul et al., 2003). Binding sites for Pep-13 peptide elicitor of *Phytophthora sojae* in the plasma membrane of parsley cells (Nennstiel et al., 1998), AVR9 peptide elicitor of *Cladosporium fulvum* in tomato (Nennsteil et al., 1998), and Pgt-glycoprotein elicitor of *Puccinia graminis* in wheat (Wendehenne et al., 1995) have been identified. It is possible that such specific binding sites may not be present in susceptible plants; however, experimental evidences for this possibility are available only in very few cases. Xylanase elicitors trigger defense responses only in specific tomato and tobacco cultivars. Binding sites for these xylanase elicitors have been reported to be present only in those tomato and tobacco cultivars, which respond to the xylanase elicitors (Hanania and Avni, 1997). The resistance (*R*) genes themselves may encode receptors (Ron and Avni, 2004). Tomato *Ve* disease resistance genes encode cell surface-like receptors (Kawchuck et al., 2001). The *Arabidopsis* resistance gene, *NHL3*, encodes a plasma membrane receptor protein (Varet et al., 2003).

It is also possible that the receptor sites may be present in the susceptible plants, but the binding would have been inhibited by some metabolites. The activity of elicitor from *Phytophthora sojae* was shown to be inhibited by certain methyl sugar derivatives, which were presumed to act by competing for elicitor-binding sites (Ayers et al., 1976). Methyl glycosides inhibited the activity of elicitor from *P. infestans* presumably by competing for the binding site (Marcan et al., 1979).

2.32.10 Speed of Expression of Signal Transduction System May Determine Susceptibility or Resistance

Pathogen signals, specifically nonspecific general elicitors, induce defense responses even in susceptible interactions (Faize et al., 2004). The signal transduction systems detected in resistant interactions are also detected in susceptible interactions. However, the speed with which the signaling pathway is expressed in the resistant interaction may be different from the susceptible interaction. SA accumulates rapidly within 2–3 days after inoculation with *Venturia nashicola* in resistant pear cultivar. By contrast, SA level did not increase till 5 days after inoculation with the pathogen in the susceptible cultivar; but at 7 days after inoculation, SA accumulated in the susceptible interaction much more than in the resistant interaction (Figure 2.29; Faize et al., 2004). Accumulation of SA triggers the SA-dependent signaling system, which is the key signal transduction pathway triggering expression of defense genes. Delayed expression of this pathway may delay the accumulation of defense compounds, which may facilitate the pathogen to overcome this defense response and lead to the disease development.

2.32.11 Reduced Accumulation of Signals May Lead to Susceptibility

In *Arabidopsis*, SA accumulates in both susceptible and resistant plant–pathogen interactions (Zhou et al., 1998b; Cameron et al., 1999). However, only reduced levels of SA accumulation

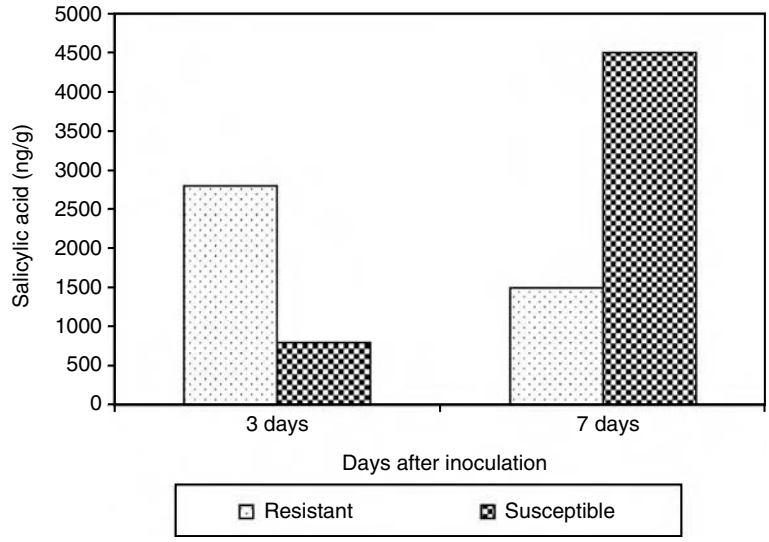

FIGURE 2.29 Accumulation of salicylic acid in pear leaves of resistant and susceptible cultivars inoculated with *Venturia nashicola*. (Adapted from Faize, M., Faize, L., Ishizaka, M., and Ishii, H., *Physiol. Mol. Plant Pathol.*, 64, 319, 2004.)

were observed in the susceptible reaction, and SA-inducible phytoalexin and PR proteins also accumulated only at reduced levels in the susceptible interaction compared with that in the resistant interaction (Zhou et al., 1998b). SA accumulation in compatible interaction did not lead to SAR, which was commonly detected in incompatible interactions (Cameron et al., 1999). These results suggest that the SA signal may be induced in both susceptible and resistant responses, and the reduced accumulation of the SA signal in the compatible reaction would not be sufficient to trigger the defense reaction. A threshold level of signal accumulation may be needed to trigger defense pathway. It was proved by the studies of Yalpani et al. (1991) in tobacco. Excised healthy leaves were fed SA for 72 h through the cut petiole and PR-1 protein levels were analyzed in opposite half-leaves. The level of SA in a leaf was proportional to the concentration of SA in the solution in which the petiole was immersed. Induction of PR-1 proteins was positively correlated with leaf SA. The average basal level of SA in control leaves was 34 ng of SA per gram fresh weight. A 59% increase in tissue SA levels, to 54 ng/g fresh weight, caused detectable induction of PR-1a in the extracellullar fluid. It clearly demonstrated that a threshold concentration of SA is needed to induce the expression of the defense gene, *PR-1*.

2.32.12 Elicitors May Induce Genes Involved in Suppression of Defense-Related Genes in Susceptible Interactions

Although elicitors have been shown to induce several defense-related genes in both resistant and susceptible interactions, they may also induce susceptibility-related genes. An elicitor of the rice sheath blight pathogen *Rhizoctonia solani* induced production of H_2O_2 and O_2^- in cultured rice cells within 10 min after elicitor treatment (Velazhahan and Vidhyasekaran, 1999). The elicitor also increased ascorbic acid content in the rice cells (Figure 2.30; Velazhahan and Vidhyasekaran, 1999).

Ascorbic acid plays an important role in the ROS-scavenging pathways in plants, which include ascorbate–glutathione cycle and catalase (Smirnoff, 2000; Barth et al., 2004). Ascorbic acid is an antioxidant and the antioxidant suppresses the oxidative burst (Vera-Estrella

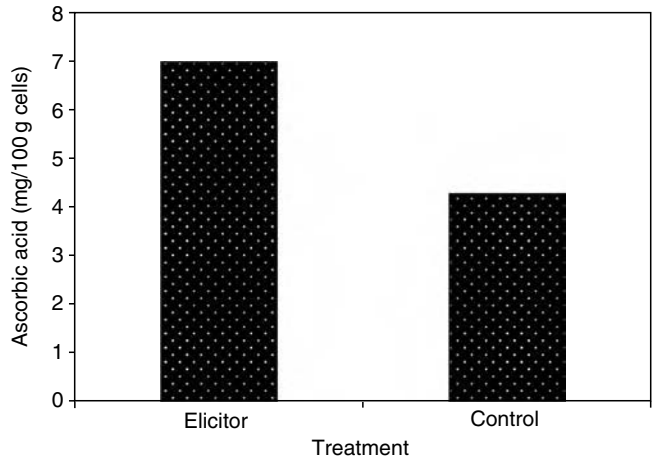

FIGURE 2.30 Ascorbic acid content in rice cells treated with *Rhizoctonia solani* elicitor. (Adapted from Velazhahan, R. and Vidhyasekaran, P., *Acta Phytopathol. Entomol. Hungarica*, 34, 187, 1999.)

et al., 1993). Ascorbic acid reacts rapidly with O_2^- and helps to remove H_2O_2 (Bradley et al., 1992). Suppression of ROS by ascorbic acid may result in suppression of the defense gene activation. Ascorbic acid reduced the elicitor-inducible phenolic synthesis in rice (Velazhahan and Vidhyasekaran, 1999) and tomato cells (Vera-Estrella et al., 1993). Ascorbic acid inhibited the oxidation of phenolics in epicotyls of *Vigna angularis* (Takahama, 1993) and spinach leaves (Takahama and Oniki, 1992). The oxidation of coniferyl alcohol by peroxidase leads to synthesis of lignin, another important defense-related compound (Ye et al., 1990).

Ascorbic acid is a cosubstrate of many enzymes, such as ascorbate peroxidase, which degrades H_2O_2, and 2-oxoacid-dependent dioxygenases, which are involved in the biosynthesis of ABA (Arrigoni and Du Tullio, 2002). Ascorbic acid is a cofactor for 1-aminocyclopropane-1-carboxylate oxidase that forms ET (Dong et al., 1992). By regulating H_2O_2, ABA, and ET, the ascorbic acid would have induced susceptibility.

Pastori et al. (2003) showed that many defense genes, particularly those that encode PR proteins, were activated in the *Arabidopsis* mutant [*vtc1* (vitamin c-1)] deficient in biosynthesis of ascorbic acid. ABA is an important signal molecule inducing *PR* genes in many plants including rice (Agrawal et al., 2001). ABA contents were significantly higher in *vtc1* than in wild type (Pastori et al., 2003). The elevated levels of ascorbic acid in the wild plants would have suppressed the induction of defense genes, probably by suppressing ABA synthesis, conferring susceptibility to pathogens.

The *Arabidopsis vtc1* mutant deficient in ascorbic acid showed increased resistance to the *Peronospora parasitica* (Figure 2.31; Barth et al., 2004). When the ascorbate-deficient mutants were inoculated with the bacterial pathogen, induction of PR proteins PR-1 and PR-5 was significantly higher than that in the wild type, which has high ascorbic acid content (Barth et al., 2004). Elevated SA levels were also detected in the ascorbic acid-deficient mutant (Barth et al., 2004). These results suggest that ascorbic acid is involved in suppression of defense genes.

This type of dual functions of elicitors in inducing susceptibility and resistance has been suggested for the fungal elicitor 1,3-1,6-glucan (Mithöfer et al., 1996a) and a yeast glycopeptide elicitor (Basse and Boller, 1992). The bacterial elicitor harpin also shows this type of dual functions (Wei et al., 1992; Vidhyasekaran, 2002). These results suggest that the elicitors may induce several genes and not exclusively defense genes. When tobacco-cultured cells were treated with the elicitor cryptogein, 20 polypeptides increased in intensity, whereas the amounts of three other polypeptides decreased (Suty et al., 1995). These observations

FIGURE 2.31 Role of ascorbic acid in inducing susceptibility to *Peronospora parasitica* in *Arabidopsis*. (Adapted from Barth, C., Moeder, W., Klessig, D.F., and Conklin, P.L., *Plant Physiol*., 134, 1784, 2004.)

suggest that the elicitor may induce susceptibility-related responses in susceptible interaction, nullifying the induced defense-related responses in these plants.

2.32.13 Suppressors Negating Elicitor-Induced Defense Responses in Susceptible Interactions

Fungal pathogens may produce two types of signal molecules; the first one is the elicitor triggering defense responses and the other one is the suppressor, which suppresses the elicitor-induced defense genes. The action of suppressor may result in disease (susceptibility). The suppressors inhibit or delay active defense mechanisms of host plants. This delay would provide the pathogen with sufficient time to enter host tissues and establish successful colonization (Kessmann and Barz, 1986; Arase et al., 1989). Suppressors have been detected in exudates of the bean rust fungus (*Uromyces phaseoli* var. *typica*), which suppressed the deposition of silicon-containing deposits in French bean leaves, which would otherwise prevent the development of the first haustorium (Heath, 1980, 1981).

The pea (*Pisum sativum*) pathogen *Mycosphaerella pinodes* secretes two well-characterized fungal signal molecules, a glycoprotein elicitor and a polypeptide suppressor. Both these molecules could be detected in the spore germination fluids (Shiraishi et al., 1978; Oku et al., 1980). The molecular weight of the elicitor was approximately 70,000 Da, whereas that of the suppressor was 5,000 Da. The glycoprotein elicitor was shown to trigger several defense-related responses in pea, including activation of genes leading to generation of superoxide (Kiba et al., 1997), protein phosphorylation (Toyoda et al., 1992, 1993b), and accumulation of PR proteins (Yoshioka et al., 1992b) and phytoalexins (Yamada et al., 1989; Toyoda et al., 2000). The suppressor of *M. pinodes* was purified into two active components. These two suppressors were identified as GalNAc-*O*-Ser-Ser-Gly (supprescin A) and Gal-GalNAc-*O*-Ser-Ser-Gly-Asp-Glu-Thr (supprescin B) (Shiraishi et al., 1992). The suppressors inhibit the defense signaling evoked by *M. pinodes* elicitors, including cell wall- and plasma membrane-ATPases, protein kinases, and phosphoinositide metabolism (Toyoda et al., 1992; Shiraishi et al., 1997).

The concomitant presence of the suppressor with the elicitor negates the activity of the elicitor (Hiramatsu et al., 1986). Pea epicotyl segments were treated with the elicitor alone or in the concomitant presence of the suppressor (elicitor + suppressor) (Yamada et al., 1989). The apparent activation of pisatin biosynthesis was initiated approximately 6 h after treatment with the elicitor, and pisatin accumulation peaked at about 36 h, followed by a gradual decline. In presence of the suppressor, however, the apparent activation of pisatin biosynthesis was delayed by 6–9 h compared with the treatment with elicitor alone, although the pattern of pisatin accumulation had similar kinetics (Yamada et al., 1989). The activation of PAL activity had similar kinetics in elicitor-treated and elicitor plus suppressor-treated tissues, except for a delay of about 6 h as observed in the pattern of pisatin biosynthesis (Yamada et al., 1989). Elicitor treatment increased pea PAL mRNA at about 1 h after elicitor treatment, whereas a dramatic increase in the PAL mRNA was first observed about 4 h after elicitor plus suppressor treatment (Yamada et al., 1989). A significant increase in the chalcone synthase (CHS) mRNA was observed about 1 h after elicitor treatment and about 4 h after elicitor plus suppressor treatment, which is a pattern very similar to that observed in PAL mRNA (Yamada et al., 1989). These results suggest that the suppressors may delay the expression of defense genes induced by the elicitor. The suppressor may be acting at or before the transcriptional level for the genes coding for the key enzymes, PAL and CHS, leading to phytoalexin production. The limited time of the delay of the defense reactions in elicitor plus suppressor-treated tissues suggests that the suppressor would have been degraded by the host. This conclusion is supported by the fact that the subsequent addition of fresh suppressor 3 h after the initial treatment with elicitor plus suppressor treatment further delayed the activation of pisatin biosynthesis (Yamada et al., 1989). It also suggests that duration of suppressor activity is limited only to a few hours, and factors limiting the duration of suppressor activity may reside in the host cells. Proteinase K-treated suppressor loses its activity dramatically (Yamada et al., 1989), and host cells may have the proteolytic enzymes to inactivate the suppressor.

The suppressor isolated from *M. pinodes* markedly inhibits the ATPase activity in pea plasma membranes *in vitro* in an uncompetitive manner, similar to an inhibitor of P-type ATPase, orthovanadate (Yoshioka et al., 1990; Shiraishi et al., 1991). In pea epicotyl tissues, orthovanadate suppresses the accumulation of pisatin that is induced by the fungal elicitor (Yoshioka et al., 1990). It appears that the primary site of action of the suppressor may be the ATPase in the pea plasma membranes (Yoshioka et al., 1990; Shiraishi et al., 1991; Kiba et al., 1995). Orthovanadate delayed accumulation of mRNAs encoding PAL and chalcone synthase in pea epicotyls induced by the elicitor from *M. pinodes* (Yoshioka et al., 1992a). Orthovanadate acted in a manner similar to the fungal suppressor (Yamada et al., 1989; Yoshioka et al., 1990).

The activity of an ATPase in the pea plasma membranes, which is markedly suppressed by 6 h, recovers within 9 h after the start of the treatment with the suppressor from *M. pinodes* (Shiraishi et al., 1991). Accumulation of mRNA for a putative P-type ATPase was not affected by the treatment with orthovanadate. The inhibition of a P-type ATPase might cause a temporary suppression of expression of the PAL and CHS genes that are responsible for production of pisatin. Expression of these genes may gradually recover as a result of the biosynthesis of new ATPase molecules from the accumulated transcripts (Yoshioka et al., 1992a).

The suppressor delays the elicitor-induced superoxide generation in pea cells (Kiba et al., 1997). The suppressor induced a *OPR-3* gene in the compatible interaction in pea. The *OPR-3* gene encodes an octadecanoid intermediate, suggesting that suppressor induced JA pathway, probably by antagonizing the elicitor-induced SA-dependent defense responses (Ishiga et al., 2002).

The *M. pinodes* suppressor inhibited the elicitor-induced activation of a myelin basic (MBP)-dependent MAPK in pea (Uppalapati et al., 2004), and MAP kinases are known to be involved in the elicitor-induced disease resistance responses in plants (Zhang and Klessig, 2001; Suzuki, 2002). Inactivation or inhibition of MAPK pathways may be a virulence strategy commonly adapted by fungal pathogens (Uppalapati et al., 2004).

A suppressor has been isolated from the culture filtrate of *Ascochyta rabiei*, the pathogen of chickpea (*Cicer arietinum*). The suppressor compound could be precipitated from the culture medium by trichloroacetic acid or fractionated with ammonium sulfate indicating that it is a protein. The suppressor had a molecular weight of less than 10,000 and inhibited accumulation of constitutive phenolics like biochanin A, biochanin A 7-0-gluoside-6″-0-malonate (BGM), formononetin, and formononetin 7-0-glucoside-6″-0-malonate (FGM) and elicitor-induced pterocarpan phytoalexins like medicarpin and maackiain (Kessmann and Barz, 1986).

Suppressor has been isolated from *Phytophthora infestans*. It has been characterized as glucans containing β-1-3 and β-1-6 linkages and 17–23 glucose units. The glucans from both mycelia and zoospores contained a nonanionic glucan and an anionic glucan, one of two residues of the latter were esterified with a phosphoryl monoester. The anionic glucan was more active than nonanionic glucan (Doke et al., 1979). Suppressor could be isolated from both compatible and incompatible races of the pathogen. There was no significant difference between compatible and incompatible races in the amount of suppressor in the germination fluid of cystospores of the fungus. However, those suppressors were qualitatively different. Suppressor from race 4 (incompatible with the cultivar Kennebec) was less active than that from compatible race 1.2.3.4, in suppressing the defense action induced by the elicitor in the cvr. Kennebec (Doke et al., 1979).

Pretreatment of protoplasts prepared from nine potato cultivars that have different resistant genes with suppressors from seven races of *P. infestans* suppressed the hypersensitive reaction of protoplasts elicited by elicitors isolated from the pathogen. Greater suppressive activity of the suppressor was characteristic of the compatible relationships between protoplasts and races used as a source of suppressor (Doke and Tomiyama, 1980a,b).

It is yet to be assessed whether suppressors act by competition for elicitor-binding sites or by the induction of a separate response that overcomes the consequences of elicitor action. The suppressor isolated from *M. pinodes* caused only a delay in the accumulation of PAL and CHS transcripts in response to the elicitors but did not affect their final levels (Yamada et al., 1989). It suggests that the suppressor does not compete for elicitor receptors but acts at a later stage in the signal pathway (Dixon and Lamb, 1990). *Ascochyta rabiei* suppressor inhibited the synthesis of elicitor-induced phytoalexins as well as the constitutive phenolics, suggesting the existence of sites of action at least partially different from those of the elicitor (Kessmann and Barz, 1986).

Suppressor may be present as a part of elicitor itself (Basse and Boller, 1992). A suppressor has been isolated from the glycopeptide elicitors of yeast extract. When the elicitor was digested with endo-β-*N*-acetyl-glucosaminidase H, the suppressor activity was detected in the released oligosaccharides. Periodate oxidation completely destroyed the suppressor activity. Carbohydrates released from the yeast extract-elicitor by *N*-glycanase also showed suppressor activity. These observations suggest that hydrolysis of the elicitor-active glycopeptides into the glyco-and-peptide parts by endoglycanases yields oligosaccharides that act as suppressors of the activity (Basse and Boller, 1992). α-Mannosidase destroyed the suppressor activity indicating that mannose residues are important for the suppressor activity. It has also been shown that the mannose residues are important for the elicitor activity. It has been well documented that N-linked glycans are essential for elicitor activity, but they act as suppressors of the same activity when released from glycopeptides (Basse and Boller, 1992).

The suppressor from the yeast extract-elicitor inhibited induction of ET biosynthesis and PAL in tomato cells by the elicitor from the yeast extract. It suggests that the elicitor and suppressors may compete for a single recognition site (Basse and Boller, 1992). When elicitor from *Phytophthora sojae* was employed to induce ET biosynthesis, the suppressors from yeast extract-elicitor did not suppress the induction in tomato cells. The active compounds in *P. sojae* elicitor are proteinaceous in nature and differ from the yeast extract-derived elicitor. The results suggest that the suppressor activity is highly specific, and that tomato cells contain at least two distinct elicitor recognition sites with different specificities (Basse and Boller, 1992).

Enzymatic nature of suppressor produced by *Colletotrichum* spp. has been reported (Siegrist and Kauss, 1990). Chitin deacetylase is produced by several races of *Colletotrichum lindemuthianum* (Kauss et al., 1983) and *C. lagenarium* (Kauss and Bauch, 1988). The enzyme produces chitosan from chitin. The elicitor activity of chitin oligomers arising from chitinase action may be destroyed by chitin deacetylase, as fragments arising from the action of chitinase provided a better substrate than crystalline chitin for the deacetylase. This enzyme was detected in cucumber leaves infected with *C. lagenarium* also (Siegrist and Kauss, 1990). These studies suggest that the suppressors may play an important role in conferring susceptibility and disease development.

2.32.14 Susceptible Plants May Have Suppressors to Suppress Action of Fungal Elicitors

Suppressors of host origin has been reported in tomato (Peever and Higgins, 1989; Basse et al., 1992), wheat (Moerschbacher et al., 1990), and pea (Murakami et al., 1997). Co-injection of *Cladosporium fulvum* elicitor and IFs from *C. fulvum*-infected tomato consistently resulted in complete suppression of elicitor-induced necrosis and callose deposition in the injected panels. Several preparations of IFs from uninfected plants also suppressed elicitor-induced necrosis. IFs from rust-infected bean also suppressed the induction of necrosis by the elicitor when co-injected into tomato leaves (Peever and Higgins, 1989).

The presence of suppressor activity in some preparations of IFs from uninfected, healthy tomato indicates that it may be a host-produced factor (Peever and Higgins, 1989). The suppressor may not be an enzyme (Lu and Higgins, 1993). A pectate digest, produced from sodium polypectate by pectinase, suppressed the elicitor-induced necrosis. Co-injection of the elicitor with active pectinase resulted in suppression. Pectinase digestion of host cell walls would have released the suppressor active molecules (Lu and Higgins, 1993). The results suggest that the suppressor may be a pectin fragment.

Wiethölter et al. (2003) have demonstrated that pectic fragments produced during host cell wall degradation can act as endogenous suppressors of the defense response in wheat leaves inoculated with the stem rust fungus *Puccinia graminis* f. sp. *tritici*. Homogalacturonans were isolated from cell walls of the susceptible wheat cultivars Prelude and Marquis and from near-isogenic lines of both cultivars containing the *Sr5*-gene for hypersensitive rust resistance. The homogalacturonans of the susceptible wheat lines had nonrandom and more blockwise distribution of the methyl esters, whereas the homogalacturonans of the resistant lines had random distribution of the methyl esters (Wiethölter et al., 2003). The homogalacturonans isolated from susceptible wheat lines suppressed the HR development in the resistant lines. The results suggest that the homogalacturonans of the susceptible lines may act as suppressors of elicitor action, resulting in disease development.

2.32.15 Downregulation of Functions of Elicitors in Susceptible Interactions

Elicitor molecules may be active in resistant plants and inactive in susceptible plants. In some plant–pathogen interactions, it has been demonstrated that the elicitor expression in planta

TABLE 2.8
Downregulation of Expression of the Elicitin Gene *inf1* in *Phytophthora infestans*-Infected Leaves

Days after Inoculation with *Phytophthora infestans*	Intensity of Expression of *inf1* in Potato Leaves
1	−[a]
2	−[a]
3	+[b]
4	++[b]
5	+++[b]
6	++++[b]
7	++[b]
8	++[b]

Source: Adapted from Kamoun, S., van West, P., de Jong, A.J., de Groot, K.E., Vleeshouwers, V.G.A.A., and Govers, F., *Mol. Plant Microbe Interact*., 10, 13, 1997b.

[a] − indicates no expression.
[b] +, ++, +++, and ++++ indicate the increasing degrees of gene expression.

may be downregulated during pathogenesis. INF1 is the elicitor produced by the potato late blight pathogen, *Phytophthora infestans*. It induces HR in fully resistant tobacco plants; but did not induce any necrosis symptoms. When compatible potato plants were inoculated with *P. infestans*, a gene encoding the protein elicitor of *P. infestans* was found to be downregulated in the plants (Kamoun et al., 1997b). The *inf1* mRNA was first detected at day 3 after inoculation and reached the highest level at days 5 and 6. Subsequently, at days 7 and 8, the level of *inf1* mRNA decreased (Table 2.8; Kamoun et al., 1997b). The results suggest that the expression of fungal elicitor gene in the susceptible host may be downregulated, resulting in disease development.

2.32.16 Activation of an Unsuitable Signaling System for Induction of Defense Responses May Lead to Susceptibility

Perception of pathogen signals by plants involves a complex suite of cellular responses that involve cross talk of numerous signal transduction pathways culminating in resistance or disease (Uppalapati et al., 2004). Pathogen signals may induce defense responses in both susceptible and resistant interactions through activation of various signaling systems. However, expression of a signaling system, which may not be suitable to induce a specific set of defense genes needed for induction of disease resistance, may result in susceptibility. *Arabidopsis thaliana* showed resistance or susceptibility to various pathogens depending on the type of the signaling system expressed in those plants (Table 2.9).

In *Arabidopsis*, activation of SA-dependent defense mechanisms enhances the level of resistance against *Hyaloperonospora* (*Peronospora*) *parasitica* and turnip crinkle virus (Uknes et al., 1993; Delaney et al., 1994; Kachroo et al., 2000), whereas JA- and ET-dependent defenses yield no resistance against these pathogens (Thomma et al., 1998, 1999). SA-mediated regulation of defense-related genes resulted in only susceptibility to *Botrytis cinerea* in *Arabidopsis* (Ryals et al., 1996; Govrin and Levine, 2002). To induce resistance against *B. cinerea* in *Arabidopsis*, JA-mediated defense mechanism is needed (Staswick et al., 1998; Vijayan et al., 1998). An *Arabidopsis* mutant, *coi1*, that affected the JA-response

TABLE 2.9
Resistance or Susceptibility against Pathogens Depends on Type of Induced Signaling Pathway in *Arabidopsis*

	Signaling Pathways Resulting in	
Pathogen	**Susceptibility**	**Resistance**
Hyaloperonospora parasitica	JA, ET	SA, ABA
Botrytis cinerea	SA	JA, ET, JA/ET
Turnip crinkle virus	JA, ET	SA
Alternaria brassicicola	SA	JA, ET, ABA
Plectosphaerella cucumerina	SA	JA, ET, ABA
Erwinia carotovora	SA	JA/ET
Erysiphe orontii	JA/ET	SA
Pseudomonas syringae	JA/ET	SA
Fusarium oxysporum	ABA	JA, ET, JA/ET

Note: SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ABA, abscisic acid.

pathway, showed enhanced susceptibility to infection by *Alternaria brassicicola* and *B. cinerea* but not to *Hyaloperonospora parasitica. NahG* and *npr1-1* are *Arabidopsis* genotypes that are blocked in their response to SA, either because of expression of a chimeric transcription unit encoding a SA hydroxylase (for *NahG* plants) or to a point mutation in an IκB-like signal transduction component acting downstream of SA (for *npr1-1* mutants). These mutants showed enhanced susceptibility to *H. parasitica* but not to *A. brassicicola* and *B. cinerea* (Thomma et al., 1998). Resistance to *H. parasitica* was boosted by external application of the SA-mimicking 2,6-dichloroisonicotinic acid (INA) (Delaney et al., 1994) but not by MeJA, whereas treatment with MeJA but not INA elevated resistance to *A. brassicicola* (Thomma et al., 1998).

JA- and ET-dependent defense mechanisms in *Arabidopsis* contribute to resistance against *Plectosphaerella cucumerina*, *B. cinerea*, and *A. brassicicola* (Thomma et al., 1998, 1999; Berrocal-Lobo et al., 2002). Resistance to *H. parasitica*, *A. brassicicola*, and *P. cucumerina* was also shown to be dependent on ABA-dependent signaling pathway (Ton and Mauch-Mani, 2004). An intact JA/ET-signaling pathway is necessary for resistance to *B. cinerea* and the bacterial pathogen *Erwinia carotovora*, and SA-dependent signaling system was not involved in disease resistance against these two pathogens. By contrast, SA-dependent signaling pathway was responsible for induction of resistance against *Erysiphe orontii* and a bacterial pathogen *Pseudomonas syringae* in *Arabidopsis*, and JA-signaling pathway was ineffective in inducing disease resistance against the pathogens (Reuber et al., 1998; Rojo et al., 2003). Resistance of *Arabidopsis* to *B. cinerea* (Thomma et al., 1999) and *Pythium* spp. (Geraats et al., 2002) is dependent on JA-signaling system. In tomato, JA- and SA-signaling pathways behaved differently or in the same manner in conferring resistance against different pathogens of the crop (Table 2.10; Thaler et al., 2004). *Phytophthora infestans* was protected by JA pathway (Thaler et al., 2004), whereas SA pathway was ineffective in conferring protection against the pathogen in tomato (Smart et al., 2003). JA-signaling pathway was not involved in defense response against *Oidium* spp. (Thaler et al., 2004), whereas SA system protected tomato plants against the powdery mildew pathogen (Inbar et al., 1998). JA system failed to protect the tomato plants against tomato spotted wilt virus (Thaler et al., 2002), whereas SA system protected the plants against the virus (Tally et al., 1999). In contrast, both JA- and SA-signaling systems conferred resistance against *B. cinerea* (Audenaert et al., 2002; Díaz et al., 2002), *F. oxysporum* f. sp. *lycopersici* (Benhamou and

TABLE 2.10
Induction of Defense Responses against Pathogens of Tomato by Jasmonate or Salicylate Signals

Pathogen	Salicylate	Jasmonate
Phytophthora infestans	−[a]	+[b]
Botrytis cinerea	+[b]	+[b]
Cladosporium fulvum	−[a]	−[a]
Fusarium oxysporum	+[b]	+[b]
Oidium spp.	+[b]	−[a]
Pseudomonas syringae	+[b]	+[b]
Xanthomonas campestris	+[b]	+[b]
Tomato spotted wilt virus	+[b]	−[a]

Source: Adapted from Thaler, J.S., Owen, B., and Higgins, V.J., *Plant Physiol.*, 135, 530, 2004.

[a] − indicates no effect against the particular pathogen.
[b] + indicates positive induction of protection against the particular pathogen.

Belanger, 1998; Thaler et al., 2004), and two bacterial pathogens *Xanthomonas campestris* (Inbar et al., 1998; Thaler et al., 2004) and *Pseudomonas syringae* (Louws et al., 2001; Thaler et al., 2004). These studies collectively suggest that different signaling systems may operate in different plant–pathogen interactions. The activation of signaling system not suitable for induction of defense responses against the particular pathogen may result in susceptibility.

2.33 SIGNALING SYSTEMS IN SUSCEPTIBLE INTERACTIONS

2.33.1 Abscisic Acid-Signaling System

Some of the signaling systems are specifically activated during susceptible interactions. ABA-signaling system plays an important role in susceptible interactions. Exogenously applied ABA increases susceptibility of potato plants to *Phytophthora infestans* and *Cladosporium cucumerinum* (Henfling et al., 1980), soybean plants to *Phytophthora sojae* (Ward et al., 1989), tobacco plants to *Peronospora tabacina* (Salt et al., 1986), and tomato plants to *Botrytis cinerea* (Kettner and Dörffling, 1995). ABA negatively regulates SA-signaling system, which induces defense responses against several pathogens (Audenaert et al., 2002). By suppressing the SA-inducible defense mechanisms, ABA may induce susceptibility. During susceptible interactions, ABA content increases in the infected tissues (Kettner and Dörffling, 1995). A threshold ABA concentration appears to be necessary for a susceptible response of plants (Herde et al., 1999; Audenaert et al., 2002). This threshold is reached when a virulent pathogen infects a susceptible host (Audenaert et al., 2002).

Fungal pathogens are known to produce ABA in culture (Dörffling et al., 1984; Crocoll et al., 1991; Danneberg et al., 1993; Audenaert et al., 2002). In addition, it is known that endogenous ABA levels can rise upon pathogen infection (Bothe et al., 1994; Kettner and Dörffling, 1995). Hence, it can be concluded that these fungi produce ABA and induce endogenous ABA production in the plant to suppress SA-dependent defense mechanisms. ABA may also suppress ET-signaling system. In ABA-negative tomato mutants, ET level increased twofold compared with wild-type plants, suggesting the role of ABA in suppression of ET production (Sharp et al., 2000). The role of ET signals in induction of defense-related responses has been reported (Hoffman et al., 1999; Thomma et al., 1999).

ABA suppressed JA-signaling pathway, which induces expression of the defensin gene *PDF1.2* in *Arabidopsis* (Anderson et al., 2004). Treatments with MeJA and ET significantly induced the defensin *PDF1.2* gene (30-fold and 15-fold, respectively) in *Arabidopsis*, whereas ABA treatment caused 10-fold reduction at the basal transcript levels of *PDF1.2* relative to those in mock-treated plants (Anderson et al., 2004). In addition, in the presence of ABA, neither ET nor MeJA were able to induce *PDF1.2* transcript levels in *Arabidopsis* plants (Anderson et al., 2004). These results suggest that ABA may suppress various defense-signaling systems and by this action, the plants may become susceptible to pathogens.

2.33.2 Ethylene-Signaling System

Elicitors are known to induce biosynthesis of ET. The growth regulator may induce susceptibility in some host–pathogen interactions. Soybean plants expressing more ET synthesis showed increased susceptibility to the disease caused by *Phytophthora sojae*, suggesting that ET may negatively regulate the expression of defense genes (Hoffman et al., 1999). ET increased susceptibility of *Verticillium* wilt of tomato and gray mold on rose and carnation flowers (Boller, 1991). Exogenous application of ET enhances *B. cinerea* (grey mold) incidence in tomato, pepper, cucumber, bean, rose, and carnation (Boller, 1991).

2.33.3 Signal Transduction Systems May Induce Susceptibility-Related Responses

Elicitor molecules of pathogen and host origin generate signals, which are transduced by different signaling systems. In most cases, these signaling systems induce many defense-related genes. However, in susceptible interactions, these signaling systems may induce some susceptibility-related genes. *Magnaporthe grisea*, the blast pathogen, induces the JA-signaling pathway. JA induces *JAmyb* gene, which encodes a Myb (for Myelo Blastosis virus) transcription factor (Lee et al., 2001). The *JAmyb* gene was induced within 1 day after fungal infection in resistant and susceptible interactions before lesion formation. Unlike most defense-related genes that are activated faster and stronger in resistant interactions, *JAmyb* induction by the blast fungus was much higher in susceptible interactions (Lee et al., 2001). *JAmyb* was activated rapidly by JA, but not by SA or ABA. Depletion of SA in rice did not abolish but rather enhanced blast-induced *JAmyb* expression, suggesting that the *JAmyb* is related to the development of disease lesions. These results suggest that some signaling systems may trigger susceptible responses.

2.34 CONCLUSION

Plants possess an efficient surveillance system triggering defense mechanisms upon recognition of fungal pathogens attempting to invade them. The first reaction of the plant that switches on the well-organized defense responses is the perception of a molecule called elicitor generated from pathogens. Several types of elicitor molecules are secreted by the pathogen and these include COSs, chitosans, oligoglucans, glucomannans, elicitins, xylanases, proteins, glycoproteins, sphingolipids, AAs, erosterols, toxins, cellulases, and pectolyases. The fungal enzymes may also release some elicitors of host origin. These elicitor complexes may be necessary to activate various defense genes, and the network of these elicitor molecules may coordinate and enhance the expression of defense genes. The release of these elicitors at the infection site is important in triggering host defense response.

Specific receptors for each type of elicitors may be present in the plant cell membrane. Various proteins, RLKs, and lectins act as receptors for the elicitors. Some of the resistance gene products are plasma membrane receptor proteins. The perceived elicitor signal is

transduced intracellularly and intercellularly through a well-orchestrated signal transduction system. Calcium ion acts as an intracellular second messenger, coupling the primary elicitor signal to intracellular and whole-plant responses. Oscillation in cytosolic Ca^{2+} levels is perceived by various intracellular sensors to regulate a series of signaling cascades and provides information for modification of enzyme activity and gene expression needed for subsequent responses. Ca^{2+} sensors can be classified into sensor responders and sensor relay. CaM protein acts as sensor relay, whereas protein kinases are sensor responders.

Protein phosphorylation plays an important role in the reception/transduction of signals originating from pathogens. Both plant kinases and protein phosphatases are involved in protein phosphorylation. Mitogen-activated cascade is a key machinery that transduces extracellular signals into a wide range of intracellular responses. Several phospholipids including PI, IP_3, DAG, PA, LPC, ceramide, and sphingosine synthesized by the action of phospholipases play a role in signal transduction.

The plasma membrane anion channels may be essential components of early signal transduction processes in plants. The anion channels intiate or amplify plant plasma membrane depolarization, which in turn activates K^+ channels and Ca^{2+} voltage-dependent channels. Transient shifts of intracellular and apoplastic pH are essential steps in the signal transduction processes. The extracellular alkalinization combined with intracellular acidification triggers activation of defense genes. The defense-signaling pathways appear to depend on changes in the proton electrochemical gradient across the plasma membrane.

Oxidative burst involving ROS is seen within a few minutes of perception of pathogen signals in plant cells. ROS signaling may act at downstream of Ca^{2+}-signaling system involving phosphorylation/dephosphorylation cascade. NO is a reactive nitrogen species and the NO burst occurs within a few minutes of exposure to fungal elicitor. NO synthesis is tightly regulated by a signaling cascade involving Ca^{2+} influx and phosphorylation events. NO cooperates with ROS in triggering downstream events in the intracellular signal transduction pathway.

Three distinct signaling pathways are involved in intercellular and whole-plant signal transduction. These include SA-, JA-, and ET-mediated signaling systems. These pathways may not act independently and the plants may be equipped with regulatory components to control the magnitude of each of these pathways. An intricate signaling network involving these pathways fine-tunes the induction of plant defense responses. Cross talk between SA, JA, and ET pathways results in efficient transduction of the signals to trigger the transcription of multitude of defense genes, resulting in disease resistance.

Signal perception and transduction systems leading to resistance have been well studied. Similar signal transduction systems with some variance may be operative in susceptible interactions, and these variant systems lead to disease development in the susceptible interactions. The differences in the signaling systems leading to susceptibility or resistance have been summarized in Figure 2.32.

The molecular signal molecules, elicitors, are abundantly present in both virulent and avirulent pathogens. However, the release of these elicitors from the fungal cell surface in the infection court differs. The induction and accumulation of elicitor-releasing enzymes are delayed in the susceptible interactions, resulting in delayed release of elicitors. The amount of elicitors released in the susceptible interactions may also be less compared with that in resistant interactions. The nature of elicitors from compatible pathogens may differ from those from incompatible pathogens. The elicitors of compatible pathogens may be less efficient in inducing defense responses than those of incompatible pathogens. Some of the elicitors show host plant- and cultivar-specificity and they do not trigger defense responses in the susceptible interactions.

In some susceptible interactions, the elicitors are degraded by host plant enzymes to inactive derivatives in the infection court. The degraded products do not trigger defense

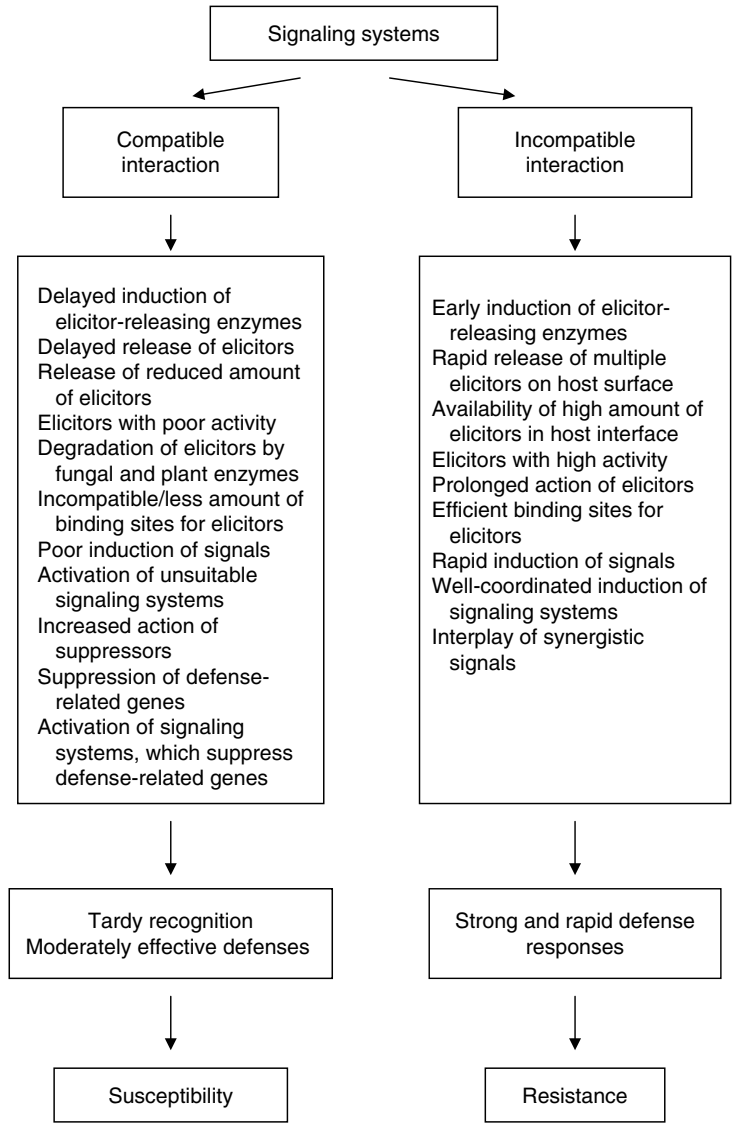

FIGURE 2.32 Differences in signaling systems leading to susceptibility or resistance.

responses. Fungal pathogens produce high amounts of pectic enzymes in infected susceptible plant tissues, and the high concentrations of enzymes degrade the elicitors of plant origin (pectic fragments) to smaller fragments, which are ineffective in inducing defense genes.

In susceptible interactions, accumulation of some signal molecules, such as SA, may be delayed and reduced. Delayed and reduced expression of such signal transduction pathways may result in susceptibility. Some elicitors in the susceptible interactions may induce susceptibility-related genes. Pathogens may also produce suppressors, besides elicitors. These suppressors negate the defense responses induced by the elicitors in the susceptible interactions. Susceptible plants themselves may contain suppressors to suppress the action of the fungal elicitors.

In some susceptible interactions, the signal transduction systems may be activated. However, the particular activated signal transduction system may not be suitable to induce

the specific defense genes needed to produce specific defense compounds to confer resistance against the specific pathogen. Some signaling systems activated by fungal elicitors confer susceptibility, rather than resistance. The ABA- and ET-signaling systems induce susceptibility in several host–pathogen systems by specifically suppressing host defense responses.

Although it is well documented that the differential perception and transduction of pathogen signals in plants may determine resistance or disease development, it is still not known why this differential reaction occurs in different cultivars. Probably the disease resistance and susceptibility genes in plants and avirulence and virulence genes in pathogens would have contributed to the differences in signal perception in resistant and susceptible interactions. The role of disease resistance and susceptibility genes in signal perception and emission will be described in the next chapter.

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3 Disease Resistance and Susceptibility Genes in Signal Perception and Emission

3.1 INTRODUCTION

Pathogens contain various elicitors to act as primary signal molecules triggering host defense genes. These elicitors include general elicitors such as oligosaccharides, proteins, glycoproteins, and lipids (Halim et al., 2004; Ning et al., 2004; Hu et al., 2005; Vidhyasekaran, 2007) and race-specific elicitors including several avirulence (*avr*) gene products (Dodds et al., 2004; Schürch et al., 2004; Shan et al., 2004; Rowland et al., 2005). Plants are endowed with several hundreds of disease resistance genes (Zhu et al., 2002; Eckardt et al., 2003; Ling et al., 2003). The disease resistance genes offer two types of resistance. The first type of resistance is called qualitative resistance, whereas the second type of resistance is called quantitative resistance (Toojinda et al., 2000). The qualitative resistance is conferred by major genes (dominant or recessive), whereas quantitative resistance is conferred by minor genes (Kousik and Ritchie, 1999; Ovesná et al., 2000). Major genes have distinct phenotypic expressions showing clear Mendelian segregation, whereas minor genes have small effects on the expression of the phenotype for resistance showing quantitative segregation (Vidhyasekaran, 2007). Two types of major genes, such as dominant and recessive genes, contribute for qualitative resistance. Recessive gene is phenotypically manifested in the homozygous state but is masked in the presence of its dominant allele (dominant gene). Usually the dominant gene produces a functional product, whereas its recessive gene does not (Vidhyasekaran, 2007).

Most of the major resistance genes show race-specific resistance following gene-for-gene hypothesis of Flor (1956, 1971). According to gene-for-gene resistance theory, there are many plant resistance (*R*) genes in a plant species against each of its pathogens and there is a corresponding avirulence (*Avr*) gene in the pathogen for every *R* gene in the host plant. When an *Avr* gene and an *R* gene of matched specificity are expressed, a strong resistance response is induced. The gene-for-gene theory has been well demonstrated in cases where plant resistance is associated with hypersensitivity. However, a clear-cut resistant phenotype expressing hypersensitivity is not seen in many major gene-mediated resistant interactions (Chisholm et al., 2000; Li et al., 2001).

There are many reports that a major gene may show resistance to a wide spectrum of races of a pathogen. Single major gene may show resistance to a wide spectrum of races of pathogens. Some non-race-specific disease resistance genes (*NDR*) have also been cloned (Century et al., 1997). Three alleles of the *Rp1* disease resistance gene, *Rp1-D21*, *Rp1-MD19*, and *Rp-NC3*, have been shown to confer a non-race-specific resistance response to rust (*Puccinia sorghi*) (Hu et al., 1996; Hulbert, 1997). The wheat stem rust resistance gene *Sr26* shows resistance to all races of the pathogen *Puccinia graminis* f. sp. *tritici* obtained worldwide

(McIntosh et al., 1995). The *R* gene *RB* cloned from *Solanum bulbocastanum* confers broad-spectrum resistance to several races of potato late blight pathogen *Phytophthora infestans* (Song et al., 2003). In some cases, a dominant resistance gene may show resistance not only against several races of a pathogen, but also against several pathogens of a host. In *Arabidopsis thaliana*, two different major disease resistance genes *RPW8.1* and *RPW8.2* individually confer resistance to four different powdery mildew pathogens, such as *Erysiphe cichoracearum*, *Erysiphe cruciferarum*, *Golvinomyces orontii* (*Erysiphe orontii*), and *Oidium lycopersici* (Xiao et al., 2001). The single major resistance gene Ry_{sto} confers resistance against four different virus diseases caused by tobacco etch virus, potato virus Y, potato virus A, and potato virus V in potato (Hinrichs-Berger et al., 2000).

The functions of dominant and recessive genes are distinctly different in triggering resistance (Cannon et al., 2002; Vogel et al., 2002; Jarosch et al., 2003). Bai et al. (2005) reported that the mechanism of resistance conferred by the dominant *Ol* genes (*Ol-1*, *Ol-3*, *Ol-4*, *Ol-5*, and *Ol-6*) conferring resistance to *Oidium neolycopersici* in tomato was associated with hypersensitive response, whereas the mechanism of resistance governed by the recessive gene *Ol-2* was associated with papillae formation. Recessive genes may also confer race-specific resistance (Leister et al., 1999; Ilag et al., 2000; Blair et al., 2003). However, some recessive genes may confer resistance against many races of a pathogen (Deslandes et al., 2002) and even against different bacterial and oomycete pathogens (Shirano et al., 2002). The *Arabidopsis* recessive gene *RRS1-R* confers resistance against several races of *Ralstonia solanacearum* (Deslandes et al., 2002), whereas another *Arabidopsis* gene *ssi4* confers resistance against the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* and the oomycete pathogen *Peronospora parasitica* (Shirano et al., 2002). In case of dominant gene-mediated resistance, the susceptible genotype corresponds to the absence of functional copy of the dominant gene and not to the presence of a susceptible allele, in some cases, no copy has been found at the allelic position in susceptible genotypes (Grant et al., 1998). In contrast, in the recessive gene-mediated resistance, the susceptibility gene may code for a host component required for the pathogenicity of the pathogen and a point mutation on this component in the recessive gene-resistant variety would confer resistance (Ndjiondjop et al., 2001; Eckardt, 2002; Piffanelli et al., 2002; Albar et al., 2003; Hückelhoven et al., 2003; Jarosch et al., 2003).

The quantitative resistance is nonhypersensitive, presumably non-race specific, and conferred by several minor genes. Minor genes have only small effects on the expression of the phenotype for resistance and hence, it is difficult to identify quantitative resistance (minor) genes (Vidhyasekaran, 2004). Attempts were made to identify the minor genes by characterizing the quantitative trait loci (QTL) associated with quantitative resistance (Geffroy et al., 2000; Manosalva et al., 2001; Thordal-Christensen et al., 2004). Recent molecular technologies have revealed that separate major and minor genes may not exist. Several QTLs have been mapped to the same chromosomal regions as major genes (Leister et al., 1996; Shen et al., 1998). Some QTLs are allelic to major resistance genes and have the same function as the major genes (Thabuis et al., 2003). The major gene, *R1*, contributing for resistance against the late blight pathogen *Phytophthora infestans* is linked to a QTL for resistance to *P. infestans* in potato (Ballvora et al., 2002). A major gene in one genetic background may function as a minor gene in another background (Ovesná et al., 2000). When many several major genes express together, they function exactly like minor genes (Kousik and Ritchie, 1999). Several major genes are clustered in a single locus (Smith and Hulbert, 2005). Fourteen maize rust resistance genes *Rp1-A* to *Rp1-N* have been mapped to a single locus on chromosome 10 in maize (Collins et al., 1999; Pataky et al., 2001). These studies suggest that both major and minor genes may function in a similar way in conferring resistance.

More than 40 disease resistance genes have been cloned from various plants and fully characterized (Martin et al., 1993b; Jones et al., 1994; Dinesh-Kumar et al., 1995; Lawrence

et al., 1995; Song et al., 1995, 2003; Wang et al., 1996; Anderson et al., 1997; Baker et al., 1997; Hammond-Kosack and Jones, 1997; Meyers et al., 1998, 2002, 2003; Yoshimura et al., 1998; Bendahmane et al., 1999; Collins et al., 1999; Gassmann et al., 1999; Chisholm et al., 2000; Dodds et al., 2001a; Bai et al., 2002; Brueggemann et al., 2002; Deslandes et al., 2002, 2003; Shen et al., 2002; Webb et al., 2002; Xu and Korban, 2002; Feuillet et al., 2003; Huang et al., 2003; Varet et al., 2003; Zhang and Gassmann, 2003; Wretblad et al., 2003; Frost et al., 2004; Schornack et al., 2004). The molecular biological studies revealed that almost all the cloned resistance (*R*) genes are directly or indirectly involved in signal perception and emission. Several studies have suggested that the resistance gene products recognize the pathogens signals. Perception of these pathogen signals activates the *R* gene-encoded protein to signal to other components in the plant cell. Emission of signals by *R* gene proteins leads to activation of downstream signal transduction pathways, ultimately leading to activation of defense genes and disease resistance (Jirage et al., 1999; Clarke et al., 2001; Rustérucci et al., 2001; Martin et al., 2003; Bennett et al., 2005). Several susceptibility genes contributing to disease development in compatible interactions have been identified (Eckardt, 2002; Vogel et al., 2002; Mengiste et al., 2003). These genes may negatively regulate the defense responses and suppress the action of *R* genes, resulting in disease development. Suppressor genes, which suppress the signaling action of *R* genes, have also been reported (Wise et al., 1996; Wilson and McMullen, 1997; Welz and Geiger, 2000; Vidhyasekaran, 2007). The complex roles of resistance and susceptibility genes in signal perception and emission during fungal pathogenesis in resistant and susceptible interactions are described in this chapter.

3.2 MOLECULAR STRUCTURE OF RESISTANCE GENES

3.2.1 LRR Domains

Several dominant disease resistance genes have been cloned and their molecular structures have been elucidated. Most of the disease resistance (*R*) genes encode intracellular proteins with leucine-rich repeat (LRR) and nucleotide-binding site (NBS) domains. The LRRs are composed of an "inner solvent-exposed" surface rim comprising β-sheets connected by an outer rim of α-helical segments. The β-sheets are stabilized by a ladder of hydrogen bonds between cysteines and asparagine side chains (Fluhr, 2001). The LRR domain is a serial repeat motif of an ~24 amino acid (aa) motif with leucines and other hydrophobic residues at regular intervals, leading to a tertiary structure resembling a curved spring (Kobe and Deisenhofer, 1994). In the central region of each repeat motif, a β-strand/β-turn structure, which has the consensus sequence XX(L)X(L)XXX, where L corresponds to conserved leucines (or other aliphatic amino acids) and X denotes the flanking hypervariable amino acids, is seen (Parniske et al., 1997; McDowell et al., 1998). The conserved leucines project into the hydrophobic core, whereas the other residues form a solvent-exposed surface involved in ligand binding (Kobe and Deisenhofer, 1994, 1999). There may be more than 15 LRRs in plant *R* genes (Fluhr, 2001), although the sugar beet nematode resistance gene $Hs1^{pro1}$ encodes protein with only seven repeats (Cai et al., 1997).

3.2.2 NBS Domains

NBS motifs have been detected in many R proteins. NBS domains have been identified in many prokaryotic and eukaryotic proteins, in which their ability to bind ATP or GTP is essential for their biological activity (Saraste et al., 1990). The NBS of R proteins has an N-terminal subdomain that contains consensus kinase 1a (P-loop), kinase 2, and kinase 3a motifs (Moffett et al., 2002). The P-loop is known to be important for binding proteins to nucleotides (Shirano et al., 2002). The C-terminal part of NBS, referred to as the ARC

(apoptosis, *R* gene product, and CED-4), subdomain. It is structurally related to regulators of animal apoptosis, including human Apaf-1 and nematode CED-4, and is conserved in plant NBS–LRR proteins (van der Biezen and Jones, 1998a).

The N-terminus of NBS–LRR proteins is either a Toll and interleukin receptor (TIR) domain or a loosely predicted coiled coil (CC). CC domains are an oligomerization motif of helical structures that are made up of bundles containing two to five helices. The CC structure shows a heptad repeat where the seven positions are labeled "a" through "g". Residues "a" and "d" tend to be hydrophobic, and the residues at the "e" and "g" positions are charged or polar (Fluhr, 2001). Coiled-coil structures form homo- or heterooligomeric associations facilitating interactions between proteins (Torii et al., 1998).

Some resistance genes encode proteins without LRR or NBS domains. Their structure is variable and described later.

3.3 CLASSIFICATION OF RESISTANCE GENES BASED ON MOLECULAR STRUCTURE OF *R* GENE-ENCODED PROTEINS

3.3.1 Resistance Genes Encoding TIR–NBS–LRR Proteins

On the basis of molecular structures, dominant *R* genes can be classified into four broad classes such as (i) resistance genes encoding TIR–NBS–LRR proteins, (ii) resistance genes encoding non-TIR–NBS–LRR proteins, (iii) resistance genes encoding LRR proteins lacking NBS domain, and (iv) other types of *R* genes encoding proteins lacking LRR proteins. Molecular structure of some recessive genes has also been identified.

The first class of R proteins includes cytoplasmic receptor-like proteins that contain N-terminus TIR, central NBS, and C-terminal LRR motif (Table 3.1). Various *R* genes in tobacco, flax, and *Arabidopsis* belong to this class. Tobacco *N* gene conferring resistance against tobacco mosaic virus (TMV) (Whitham et al., 1994; Dinesh-Kumar et al., 1995, 2000;

TABLE 3.1
***R* Gene-Encoded TIR–NBS–LRR Proteins**

R Gene Encoding TIR–NBS–LRR Protein	Host Plant	Resistance to Pathogen	References
L (alleles of L: *L1*, *L2*, *L3*, *L4*, *L5*, *L6*, *L7*, *L8*, *L9*, *L10*, *L11*, *LH*)	Flax	*Melampsora lini*	Lawrence et al. (1995); Ellis et al. (1995)
M	Flax	*Melampsora lini*	Anderson et al. (1997)
N	Flax	*Melampsora lini*	Dodds et al. (2001b)
P, *P2*	Flax	*Melampsora lini*	Dodds et al. (2001a)
N	Tobacco	Tobacco mosaic virus	Whitham et al. (1994); Dinesh-Kumar et al. (1995)
Bs4	Tomato	*Xanthomonas campestris* pv. *vesicatoria*	Schornack et al. (2004)
RPP1	*Arabidopsis*	*Hyaloperonospora parasitica*	Botella et al. (1998)
RPP4	*Arabidopsis*	*Hyaloperonospora parasitica*	van der Biezen et al. (2002)
RPP5	*Arabidopsis*	*Hyaloperonospora parasitica*	Parker et al. (1997)
RPS4	*Arabidopsis*	*Pseudomonas syringae*	Gassmann et al. (1999)
SNC1	*Arabidopsis*	*Pseudomonas syringae*	Wise (2000)
RAC1	*Arabidopsis*	*Albugo candida*	Borhan et al. (2004)
RPS4	*Arabidopsis*	*Pseudomonas syringae*	Gassmann et al. (1999)

Dinesh-Kumar and Baker, 2000), flax rust resistance genes *L* (12 alleles, *L1* to *L11* and *LH*), *M*, *N*, and *P* (Ellis et al., 1995, 1999; Lawrence et al., 1995; Anderson et al., 1997; Gassmann et al., 1999; Dodds et al., 2001a,b) and *Arabidopsis* genes *RPP1*, *RPP4*, *RPP5*, *RAC1*, and *SNC1* (Lawrence et al., 1995; Parker et al., 1997; Gassman et al., 1999; van der Biezen et al., 2002; Meyers et al., 2003; Borhan et al., 2004; Yang and Hua, 2004) belong to the TIR–NBS–LRR class (Table 3.1). *RPS4* is the disease resistance gene of *Arabidopsis thaliana* specifying resistance to strains of *Pseudomonas syringae* pv. *tomato* expressing *avrRps4*. It encodes a predicted protein of 1217 amino acids that contains TIR–NBS–LRR domains (Gassmann et al., 1999). The tomato (*Lycopersicon esculentum*) resistance protein Bs4 is a predicted non-nuclear TIR–NBS–LRR protein (Schornack et al., 2004). The *Bs4* allele was detected in *Lycopersicon pennelli*, which was >98% identical to *L. esculentum Bs4* (Schornack et al., 2004).

3.3.2 Resistance Genes Encoding Non-TIR–NBS–LRR Proteins

The resistance gene-encoded NBS–LRR proteins without a TIR are called non-TIR–NBS–LRR proteins (Nobuta et al., 2005). Most of those R proteins lacking a TIR have a coiled-coil (CC) motif in the N-terminal region (Pan et al., 2000a,b). A subset of non-TIR–NBS–LRRs has a leucine zipper (LZ), a specific example of the coiled-coil structure consisting of heptad repeat sequences (LX6L) with interspersed hydrophobic residues (Alber, 1992) and this group of proteins is called LZ–NBS–LRR proteins.

TIR- and non-TIR–NBS–LRR sequences are distinguishable by amino acid motifs internal to their NBS domains. The motifs P-loop, Kin-1a, and GLPLA signatures are present in both classes. However, the motifs RNBS-A-TIR (LQKKLLSKLL) and RNBS-D-TIR (FLHIACFF) are found exclusively in the TIR class and RNBS-A-non-TIR (FDLxAWVCVSQxF) and RNBS-D-non-TIR (CFLYCALFPED) (the one-letter amino acid codes used are: L-leucine, Q-glutamine, K-lysine, S-serine, F-phenylalanine, H-histidine, I-isoleucine, A-alanine, C-cysteine, D-aspartate, W-tryptophan, V-valine, Y-tyrosine, P-proline, E-glutamate, and x-any amino acid) are found exclusively in the non-TIR class (Meyers et al., 1999). The two classes can be distinguished with 95% accuracy by the final amino acid in motif Kin-2; a tryptophan (W) in non-TIRs and an aspartic acid (D) in TIRs (Meyers et al., 1999).

Several *R* genes in wheat, barley, rice, maize, potato, tomato, pepper, lettuce, and *Arabidopsis* belong to the class (Table 3.2). Wheat *R* gene *Lr10* conferring resistance to leaf rust pathogen *Puccinia triticina* belongs to this class (Feuillet et al., 1997, 2003). Another wheat resistance gene *Lr21* encodes a protein containing a conserved NBS domain and 13 imperfect LRRs, and a unique 151-amino acid sequence missing from known NBS–LRR proteins at the N-terminus (Huang et al., 2003). Barley *R* genes *Mla1*, *Mla6*, *Mla12*, and *Mla13* (Halterman et al., 2001, 2003; Zhou et al., 2001) conferring resistance to powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* belong to this class. The *Arabidopsis* R proteins belonging to CC–NBS–LRR class include RPM1 and RPP13 (Mindrinos et al., 1994; Parker et al., 1997; Tornero et al., 2002a; Hubert et al., 2003; Meyers et al., 2003). The *A. thaliana* resistance gene *RCY1* encodes a 104 kDa CC–NBS–LRR-type protein and confers resistance against yellow strain of cucumber mosaic virus (Takahashi et al., 2002). The late blight resistance gene *RB* cloned from *Solanum bulbocastanum* belongs to CC–NBS–LRR class (Song et al., 2003).

The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the LZ–NBS–LRR class (Ballvora et al., 2002). The *Arabidopsis* genes *RPS2*, *RPS5*, *RPP8*, and *HRT* also belong to LZ–NBS–LRR class (Warren et al., 1998; Cooley et al., 2000; Tao et al., 2000). The primary structure of potato virus X (PVX) resistance gene *Rx* in *Arabidopsis* is similar to that of the NBS–LRR class of R proteins (Bendahmane et al., 1999, 2002; Moffett et al., 2002). A putative leucine zipper is present in the N-terminal region of Rx (Bendahmane et al., 1999). The putative NBS domain of Rx comprises P-loop, kinase 2, and kinase 3a motifs. The NBS is followed by a domain with unknown function that includes GLPL,

TABLE 3.2
***R* Gene-Encoded Non-TIR–NBS–LRR Proteins**

R Gene	Domain Structure of R Protein	Host Plant	Resistance to Pathogen	References
Lr10	CC–NBS–LRR	Wheat	*Puccinia triticina*	Feuillet et al. (2003)
Mla1	CC–NBS–LRR	Barley	*Blumeria graminis* f. sp. *hordei*	Halterman et al. (2001); Zhou et al. (2001)
Mla6	CC–NBS–LRR	Barley	*Blumeria graminis* f. sp. *hordei*	Shen et al. (2003)
Mla12	CC–NBS–LRR	Barley	*Blumeria graminis* f. sp. *hordei*	Wei et al. (1999); Shen et al. (2003)
Mla13	CC–NBS–LRR	Barley	*Blumeria graminis* f. sp. *hordei*	Halterman et al. (2003)
RB	CC–NBS–LRR	Potato	*Phytophthora infestans*	Song et al. (2003)
DM3	CC–NBS–LRR	Lettuce	*Bremia lactuca*	Shen et al. (2002)
RPP13	CC–NBS–LRR	*Arabidopsis*	*Hyaloperonospora parasitica*	Wise (2000)
RPM1	CC–NBS–LRR	*Arabidopsis*	*Pseudomonas syringae* pv. *maculicola*	Grant et al. (1995); Boyes et al. (1998)
RCY1	CC–NBS–LRR	*Arabidopsis*	Cucumber mosaic virus	Takahashi et al. (2002)
Rx	LZ–NBS–LRR	Potato	Potato virus X	Bendahmane et al. (1999)
RPP7/8	LZ–NBS–LRR	*Arabidopsis*	*Hyaloperonospora parasitica*	Cooley et al. (2000)
HRT	LZ–NBS–LRR	*Arabidopsis*	Turnip crinkle virus	Cooley et al. (2000)
RPS2	LZ–NBS–LRR	*Arabidopsis*	*Pseudomonas syringae*	Tao et al. (2000)
RPS5	LZ–NBS–LRR	*Arabidopsis*	*Pseudomonas syringae*	Warren et al. (1998)
Prf	LZ–NBS–LRR	Tomato	*Pseudomonas syringae* pv. *tomato*	Salmeron et al. (1996)
R1	LZ–NBS–LRR	Potato	*Phytophthora infestans*	Ballvora et al. (2002)
Bs2	NBS–LRR	Pepper	*Xanthomonas campestris* pv. *vesicatoria*	Tai et al. (1999)
Xa1	NBS–LRR	Rice	*Xanthomonas oryzae* pv. *oryzae*	Yoshimura et al. (1998)
Pi-b	NBS–LRR	Rice	*Magnaporthe grisea*	Wang et al. (1999)
Lr21	NBS–LRR	Wheat	*Puccinia triticina*	Huang et al. (2003)
Rp3	NBS–LRR	Maize	*Puccinia sorghi*	Webb et al. (2002)
Rp1-D	NBS–LRR	Maize	*Puccinia sorghi*	Collins et al. (1999)
I2	NBS–LRR	Tomato	*Fusarium oxysporum*	Ori et al. (1997)
Bs4	NBS–LRR	Tomato	*X. campestris* pv. *vesicatoria*	Schornack et al. (2004)
SW5	NBS–LRR	Tomato	Tomato spotted wilt virus	Brommonschenkel et al. (2000)

CFLY, and MHD motifs. The putative LRR domain of Rx comprises 14–16 imperfect copies of LRR (Bendahmane et al., 1999).

The rice bacterial resistance gene *Xa1* encodes an NBS–LRR protein (Yoshimura et al., 1998). The LRR of Xa1 is composed of six almost perfect repeats, each 93 amino acids long. In each repeat unit, six occurrences of a consensus sequence (LXXLXL/IXXN/CXX) were found. In addition, there was a sequence GHGEDG, matching the corresponding consensus NBS sequence GXGXXG (Yoshimura et al., 1998). The putative protein encoded by the pepper *Bs2* gene conferring resistance against the bacterial spot pathogen *Xanthomonas campestris* pv. *vesicatoria* has a putative NBS domain consisting of P-loop, kinase 2, and kinase 3a sequences followed by a domain with unknown function including sequences similar to the sequence motifs GLPL, CFLY, and MHD. The putative LRR domain of the Bs2 has 14–15 imperfect copies of the repeat. Bs2 has a hydrophobic N terminus containing a consensus sequence for mitochondrial sorting and is similar to the human apoptotic protease activating factor-1 (APAF-1) through its central NBS domain. Bs2 does not contain an apparent leucine zipper (LX6L) motif or TIR motif (Tai et al., 1999).

The rice blast resistance gene *Pi-b* encodes a polypeptide of 1251 amino acids (Wang et al., 1999). The protein contains a predicted NBS and the amino acid sequences GMGGLGKTT

(432–440), KSCLIVDDF (520–529), and TSRIIVTTRKENI (549–561) corresponding to the kinase 1a (P-loop), 2, and 3a consensus motifs, respectively. A duplication of the kinase 1a, 2, and 3a motifs of the NBS region was found in the N-terminal half of the Pib protein. The C-terminal region of the Pib protein is composed of 17 imperfect LRRs. Eight cysteine residues were clustered in LRR7 and LRR8 (Wang et al., 1999). The R protein does not have any distinct transmembrane domain. Thus, the *Pi-b* gene belongs to the NBS–LRR class of *R* genes and is predicted to encode a cytoplasmic protein.

The tomato *I2C-1* and *I2C-2* genes confer resistance to the wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici*. The genes were cloned and they were found to encode NBS–LLR proteins. The C-terminal region of I2C-1 and I2C-2 proteins was leucine-rich and arranged as repeats (Ori et al., 1997). The central conserved region of these proteins includes a putative NBS (Ori et al., 1997). In maize, the *Rp3* gene confers resistance to common rust caused by *Puccinia sorghi*. The gene encodes a NBS–LRR protein (Webb et al., 2002). Another maize common rust resistance gene, *Rp1-D*, belongs to the NBS–LRR class of *R* genes (Collins et al., 1999). The *Rp1-D* gene does not encode an N-terminal TIR (Collins et al., 1999). The tomato *R* gene *SW5* conferring resistance to tomato spotted wilt virus also belongs to NBS–LRR class (Brommonschenkel et al., 2000).

In addition to the abovementioned domains, all *R* genes that have cytoplasmic LRRs, contain a highly conserved domain of unknown function called GLPLAL between the NBS and LRR domains (Dinesh-Kumar et al., 2000).

3.3.3 Resistance Genes Encoding LRR Proteins Lacking NBS Domain

Some of the *R* genes encode LRR proteins without characteristic NBS domain (Table 3.3). These LRR proteins may have transmembrane (TM) domains. The LRR domain may have receptor function, whereas TM domain may participate in signal transduction.

The *R* genes encoding receptor-like LRRs with transmembrane domains include apple *Vfa* genes (Xu and Korban, 2002). A cluster of four receptor-like genes (*Vfa1*, *Vfa2*, *Vfa3*, and *Vfa4*) resides in the *Vf* locus of crab apple (*Malus floribunda*) and these genes confer resistance to the apple scab pathogen *Venturia inaequalis* (Xu and Korban, 2002). The *R* genes encode proteins with extracellular LRRs and transmembrane (TM) domains (Xu and Korban, 2002). The resistance genes belonging to the LRR–TM class first recognize the

TABLE 3.3
***R* Gene-Encoded LRR Proteins Lacking NBS Domain**

R Gene	Domain Structure of R Protein	Host Plant	Resistance to Pathogen	References
Cf2/5	LRR–TM	Tomato	*Cladosporium fulvum*	Dixon et al. (1996)
Cf4/9	LRR–TM	Tomato	*Cladosporium fulvum*	Jones et al. (1994); Thomas et al. (1997)
RPP27	LRR–TM	*Arabidopsis*	*Hyaloperonospora parasitica*	Tör et al. (2004)
Vfa1, *Vfa2*, *Vfa3*, *Vfa4*	LRR–TM	Apple	*Venturia inaequalis*	Xu and Korban (2002)
HcrVf2	LRR–TM	Apple	*Venturia inaequalis*	Belfanti et al. (2004)
Xa21	LRR–Kinase	Rice	*Xanthomonas oryzae* pv. *oryzae*	Song et al. (1995)
Ve1	LRR–LZ-like sequences	Tomato	*Verticillium albo-atrum*	Kawchuck et al. (2001)
Ve2	LRR–PEST sequences	Tomato	*Verticillium albo-atrum*	Kawchuck et al. (2001)
Xa21D	LRR	Rice	*Xanthomonas oryzae* pv. *oryzae*	Wang et al. (1998)

elicitor of a specific pathogen and then pass down this signal to such elements as protein kinases to induce a plant defense system (Xu and Korban, 2002).

This class includes apple *HcrVf2* gene for resistance to the scab pathogen *V. inaequalis* (Belfanti et al., 2004). It also includes the tomato *R* genes *Cf2*/*Cf5*, (Dixon et al., 1996), *Cf9* (Jones et al., 1994; Kruijt et al., 2005), and *Cf4* (Thomas et al., 1997). The *Cf* genes encode membrane-anchored, extracellular LRR glycoproteins (Thomas et al., 1997). The large extracytoplasmic LRR domains and putative transmembrane (TM) receptor domains (LRR–TM proteins) are the important components in the R proteins involved in signal transduction (Table 3.3).

Another *R* gene included in this group is *RPP27*, which confers resistance to *Hyaloperonospora parasitica* in *Arabidopsis*. The gene encodes a predicted protein of 1044 amino acids (molecular mass of 116.9 kDa) with structural similarity to tomato *Cf9* and *Cf2* genes (Tör et al., 2004). The protein consists of seven domains: an initial signal peptide (domain A), followed by two LRR domains (domains B and C), a variable region (domain D), an acidic region (domain E), a predicted transmembrane domain (domain F), and a cytoplasmic tail (domain G). Domain C constitutes the majority of the predicted RPP27 protein and consists of 30 imperfect copies of extracellular LRRs with a consensus sequence of LxxLxxLxxLxxLxxNxLSGxIPxx (Tör et al., 2004). The rice bacterial blight resistance gene *Xa21* encodes protein consisting of a putative transmembrane receptor with an extracellular LRR domain and an intracellular serine/threonine kinase domain (Song et al., 1995, 1997, 1998).

The tomato *Verticillium* wilt-resistance genes *Ve1* and *Ve2* seem to encode R proteins which are cell-surface glycoproteins with receptor-mediated endocytosis-like signals and leucine zipper or PEST sequences [PEST, region rich in proline (P), glutamate (E), serine (S), and threonine (T)] (Kawchuck et al., 2001). The primary structure of Ve1 and Ve2 included a hydrophobic N-terminal signal peptide, LRRs containing 28 or 35 potential glycosylation sites, a hydrophobic membrane-spanning domain, and a C-terminal domain with the mammalian E/DXXXLϕ or YXXϕ endocytosis signals (ϕ is an amino acid with a hydrophobic side chain; E-glutamate, D-aspartate, L-leucine, Y-tyrosine, X-unknown amino acid). A leucine zipper-like sequence occurs in the hydrophobic N-terminal signal peptide of Ve1 and proline–glutamate–serine–threonine (PEST)-like sequence resides in the C-terminal domain of Ve2 (Kawchuck et al., 2001).

An LRR resistance gene with LRR domain, but without TM and kinase domains has been detected in rice. The rice bacterial blight resistance gene *Xa21D*, a *Xa21* gene family member, has been cloned (Wang et al., 1998). It encodes a receptor-like protein carrying LRR domain. It lacks transmembrane (TM) and kinase domains (Wang et al., 1998).

3.3.4 Resistance Genes Encoding Proteins Lacking LRR Domain

3.3.4.1 LRD Proteins

There are several *R* genes encoding proteins, which lack LRR domain (Table 3.4). In this group of R proteins, several classes of R proteins are recognized. *Pi-ta* is the resistance gene cloned from the rice blast pathogen *Magnaporthe grisea* (Bryan et al., 2000). It is a single copy gene encoding a putative cytoplasmic protein of 928 amino acids with a centrally localized NBS domain and a leucine-rich domain (LRD) at the C-terminus. Pi-ta contains 16.4% leucine within this LRD region. Although Pi-ta is most similar to R proteins of the NBS–LRR class, the Pi-ta LRD has areas of LXXLXXL motifs, but does not contain LRRs (Jia et al., 2000).

3.3.4.2 Intracellular Protein Kinases

A class of R proteins lacking LRR domain includes intracellular protein kinases. The tomato resistance gene *Pto* lacks LRR domain; it encodes serine/threonine kinase (Martin et al.,

TABLE 3.4
***R* Gene-Encoded Proteins Lacking LRR Domain**

R Gene	Domain Structure of R Protein	Host Plant	Resistance to Pathogen	References
Pi-ta	NBS–LRD	Rice	*Magnaporthe grisea*	Bryan et al. (2000)
Pto	Serine/threonine protein kinase	Tomato	*Pseudomonas syringae* pv. *tomato*	Martin et al. (1993b); Loh and Martin (1995)
PBS1	Protein kinase	*Arabidopsis*		Swiderski and Innes (2001)
Rpg1	Receptor kinase-like protein with two tandem kinase domains	Barley	*Puccinia graminis* f. sp. *tritici*	Brueggemann et al. (2002); Rostoks et al. (2004)
RPW8.1, *RPW8.2*	CC-transmembrane (TM) protein	*Arabidopsis*	*Erysiphe cichoracearum, E. cruciferarum, E. orontii, Oidium lycopersici*	Xiao et al. (2001, 2003)
LM1	Two transmembrane (TM) motifs	Brassica nigra	*Leptosphaeria maculans*	Wretblad et al. (2003)
Asc1	Protein with multiple transmembrane (TM) domains	Tomato	*Alternaria alternata* f. sp. *lycopersici*	Brandwagt et al. (2002); Spassieva et al. (2002)
RTM1	Lectin-type protein	*Arabidopsis*	Tobacco etch virus	Chisholm et al. (2000)
RTM2	Heat shock protein-like protein	*Arabidopsis*	Tobacco etch virus	Whitham et al. (2000)
HM1	NADPH-dependent reductase	Maize	*Cochliobolus carbonum*	Johal and Briggs (1992)
At1, *At2*	Photorespiratory peroxisomal enzyme proteins glyoxalate aminotransferases	Melon	*Pseudoperonospora cubensis*	Taler et al. (2004)

1993a,b; Loh and Martin, 1995). However, an LRR–NBS-containing protein, Prf is necessary for *Pto* function (Salmeron et al., 1996). *PBS1* from *Arabidopsis* (Swiderski and Innes, 2001) is also a serine/threonine protein kinase that provides resistance to *Pseudomonas syringae*. PBS1 belongs to a distinct subfamily of protein kinases that contains no other members of known function. The Pto kinase of tomato does not fall in the same subfamily as PBS1 and is only 42% identical in the kinase domain (Swiderski and Innes, 2001).

The barley stem rust (*Puccinia graminis* f. sp. *tritici*) resistance gene *Rpg1* encodes a receptor kinase-like protein with two tandem protein kinase domains. It is a novel structure for a plant disease resistance gene (Brueggemann et al., 2002). The *Rpg1* gene has an N-terminal domain that does not resemble any previously described receptor (Brueggemann et al., 2002).

3.3.4.3 Transmembrane Proteins

The *Arabidopsis* disease resistance genes *RPW8.1* and *RPW8.2* encode another type of R proteins. These genes encode small, basic proteins with a putative N-terminal transmembrane domain and a coiled-coil domain (Xiao et al., 2001, 2003). They lack the NBS and LRR domains (Dangl and Jones, 2001).

LM1 gene cloned from black mustard (*Brassica nigra*) encodes another new type of protein having two predicted transmembrane motifs (Wretblad et al., 2003). The gene confers resistance against *Leptosphaeria maculans* (Wretblad et al., 2003). The tomato *R* gene *Asc1*

encodes another transmembrane protein, which confers insensitivity to the toxin produced by the pathogen *Alternaria alternata* f. sp. *lycopersici* and resistance to the pathogen (Brandwagt et al., 2002). The gene was cloned from mRNA purified from tomato genotype *Asc1/Asc* leaf total RNA (Spassieva et al., 2002). The gene codes a protein with multiple transmembrane domains and a highly conserved motif designated as the Lag1p motif. *Asc1* confers insensitivity to AAL-toxin that inhibits the enzyme sphinganine *N*-acyltransferase involved in sphingolipid metabolism leading to programmed cell death (Spassieva et al., 2002).

3.3.4.4 Lectin-Type Proteins

Another *Arabidopsis R* gene is *RTM1*, which is necessary for restriction of long-distance movement of tobacco etch virus without causing a hypersensitive response. *RTM1* gene was isolated by map-based cloning and the deduced gene product was similar to jacalin, a D-galactose-specific lectin from *Artocarpus integrifolia* (Chisholm et al., 2000). Jacalin is synthesized as a prepropeptide that is proteolytically processed to form two mature polypeptides, a 133-aa α-chain and a 20- to 21-aa β-chain, which remain associated in the folded protein. The majority of RTM1 sequence is similar to the jacalin α-chain, although the N terminus of RTM1 exhibits similarity with the C-terminal half of the β-chain (Chisholm et al., 2000).

3.3.4.5 Heat Shock Protein-Like Proteins

Another *Arabidopsis R* gene, *RTM2*, which is also necessary for long-distance movement of tobacco etch virus, has been cloned (Whitham et al., 2000). The deduced RTM2 protein contains several domains, including an N-terminal region with similarity to plant small heat shock proteins (HSPs) (Whitham et al., 2000; Chisholm et al., 2001). Unlike most other small HSPs, RTM2 has an extended C-terminus that includes a predicted transmembrane domain (Whitham et al., 2000).

3.3.4.6 NADPH-Dependent Reductase-Type Protein

Another type of *R* genes is maize *HM1* gene that encodes a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase, which inactivates toxin produced by pathogen (*Cochliobolus carbonum*) (Johal and Briggs, 1992).

3.3.4.7 Plant *eR* Genes Encoding Photorespiratory Peroxisomal Enzyme Proteins

Two novel genes, *At1* and *At2*, have been cloned from the wild melon line P1 124111F and they are called enzymatic resistance (*eR*) genes (Taler et al., 2004). Unlike other plant disease resistance genes, which confer an ability to resist infection by pathogens expressing corresponding avirulence genes, the resistance of the wild melon to *Pseudoperonospora cubensis* was controlled by enhanced expression of the *eR* genes, *At1* and *At2*. These constitutively expressed genes encode the photorespiratory peroxisomal enzyme proteins glyoxylate aminotransferases. These genes were expressed at low level in the susceptible lines. Enhanced expression was observed in resistant lines (Taler et al., 2004).

3.4 MOLECULAR STRUCTURE OF RECESSIVE GENES

3.4.1 Barley *mlo* Gene

Recessive genes conferring high level of resistance have also been cloned (Table 3.5). The recessive *mlo* mutation in barley confers broad-spectrum (race-nonspecific) resistance to

TABLE 3.5
Molecular Structure of Recessive Genes

Recessive Gene	Molecular Structure	Host Plant	Resistance to Pathogen	References
mlo	Calmodulin-binding transmembrane (TM) protein	Barley	*Blumeria graminis* f. sp. *hordei*	Büschges et al. (1997); Kim et al. (2002); Piffanelli et al. (2002)
pmr6	Pectate lyase-like protein	*Arabidopsis*	*Erysiphe cichoracearum*	Vogel et al. (2002)
RRS1-R	TIR–NBS–LRR–WRKY	*Arabidopsis*	*Ralstonia solanacearum*	Lahaye (2002); Deslandes et al. (2002)
ssi4	TIR–NBS–LRR	*Arabidopsis*	*Pseudomonas syringae* pv. *maculicola* and *Hyaloperonospora parasitica*	Shirano et al. (2002)

several isolates of *Blumeria graminis* f. sp. *hordei*, and the *Mlo* gene encodes a novel class of plant-specific integral membrane proteins anchored in the plasma membrane by seven transmembrane (TM) domains (Büschges et al., 1997; Devoto et al., 1999). MLO is a novel calmodulin-binding protein (Kim et al., 2002). *Mlo* is hypothesized to be a negative regulator of defense responses such that the null *mlo* alleles mediate resistance by allowing abnormal defense responses (Büschges et al., 1997; Király et al., 2002; Piffanelli et al., 2002).

3.4.2 *Arabidopsis PMR6* Gene

An *Arabidopsis* gene *PMR6* was cloned and found to encode a pectate lyase-like protein. The protein sequence contains a predicted N-terminal endoplasmic reticulum transport domain and a predicted C-terminal glycosyl-phosphatidylinositol modification, which in other proteins has been shown to function as an anchor to the plasma membrane surface (Vogel et al., 2002). The powdery mildew (*Erysiphe cichoracearum*) disease resistance was associated with a recessive, loss of function mutation in *PMR6*. The recessive gene *pmr6* confers disease resistance by inhibiting growth and reproduction of *E. cichoracearum* (Vogel et al., 2002).

3.4.3 *Arabidopsis RRS1-R* Gene

The *Arabidopsis* recessive gene *RRS1-R* (Lahaye, 2002) confers resistance to several races of *Ralstonia solanacearum* (Deslandes et al., 2002). *RRS-R* gene from *Arabidopsis thaliana* encodes a protein whose structure combines the TIR–NBS–LRR domains and a WRKY motif characteristic of some plant transcriptional factors (Deslandes et al., 2002, 2003).

3.4.4 *Arabidopsis ssi4* Gene

Another recessive gene in *Arabidopsis*, *ssi4*, confers constitutive expression of *PR* (pathogenesis-related) genes, induces salicylic acid accumulation, and enhances resistance to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* and the oomycete pathogen *Hyaloperonospora* (= *Peronospora*) *parasitica* (Shirano et al., 2002). The gene *ssi4* has been cloned through map-based cloning method and was found to encode a TIR–NBS–LRR protein (Shirano et al., 2002).

3.5 PERCEPTION OF PATHOGEN SIGNALS BY RESISTANCE GENES

3.5.1 Functions of Different Domains of R Proteins in Pathogen Recognition

3.5.1.1 LRR Domain

LRR proteins are the common domains of many *R* genes. It has been well demonstrated that LRR proteins from animals and fungi mediate specific protein–protein interactions or ligand binding (Kobe and Deisenhofer, 1993, 1995; Jones and Jones, 1997). LRR structures mediate protein–protein interaction and are the major determinants of recognition specificity (Kobe and Deisenhofer, 1999). The LRR domain of R proteins might contribute to the recognition of diverse pathogen-derived ligands (Tameling et al., 2002).

A single amino acid difference in the LRR domain distinguished susceptible and resistant alleles of the rice blast resistance gene *Pi-ta* (Bryan et al., 2000), suggesting the importance of LRR domain in inducing defense response in rice plants. The tomato *R* genes *Cf4* and *Cf9*, which confer resistance to *Cladosporium fulvum*, contain an extracellular LRR domain. By domain swapping, a number of LRRs have been shown to be essential for both Cf4 and Cf9 function (Van der Hoorn et al., 2001; Wulff et al., 2001). Loss of a repeated unit within the LRR coding region resulted in loss of function of the flax rust resistance gene *M* (Anderson et al., 1997). Substitution of serine for proline at position 619 at the beginning of the second LRR in the LRR domain of the tobacco *R* gene *N* encoded proteins leads to loss of N function (Dinesh-Kumar et al., 2000). A mutation, which causes a glutamate-to-lysine in the LRR domain of the *Arabidopsis RPS5* gene-encoded protein, resulted in loss of resistance-inducing ability of the *R* gene against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Warren et al., 1998). These results suggest the importance of LRR domain in *R* gene-mediated gene-for-gene resistance system.

LRR is an important determinant of pathogen specificity of the *R* gene. LRR-encoding region is the most variable part of resistance genes (Botella et al., 1998; McDowell et al., 1998; Ellis et al., 1999). Resistance genes tend to be clustered in the genome (Michelmore and Meyers, 1998). *R* loci may be single genes with multiple alleles. For example, the *L* locus in flax contains 13 alleles. The *R* genes show varying levels of recombination between the component genes. Genes within a single cluster can determine resistance to very different pathogens (Michelmore and Meyers, 1998). LRRs have been shown to have considerable variation among members within clustered *R* gene families (Meyers et al., 1998; Dinesh-Kumar et al., 2000). This variation in LRRs correlates directly with new specificities in case of flax *L* alleles (Meyers et al., 1998; Ellis et al., 1999). The flax rust resistance *L* gene alleles *L6* and *L7* differ only in the LRR region (Ellis et al., 1999). When the *L6* or *L10* TIR and NBS domains were fused to the LRR region of *L2*, the chimeric genes encoded *L2* specificity and not *L6* or *L10* specificity (Ellis et al., 1999).

The importance of LRR region of resistance proteins in the specificity of gene-for-gene interactions has been demonstrated in tomato also. The Cf4 and Cf5 resistance proteins of tomato conferring resistance to different races of *Cladosporium fulvum* consist of only a membrane-bound extracellular LRR domain. The specificity difference between the two R proteins was found within the 459 amino acids of the N-terminal half of the molecule (Thomas et al., 1997). The majority of these differences were detected in the conserved β-strand/β-turn structural motif of LRR units (Parniske et al., 1997). In the *P* locus of flax, six amino acid changes within the β-strand/β-turn motif of the LRR were responsible for the different specificities of *P* and *P2* (Dodds et al., 2001a). These results demonstrate the importance of LRR domains in determining race specificity of *R* genes.

3.5.1.2 NBS Domain

NBS motifs have been detected in many R proteins. NBS domains have been identified in many prokaryotic and eukaryotic proteins, in which their ability to bind ATP or GTP is

essential for their biological activity (Saraste et al., 1990). The NBS of R proteins has an N-terminal subdomain that contains consensus kinase 1a (P-loop), 2, and 3a motifs (Moffett et al., 2002). The kinase 1a motif forms a glycine-rich flexible loop containing an invariant lysine residue involved in binding the phosphates of the nucleotide (Saraste et al., 1990; Traut, 1994). The P-loop is known to be important for binding proteins to nucleotides (Shirano et al., 2002). The kinase-2 motif has an invariant aspartate that coordinates the divalent metal ion required for phosphotransfer reactions. The kinase 3a motif is involved in binding the purine base or pentose of the nucleotide (Traut, 1994).

NBS domain appears to be important in inducing the activity of *R* gene. The *Arabidopsis* resistance gene *ssi4* enhances resistance to bacterial and oomycete pathogens. Comparison between *ssi4* and the corresponding wild-type sequence revealed a single amino acid substitution in the NBS (Shirano et al., 2002). The wild-type *SSI4* gene encodes a TIR–NBS–LRR-type protein and Gly-to-Arg substitution in its NBS in the mutant *ssi4* confers enhanced disease resistance. It suggests that NBS may be important in enhancing R protein activity (Shirano et al., 2002). The *RPS2* gene confers resistance against *Pseudomonas syringae* strains carrying the avirulence gene *avrRpt2* (Tao et al., 2000). Directed mutagenesis revealed that the NBS and an N-terminal LZ motif were critical for RPS2 function (Tao et al., 2000).

NBS domain may also be important in the race specificity function of *R* genes. Several mutant and recombinant forms of *L6* were made that altered the methionine–histidine–aspartate motif conserved in the NBS domain of resistance proteins. In transgenic flax, some of these alleles were autoactive and the transgenic plants showed resistance to flax rust strains virulent to wild-type *L6* plants. Thus, a change in structure of NBS domain converts race specificity of *L6* gene into non-race specificity (Howles et al., 2005).

NBS of R proteins forms a functional nucleotide-binding pocket (Tameling et al., 2002). However, the ability of plant R proteins to bind nucleotides is yet to be demonstrated. NBS domains are capable of binding and hydrolyzing ATP (Tameling et al., 2002) and act as ATPases (Dinesh-Kumar et al., 2000). The NBS may be involved in signal transduction cascades through phosphorylation/dephosphorylation events with either ATP or GTP (Dangl and Jones, 2001).

3.5.1.3 TIR Domain

TIR domain has a homology with the cytoplasmic signaling domains of animal innate immunity factors, *Drosophila* Toll and mammalian interleukin-1 receptor proteins (Rock et al., 1998; Kopp and Medzhitov, 1999). LRR region does not exclusively control differences in allelic specificity (Ellis et al., 1999). Although *L6* and *L7* encode distinct resistance specificities, they have identical LRR regions. However, there were 11 amino acid differences between L6 and L7 proteins and all were located within the first 208 amino acids of the TIR region (Ellis et al., 1999). In flax rust resistance, replacement of TIR-encoding region of the *L6* allele of the *R* gene *L* with the corresponding regions of *L2* or *LH* allele by recombination changed the specificity of the allele from *L6* to *L7* (Luck et al., 2000). These results suggest that TIR also may be involved in conferring specificity to some *R* genes. The TIR domain appears to be essential for *N* gene-mediated TMV resistance in tobacco. Changes in amino acids that affected TIR-dependent signaling caused either partial or full loss of *N* gene function (Dinesh-Kumar et al., 2000).

3.5.1.4 CC Domain

CC domain may be involved in the recognition of Avr gene product. The *Arabidopsis R* gene *RPW8* confers broad-spectrum resistance to various powdery mildew pathogens, such as *Erysiphe cichoracearum*, *Golvinomyces orontii* (= *Erysiphe orontii*), and *Oidium lycopersici* (Xiao et al., 2001, 2003). The *R* gene-coded protein contains CC domains that appear at the C-terminal of a predicted transmembrane domain or signal peptide domain (Xiao et al., 2001).

In this case, the CC domain may be analogous to the function played by the LRR domain and could act directly in pattern recognition of an avirulence factor common to many mildew pathogens (Fluhr, 2001).

3.5.1.5 C-Terminal Non-LRR Region

C-terminal non-LRR region (CT) also may be involved in race specificity determination. The CT region of barley powdery mildew resistance genes *Mla1* and *Mla6* (Shen et al., 2003) and that of flax rust resistance gene *P* (Dodds et al., 2001a) have been shown to be involved in determining race specificity of the *R* genes. Recombinant forms of the race-specific flax rust resistance gene *L6* in which the short C-terminal to the LRR region was altered, showed nonspecific resistance to the strains of flax rust pathogen, suggesting the importance of the CT region in conferring race specificity to the *R* gene (Howles et al., 2005). A series of reciprocal domains swaps between the barley R proteins MLA1 and MLA6 identified in each protein recognition domain for cognate powdery mildew fungus avirulence genes (*AvrMla1* and *AvrMla6*). These domains were within the CT part (Shen et al., 2003).

3.5.1.6 C-Terminus Transcriptional Activation Domain

A recessive gene from *Arabidopsis* encoded R protein, *RRS1-R*, contains a putative transcriptional activation domain, besides TIR–NBS–LRR domains (Deslandes et al., 2002). The *RRS1-R* may have a dual function. The NH2 terminus may bind a pathogen-derived signal by means of the LRR motifs known to mediate protein–protein interaction. This recognition event may lead to the activation of the C-terminus WRKY transcriptional factor domain. This, in turn, would activate a signaling cascade, leading to disease resistance response (Deslandes et al., 2002). WRKY factors play an important role in signal transduction systems. Induction of accumulation of defense-related *PR* gene-encoded pathogenesis-related proteins has been shown to occur downstream of action of certain WRKY factors (Yang et al., 1999). The WRKY factors regulate the expression of NPR1, a positive regulator of inducible plant disease resistance (Yu et al., 2001). WRKY transcription factors act as potential common regulators of genes in the PR-1 regulon (Ryals et al., 1996).

3.5.1.7 Protein Kinase Domain

Another class of *R* genes encodes proteins with protein kinase domain (Martin et al., 1993a,b; He et al., 2000). These *R* genes include tomato *Pto* (Martin et al., 1993b), barley *Rpg1* (Brueggemann et al., 2002), and *Arabidopsis PBS1* (Swiderski and Innes, 2001) genes. The protein kinase domain may play a role in protein phosphorylation. Protein phosphorylation is a key component in pathogen recognition (Martin et al., 1993b). Some of the protein kinases are known to act as receptor sites for the pathogen signals (Shiu and Bleecker, 2001).

3.5.1.8 Transmembrane Domain

A group of R proteins contains transmembrane proteins, besides LRR domains. The LRR domain may participate in recognition of pathogen signals and TM may pass down this signal to induce plant defense responses (Xu and Korban, 2002). The genes encoding transmembrane proteins include *RPP27* in *Arabidopsis* (Tör et al., 2004), *Cf2*, *Cf4*, *Cf5*, and *Cf9* in tomato (Jones et al., 1994; Dixon et al., 1996; Thomas et al., 1997), *Ve1* and *Ve2* genes in tomato (Kawchuck et al., 2001), and *HcrVf2*, *Vfa1*, *Vfa2*, *Vfa3*, and *Vfa4* genes in apple (Xu and Korban, 2002; Belfanti et al., 2004), *Asc1* in tomato (Spassieva et al., 2002), *LM1* in *B. nigra* (Wretblad et al., 2003), *RPW8.1* and *RPW8.2* in *Arabidopsis* (Xiao et al., 2003) and these *R* genes may have a function in *R* gene-mediated signal transduction.

3.5.1.9 Calmodulin-Binding Protein

Another gene isolated from barley and rice, *Mlo* is a calmodulin (CAM)-binding protein (Kim et al., 2002). Influx of Ca^{2+} is an early event in the signaling cascades that trigger plant defense responses and Ca^{2+} signals are mediated by calmodulin (Kim et al., 2002). The rice *Mlo* (OsMlo) has been shown to bind soybean calmodulin isoform-1 (SCam-1) in a Ca^{2+}-dependent manner. A 20-amino acid calmodulin-binding domain (CaMBD) in the OsMlo C-terminal cytoplasmic tail has been shown to be necessary and sufficient for Ca^{2+}-dependent CaM complex formation. Expression of *OsMlo* was strongly induced by defense signaling molecules (Kim et al., 2002). These observations suggest that the *R* gene, *Mlo*, may operate through Ca^{2+} signaling system and trigger the defense genes.

3.5.1.10 Lectin-Type Protein

Arabidopsis RTM1 gene encodes a lectin-type protein (Chisholm et al., 2000). Lectins are known to act as receptors for pathogen signals (Baureithel et al., 1994). However, the function of *RTM1* gene in pathogen perception has not been demonstrated.

3.5.1.11 Heat Shock Protein (HSP)-Like Protein

Another *Arabidopsis R* gene, *RTM2*, encodes an HSP-like protein (Whitham et al., 2000). Some HSP-like proteins have been demonstrated to play a regulatory role in *R* gene-mediated signal pathways (Liu et al., 2004). An HSP interacts with the LRR-C-terminal (CT) part of all tested barley R proteins (Bieri et al., 2004). The function of HSP in the signaling system is described in the later part of this chapter.

3.5.2 *R* Gene Product May Act as a Receptor That Recognizes an *AVR* Gene Product

According to the gene-for-gene hypothesis (Flor, 1971), the defense responses are often activated by the action of a plant resistance (*R*) gene and a pathogen avirulence (*AVR*) gene. The molecular basis of gene-for-gene interactions is explained by a ligand and receptor model. The *R* gene product may act as a receptor that recognizes a ligand, or elicitor, produced directly or indirectly by the pathogen's *AVR* gene. Several *AVR* gene-encoded products have been identified as elicitors (De Wit, 1995; Rohe et al., 1995; Kruijt et al., 2005). The structures of *R* genes support the ligand–receptor model (Kobe and Deisenhofer, 1999). On perception of the pathogen signal, the host plant defense response is activated.

Direct interaction between pathogen's *Avr* gene product and plant's *R* gene product has been reported only in a few plant–pathogen systems. An elicitor called AVR-Pita is an *Avr* gene product of the rice blast pathogen *Magnaporthe grisea*. The Avr-Pita is a genotype-specific elicitor and it acts inside cells of resistant *Pi-ta* rice (Jia et al., 2000). Avr-Pita was predicted to encode a metalloprotease with an N-terminal secretory signal and pro-protein sequences. AVR-$Pita_{176}$ lacked the secretory and pro-protein sequences. Transient expression of *AVR-Pita*$_{176}$ inside plant cells resulted in a *Pi-ta*-dependent resistance response. AVR-$Pita_{176}$ was shown to bind specifically to the LRD of the Pi-ta protein, both in the yeast two-hybrid system and an *in vitro* binding assay. Single amino acid substitutions in the Pi-ta LRD or in the AVR-$Pita_{176}$ protease motif that resulted in loss of resistance in the plant also disrupted the physical interaction, both in yeast and *in vitro*. These results suggest that the Avr-$Pita_{176}$ protein binds directly to the Pi-ta LRD region inside the plant cell to initiate defense response (Jia et al., 2000).

Physical interaction between *R* gene products and *avr* gene products has also been described in bacterial diseases. Pto is a serine/threonine kinase gene product of tomato plants showing resistance to the tomato bacterial speck pathogen *Pseudomonas syringae* pv. *tomato* and it lacks an LRR domain. The avrPto and avrPtoB are the *avr* gene products of this pathogen (Lin and Martin, 2005). Physical interaction between Pto and avrPto proteins has been demonstrated with the yeast two-hybrid system (Scofield et al., 1996; Tang et al., 1996; Lin and Martin, 2005). The susceptible tomato cultivar Alisa Craig contained an allele of *Pto* which encodes an active Pto kinase; however, the allele failed to bind avrPto in the yeast two-hybrid system (Tang et al., 1996; Jia et al., 1997). A single amino acid residue, Thr204 of the Pto kinase determines the recognition specificity for avrPto (Frederick et al., 1998) and the susceptible allele of *Pto* may differ slightly from the resistance allele. These results suggest that specific binding of *R* and *avr* gene products is essential for activation of the host defense responses.

The binding of the *Arabidopsis thaliana* resistance gene *RPS2* encoded protein and its corresponding *Pseudomonas syringae* avirulence gene *avrRpt2* product has been reported (Leister and Katagiri, 2000). It is suggested that AvrRpt2 protein is transported into plant cells, in which it interacts with and is recognized by the RPS2 protein-based surveillance system (Tao et al., 2000). It has been demonstrated that direct expression of *avrRpt2* in plant cells is sufficient to induce *RPS2*-dependent responses and RPS2 is localized in the plant cytoplasm. AvrRpt2 and RPS2 form an immunoprecipitable complex in the plant cell (Tao et al., 2000). These observations suggest that the pathogen signal is recognized by plant *R* gene inside the cell.

3.5.3 R Protein May Detect Binding of an AVR Protein to a Different Protein in the Plant

Although binding of Avr proteins and R proteins is suggested in the gene-for-gene systems, there are also reports that direct interaction of Avr and R proteins may be lacking in some systems. In these cases, R proteins function indirectly in the recognition process, which involves the surveillance of a host protein or a complex that is targeted by AVR products (Dangl and Jones, 2001; Mackey et al., 2002).

Instead of R and AVR protein interactions, complex protein interactions involving additional factors are involved in the R protein Cf9-the AVR protein Avr9-mediated resistance to the leaf mold pathogen *Cladosporium fulvum* in tomato (Bogdanove, 2002a). Luderer et al. (2001) provided a convincing lack of evidence for an interaction between the products of *Cf9* and *Avr9* genes using a variety of binding assays. A high-affinity binding site (HABS) for ^{125}I-labeled Avr9 was identified in near-isogenic tomato lines with and without the *R* gene *Cf9*. The HABS was also detected in tobacco and other solanaceous plants (Kooman-Gersmann et al., 1996). It suggests that the binding site for Avr9 protein is not the Cf9 R protein. However, affinity of Avr9 mutants for the HABS correlated with their relative activity as defense response elicitors, suggesting a role for the binding site in Avr9 recognition (Kooman-Gersmann et al., 1998).

A three-way interaction between Avr9, HABS, and Cf9 may be involved in activation of defense responses (Luderer et al., 2001). There may be an indirect role for Cf9 in perception of Avr9. Such indirect perception has been referred to as the "guard hypothesis" (van der Biezen and Jones, 1998b; Dangl and Jones, 2001). According to this hypothesis, the Avr protein targets the third protein to promote disease and pathogen multiplication, acting as a virulence factor and the R protein in effect guards the protein. The HABS may represent a virulence target for Avr9 and the Cf9 protein may act as a "guard" to monitor the behavior of one or more host-encoded virulence proteins that are targets for AVR9 (Luderer and Joosten, 2001; Van der Hoorn et al., 2002). However, experimental evidences to prove this hypothesis

are lacking. A model involving associations of multiple proteins in the plant for pathogen recognition may be more apt to explain the molecular basis of perception of pathogen signals by plants in the race-specific interactions (Bogdanove, 2002a).

The binding site for Avr4 elicitor protein of *C. fulvum* has been reported to be different from Cf4 resistance gene product (Westerink et al., 2002). An Avr4 HABS, which exhibited all the characteristics expected for ligand–receptor interactions, such as saturability, reversibility, and specificity, has been identified. However, the Avr4 HABS appeared to originate from fungi present on infected tomato plants rather than from the tomato plants themselves (Westerink et al., 2002). Although Avr4 binds to fungal components with high affinity, this interaction was not required for elicitor activity of Avr4 in Cf4-resistant plants, because Avr4 exhibits necrosis-inducing activity when injected into resistant Cf4 leaves in the absence of fungal components (Westerink et al., 2002). It suggests that Cf4 protein may be involved in inducing resistance, not binding with the elicitor, but by involving in association with multiple proteins as additional factors. Probably, the R protein may detect binding of an Avr protein to a different protein in the plant (van der Biezen and Jones, 1998; Bogdanove, 2002a).

3.6 ACTIVATION OF R PROTEIN AND EMISSION OF SIGNALS TO OTHER COMPONENTS IN THE CELL

On perception of pathogen signals, the R proteins may be activated and become competent to signal to other components in the plant cell. Some *R* genes have also been detected in susceptible plants. The rice blast resistance gene *Pib* was detected in both resistant and susceptible rice varieties. *Pib* was expressed at a low level in intact leaves but the gene expression increased at 12 h following inoculation with both incompatible and compatible races of the blast pathogen *M. grisea* (Wang et al., 1999). Although the gene could be detected in the susceptible rice variety, no transcript of the gene could be detected (Wang et al., 1999). It suggests that some specific signal recognized by the resistant variety may be needed to activate the *R* gene transcription.

Transcription of race-specific *Mla6* and *Mla13* genes was induced several fold in the interactions with incompatible *Blumeria graminis* f. sp. *hordei* pathotypes, but not with compatible pathotypes (Figure 3.1; Halterman et al., 2003). It also suggests that specific

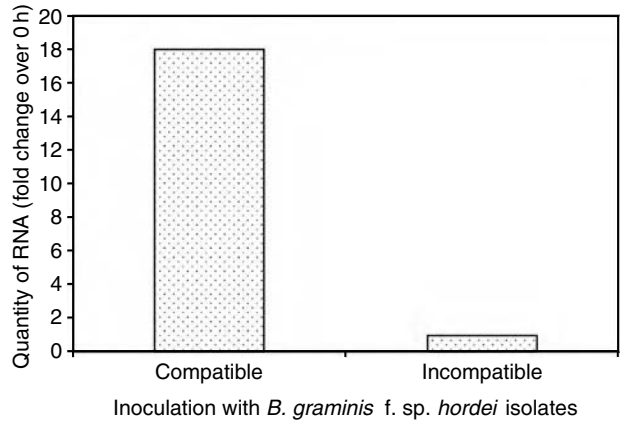

FIGURE 3.1 Transcript profiles of *Mla* in barley plants inoculated with compatible or incompatible isolates of *Blumeria graminis* f. sp. *hordei*. (Adapted from Halterman, D., Wei, F., and Wise, R.P., *Plant Physiol*., 131, 558, 2003.)

pathogen signals (probably avr proteins) may be involved in activation of transcription of *R* genes.

The mRNA for the *Xa1* gene for resistance against bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* was detected from rice leaves 5 days after inoculation with the pathogen, but was not detected in intact leaves (Yoshimura et al., 1998). It suggests that the *R* gene is not constitutively expressed and that it is activated by a signal from the pathogen.

On perception of signals, *R* gene transcription may increase, resulting in abundance of R proteins. The barley resistance gene *Mlo* transcript abundance increases in barley leaf in response to inoculation with *B. graminis* f. sp. *hordei* or *Magnaporthe grisea* (Piffanelli et al., 2002). Biotic elicitors such as the carbohydrate elicitor from the wheat powdery mildew pathogen *B. graminis* f. sp. *tritici* and abiotic elicitors such as paraquat also activated the resistance gene (Piffanelli et al., 2002). Bieri et al. (2004) suggested that a threshold for R protein-derived signal might exist to trigger effective resistance. Activation of the *R* gene by the signals may enhance the abundance of R proteins and this may facilitate in conferring disease resistance.

Structural characteristics of *Pib* gene product suggest that the R protein may interact with other proteins in a defense-reaction signal transduction pathway (Wang et al., 1999). Expression of *Pib* gene was regulated dramatically by environmental conditions such as altered temperatures and darkness (Wang et al., 1999) and also induced by chemical treatments, such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and probenazole (Wang et al., 2001). Expression of the rice *Mlo* gene was strongly induced by a fungal pathogen and by various plant defense signaling molecules (Kim et al., 2002).

Interaction between domains of the R proteins may result in activation of R proteins. Different domains of the PVX resistance gene *Rx* encoded protein were expressed either individually or in combinations (Moffett et al., 2002). In the bioassays, the coexpression of LRR and CC–NBS as separate domains resulted in elicitor-dependent disease resistance. Coexpression of CC with NBS–LRR also resulted in induction of disease resistance (Moffett et al., 2002). Similar interactions also take place in pepper R protein Bs2 (Moffett et al., 2002). The results suggest that the different domains of the R proteins may physically interact and confer resistance. These interactions are likely to be intramolecular. Recognition of pathogen signal may initiate a sequence of conformational changes in R protein that involve disruption of these intramolecular interactions. These conformational changes result in the activation of the *R* gene-encoded protein so that it becomes competent to initiate signaling that leads to disease resistance (Moffett et al., 2002).

Protein–protein interactions may occur between different R proteins. It has been suggested that the LRR region may interact with other plant proteins (Warren et al., 1998). An R protein may affect the function of other R proteins. The *RPS5* gene encodes a putative nucleotide-binding domain and 12 to 21 LRRs (Warren et al., 1998). In *Arabidopsis*, accession Columbia (Col-0) possesses the *R* gene *RPS5*, which mediates resistance to *Pseudomonas syringae* pv. *tomato* DC300 carrying the *avr* gene *avrPphB*. An *rps5* mutant, *rps5-1*, was detected in mutagenized Col-0 plants. The mutant showed susceptibility to *P. syringae* pv. *tomato* DC3000. The rps5-1 mutation was located within LRRs. The mutation affected not only the function of *RPS5*, but also several *R* genes (Warren et al., 1998). In addition to *RPS5*, the *Arabidopsis* line Col-0 possesses different *R* genes, such as *RPS2* and *RPM1*, which confer strain-specific resistance to strains of *P. syringae* pv. *tomato* carrying different *avr* genes. The *RPS2* gene confers resistance to the strain carrying *avrRpt2*, whereas *RPM1* gene confers resistance to *avrB*. The *RPS5* gene, which confers resistance against the strain carrying avrPphB, does not have any role in resistance to strains carrying *avrRpt2* or *avrB*. Hence, any mutation in *RPS5* should not have any effect on resistance conferred by *RPS2* or *RPM1* gene. However, the *rps5-1* mutant showed susceptibility not only to the strain carrying *avrPphB*, but also to those carrying *avrRpt2* and *avrB* (Warren et al., 1998). Interestingly,

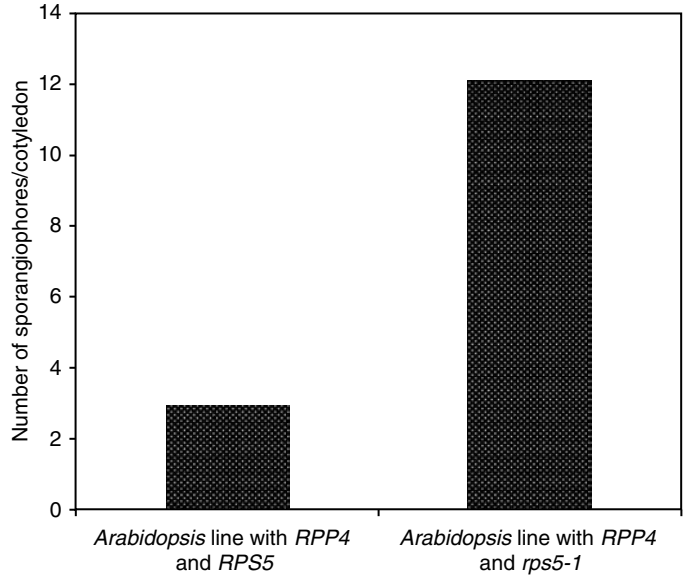

FIGURE 3.2 Effect of *rps5-1* on expression of another resistance gene *RPP4* conferring resistance to *Peronospora parasitica*. (Adapted from Warren, R.F., Henk, A., Mowery, P., Holub, E., and Innes, R.W., *Plant Cell*, 10, 1439, 1998.)

rps5-1 negatived the resistance conferred by *RPP4* gene against the downy mildew pathogen *Hyaloperonospora parasitica*. Sporulation of *P. parasitica* was enhanced in Col *RPP4-rps5-1* plants when compared with wild-type Col-0 plants expressing both *RPP4* and *RPS5* (Figure 3.2; Warren et al., 1998).

The mutant *rps5-1* also reduced the efficiency of other downy mildew resistance genes such as *RPP9*, *RPP2*, and *RPP6* (Warren et al., 1998). The *rps5-1* mutation causes a G-to-A transition, which results in a glutamate-to-lysine change at amino acid 799. The mutation was located in the LRR region and the mutation was contained in the third LRR. It partially compromised the function of several *R* genes that conferred bacterial and oomycete resistance. These results suggest that the LRR domain of the R protein RPS5 may interact with a signal transduction component of a pathway triggered by several other R proteins (Warren et al., 1998).

3.7 DOWNSTREAM COMPONENTS OF *R* GENE-SIGNALING SYSTEMS

3.7.1 Regulatory Genes (or Complementary Genes or *R* Gene-Signaling Components)

Genetically, R protein signaling appears to be integrated through a limited set of common downstream components (Bennett et al., 2005). The downstream components include various regulatory genes (Fan and Dong, 2002; Tör et al., 2002; Shen et al., 2003), which are also called complementary genes (Kalavacharla et al., 2000) or *R* gene-signaling components (Halterman et al., 2003). These genes interact with *R* genes and participate in downstream events of *R* gene-signaling (Kalavacharla et al., 2000). These genes include *EDS1* (enhanced disease susceptibility) (Falk et al., 1999; Xiao et al., 2001; Peart et al., 2002a), *NDR1* (non-race-specific disease resistance) (Century et al., 1997), *PAD4* (phytoalexin deficient 4) (Glazebrook et al., 1997; Zhou et al., 1998; Jirage et al., 1999, 2001), *RAR1* (required for Mla12 resistance1) (Shirasu et al., 1999; Halterman et al., 2003; Shen et al., 2003;

Jarosch et al., 2005), *SGT1* (suppressor of G-two allele of SKP1) (Kitagawa et al., 1999; Shirasu et al., 1999; Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002b; Peart et al., 2002b; Tör et al., 2002), *HSP90* (heat shock protein 90) (Hubert et al., 2003), *Crg* (complements resistance gene) (Kalavacharla et al., 2000), *NPR1* (nonexpressor of *PR1*) (Chern et al., 2001, 2005; Fan and Dong, 2002), and *edr1* (enhanced disease resistance 1) (Tang and Innes, 2002).

The *Arabidopsis* genes *EDS1* and *PAD4* encode lipase-like proteins that interact with each other and mediate the downstream signaling of known TIR–NBS–LRR class of R proteins but not CC–NBS–LRR class R proteins (Feys et al., 2001). Conversely, the membrane-bound protein encoded by *NDR1* is required for many CC–NBS–LRR proteins (Century et al., 1997; Aarts et al., 1998). Two CC-type resistance genes, *RPP8* and *RPP13*, function independently of *EDS1* and *NDR1* (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). The spectra of *R* genes that require *RAR1* or *SGT1* do not coincide with those of genes that use *EDS1*/*PAD4* or *NDR1*. *R* genes from both TIR–NBS–LRR or CC–NBS–LRR classes and both the signaling pathways may require *RAR1* or *SGT1* to varying degrees. Some *R* genes require *RAR1* and *SGT1*, some require *RAR1* or *SGT1* only, and some require neither (Dodds and Schwechheimer, 2002; Muskett et al., 2002; Tornero et al., 2002b).

3.7.2 EDS1–PAD4 Proteins

Defective alleles of *EDS1* (enhanced disease susceptibility) and *PAD4* (phytoalexin deficient) were identified in mutational screens for the suppression of *RPP5*-specified resistance in *Arabidopsis* (Parker et al., 1996; Zhou et al., 1998; Falk et al., 1999; Jirage et al., 1999). *EDS1* and *PAD4* are required for TIR–NBS–LRR R proteins-mediated signal transduction and not required for CC–NBS–LRR R proteins (Glazebrook et al., 1997; Aarts et al., 1998; Falk et al., 1999; Feys et al., 2001; Dodds and Schwechheimer, 2002; Peart et al., 2002a). Silencing of *EDS1* in *Nicotiana benthamiana* compromised TMV resistance mediated by *N* gene encoding TIR–NBS–LRR protein (Liu et al., 2002a,b) and transgenic *N. benthamiana* expressing tobacco *N* gene required *EDS1* for conferring resistance against TMV (Peart et al., 2002a). The potato R protein Rx (a CC–NBS–LRR protein) conferring resistance against PVX and the tomato R protein Pto (a protein kinase) conferring resistance against *Pseudomonas syringae* pv. *tomato* did not require EDS1 for their function (Peart et al., 2002a). These results suggest that *EDS1* is not required for the function of CC–NBS–LRR and protein kinase R proteins, and specifically required by TIR–NBS–LRR proteins. However, the *Arabidopsis* gene *RPW8*, which encodes R protein containing TM and CC domains without NBS–LRR domains, is also regulated by *EDS1* (Xiao et al., 2001). EDS1 is also required for a receptor-like *R* gene *Ve*-mediated resistance against *Verticillium* wilts in tomato (Hu et al., 2005). It suggests that the receptor-like *R* gene class may also require *EDS1* (Hu et al., 2005). EDS1 is required for the function of a wide range of *R* genes including *Arabidopsis* genes *RPP2*, *RPP4*, *RPP5*, *RPP21*, *RPS4*, and *RPW8*, tobacco *R* gene *N*, flax *L6* (Howles et al., 2005), and tomato genes *Bs4*, *I*, and *Ve* (Aarts et al., 1998; Xiao et al., 2001; Hu et al., 2005). It is required for mediating resistance against a broad range of fungal, oomycete, bacterial, and viral pathogens (Hu et al., 2005).

EDS1 gene was cloned from *Arabidopsis thaliana* and it was found to encode a protein that has similarity in its amino-terminal portion to the catalytic site of eukaryotic lipases (Falk et al., 1999). EDS1 homologs have been detected in tomato (Hu et al., 2005) and tobacco (Liu et al., 2002a; Peart et al., 2002a). *EDS1* may have additional early defense role that is independent of *PAD4*. *EDS1* can homodimerize and also interact with *PAD4* (Feys et al., 2001). White rust (*Albugo candida*) resistance in *Arabidopsis* conferred by *RAC1* was completely abolished by the mutation in *eds1* but was not affected by *pad4*, suggesting that EDS1 may operate independently (Borhan et al., 2004).

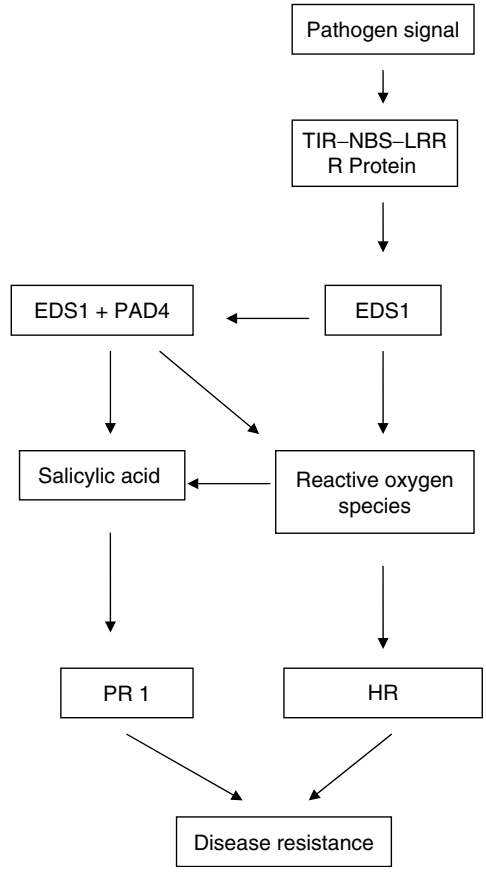

FIGURE 3.3 Function of EDS1 and PAD4 in *R* gene-mediated signaling events.

The downstream events in *EDS1*/*PAD4*-mediated signaling pathway are suggested in Figure 3.3. EDS1 may be involved in *R* gene-mediated production of reactive oxygen species and hypersensitive cell death (Levine et al., 1994, 1996). Induction of salicylic acid (SA) and expression of *PR* genes are suppressed in the *eds1-1* tomato mutant (Hu et al., 2005). Application of exogenous SA restores PR gene expression in the mutant, indicating that *EDS1* acts upstream of salicylic acid (Hu et al., 2005).

The tomato *eds1-1* mutant plants exhibited enhanced susceptibility to various pathogens, suggesting that *EDS1* may induce general resistance pathways downstream of *R* gene-mediated signaling system (Hu et al., 2005).

3.7.3 NDR1 Proteins

NDR1 is a regulatory gene detected in *Arabidopsis. NDR1* encodes a 25 kDa protein that has two putative membrane attachment domains (Century et al., 1997). *NDR1* encodes a 660 base pair open reading frame. The predicted 219-amino acid sequence suggests that NDR1 may be associated with a membrane (Century et al., 1997). Many, not all, CC–NBS–LRR *R* genes are dependent on *NDR1* (Century et al., 1995). The *Arabidopsis* genes, *RPS2*, *RPM1*, and *RPS5* are *NDR1*-dependent and operate independently of *EDS1* (Aarts et al., 1998). Absence of functional alleles of *NDR1* enhances susceptibility to various pathogens in *Arabidopsis* (Aarts et al., 1998).

3.7.4 RAR1–SGT1–HSP90 Proteins

3.7.4.1 RAR1

RAR1, a regulatory gene was first detected in barley and was found to be essential for a subset of *Mla R* gene specificities conferring resistance against specific races of *Blumeria graminis* f. sp. *hordei* (Freialdenhoven et al., 1994; Shen et al., 2003). RAR1 has been shown to be required for the function of barley powdery mildew resistance genes *Mla6*, *Mla12*, and *Mla13* (Halterman et al., 2003). Subsequently, it has been shown to be required for several *R* gene-mediated resistance responses in monocotyledonous and dicotyledonous plant species against various pathogens (Shirasu et al., 1999; Tornero et al., 2002b; Shen et al., 2003; Shirasu and Schulze-Lefert, 2003; Jarosch et al., 2005). RAR1 contributes to resistance to *Magnaporthe grisea* in barley carrying *mlo* gene (Jarosch et al., 2005). RAR1 encodes a protein with two zinc-binding domains called CHORD-I and -II (cysteine-and histidine-rich domains I and II) (Muskett et al., 2002).

Genetic requirement of RAR1 is limited to CC–NBS–LRR and TIR–NBS–LRR-type R proteins (Falk et al., 1999; Shirasu and Schulze-Lefert, 2003; Bieri et al., 2004). Dependence of these *R* genes on RAR1 may be determined by the LRR domain of the R genes. A single amino acid substitution in the LRR domain of MLA6 protein (G721D) alleviated the resistance from *RAR1* dependence (Halterman and Wise, 2004), suggesting the importance of LRR domain in R protein in its dependence on RAR1 protein to trigger resistance.

RAR1 may control steady-state levels of R proteins in plants. A reduction of NBS–LRR protein steady-state levels in nonchallenged plants appears to be a common feature of plants lacking RAR1 (Bieri et al., 2004). Transgenic barley plants expressing *Mla1* or *Mla6* were crossed with a *rar1* mutant line lacking detectable RAR1 protein. Homozygous plants expressing *Mla1* or *Mla6* genes conferring resistance to strains of *B. graminis* f. sp. *hordei* were selected and analyzed for the abundance of MLA proteins. There was a strong reduction of both MLA1 and MLA6 steady-state levels in *rar1* mutant background (Table 3.6; Bieri et al., 2004). Transgenic *Nicotiana benthamiana* lines expressing Rx gene conferring resistance to PVX were developed. Gene silencing of RAR1 by virus-induced gene silencing strongly reduced both RAR1 and Rx abundance (Table 3.6; Bieri et al., 2004). In *Arabidopsis*, the abundance of RPM1, which recognizes *Pseudomonas syringae* AvrRpm1, is also reduced in nonchallenged *rar1* mutant plants (Tornero et al., 2002b). These results suggest that RAR1 elevates R protein abundance. RAR1 may play a role to reach minimum NBS–LRR protein levels that are needed to trigger effective resistance responses (Bieri et al., 2004).

TABLE 3.6
RAR1 Elevates Rx Protein Level in *Nicotiana benthamiana* and MLA1 and MLA6 Abundance in Barley

Presence (+) or Absence (−) of RAR1 in Transgenic Plants Expressing *R* Gene	Steady-State Levels of Rx Protein in Transgenic *Nicotiana benthamiana*	Relative MLA Abundance in Transgenic Barley Plants Homozygous for the Transgene	
		Mla6	*Mla1*
+RAR1	***	**	****
−RAR1	*	*	**

Source: Adapted from Bieri, S., Mauch, S., Shen, Q.-H., Peart, J., Devoto, A., Casais, C., Ceron, F., Schulze, S., Steinbiß, H.-H., Shirasu, K., and Schulze-Lefert, P., *Plant Cell*, 16, 3480, 2004.

Note: *, **, ***, and **** indicate the increasing levels of R protein levels.

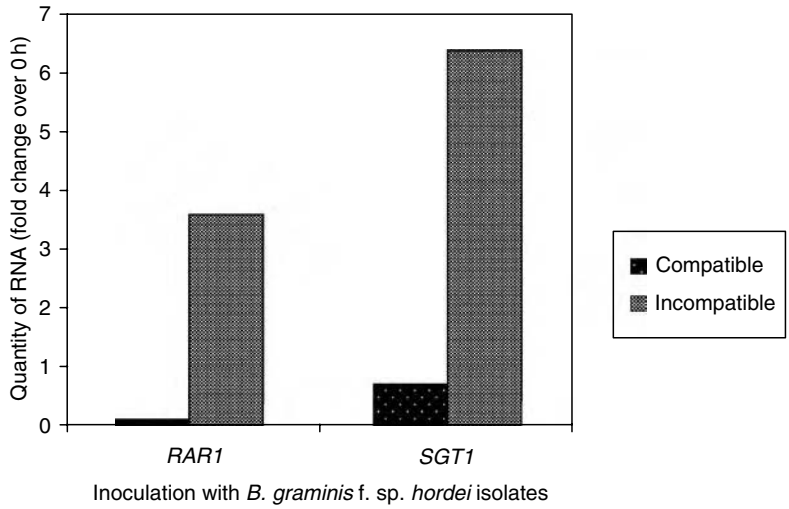

FIGURE 3.4 Transcript profiles of *RAR1* and *SGT1* in barley plants inoculated with compatible or incompatible isolates of *Blumeria graminis* f. sp. *hordei*. (Adapted from Halterman, D., Wei, F., and Wise, R.P., *Plant Physiol*., 131, 558, 2003.)

RAR1 level itself is increased during incompatible interactions to facilitate increase in *R* gene activity. The RAR1 transcript levels increased more than threefold in barley within 20 h after inoculation with the incompatible *B. graminis* f. sp. *hordei* isolate, whereas such increase was not observed in compatible interactions (Figure 3.4; Halterman et al., 2003). It suggests that increased abundance of RAR1 may increase the *R* gene activity.

3.7.4.2 SGT1

SGT1 (suppressor of G-two allele of SKP1) is another regulator protein in *R* gene-mediated signaling system. Five domains have been identified in plant SGT1 proteins and these include a tetratricopeptide repeat domain (TPR), two variable regions (VR1 and VR2), the CS motif, and SGS motif (Azevedo et al., 2002). The regulatory protein encoded by *Arabidopsis SGT1* gene, AtSGT1b, contains three tetratricopeptide repeats at the N terminus followed by a bipartite chord-containing SGT domain and an SGT-specific domain at the C-terminus (Tör et al., 2002). Resistance triggered by the barley powdery mildew resistance gene *MLA6* was significantly compromised upon transient *Sgt1* single cell silencing (Azevedo et al., 2002; Shen et al., 2003). Two highly homologous *Arabidopsis SGT1* genes, *SGT1a* and *SGT1b*, have been identified. Mutations in *SGT1b* disabled early plant defenses conferred by multiple *R* genes and loss of *SGT1b* function in resistance was not compensated for by SGT1a (Austin et al., 2002). It suggests that SGT1b is a regulatory protein taking part in *R* gene-mediated signaling system (Austin et al., 2002). A mutant in the *Arabidopsis* accession Columbia (Col-0) exhibited enhanced downy mildew susceptibility to several *Hyaloperonospora parasitica* isolates. The gene *SGT1b* complemented the mutant to wild-type resistance against all of the *Hyaloperonospora* isolates tested (Tör et al., 2002). These results suggest that SGT1 plays a key role in *R* gene-mediated disease resistance.

SGT1 plays a general role in the function of both host and nonhost resistance genes in plants (Peart et al., 2002b). It seems that SGT1 is required for resistance responses by NBS–LRR as well as by other R proteins. Transgenic *Nicotiana benthamiana* plants carrying *Rx* gene from potato encoding a CC–NBS–LRR protein that confers resistance against PVX, *N* gene from *Nicotiana glutinosa* that encodes a TIR–NBS–LRR protein conferring resistance

against TMV, and *Pto* gene from tomato which encodes a serine/threonine kinase and confers resistance against *Pseudomonas syringae* pv. *tabaci* (*avrPto*), were developed (Dangl and Jones, 2001). It was demonstrated that SGT1 was required for all the three *R* genes-mediated disease resistance in *N. benthamiana* (Peart et al., 2002b).

Arabidopsis thaliana R gene *RPW8*, which encodes a protein containing a transmembrane and CC domain and conferring resistance against powdery mildew pathogens, requires *SGT1* for its function in inducing resistance (Peart et al., 2002b). Requirement of *SGT1* for the function of tomato *R* genes *Cf4* and *Cf9* (encoding membrane-localized proteins with LRRs) conferring resistance against *Cladosporium fulvum* has also been reported (Peart et al., 2002b). Silencing of *SGT1* gene in *N. benthamiana* resulted in loss of nonhost resistance to the pepper pathogen *Xanthomonas campestris* pv. *vesicatoria* and the Brassicaceae pathogen *Pseudomonas syringae* pv. *maculicola* (Peart et al., 2002b). These results suggest the importance of SGT1 in a broad range of *R* gene signaling.

SGT1 is known to contribute to cyclic AMP pathway activity in yeast (Dubacq et al., 2002). It may exert cochaperone-like activity and may be involved in folding of R proteins into a form competent for effector recognition and activation of the resistance response (Bieri et al., 2004). It encodes an essential component of the yeast kinetochore assembly pathway where it interacts with SKP1. The SKP1 protein is a conserved subunit of Skp1-Cullin-F-box protein (SCF)-type E3 ubiquitin ligases that mediate the ubiquitylation of proteins that are targeted for degradation by the 26S proteasome (Kitagawa et al., 1999; Bachmair et al., 2001). SCF E3 ubiquitin ligases can modify protein activity (Azevedo et al., 2002). It is known that monoubiquitination regulates transcription and protein kinase activity (Pickart, 2001). SGT1 is associated with SCF-type E3 ubiquitin–ligase complexes and the 26S proteasome, which is indicative of a link to ubiquitination system-dependent processes (Azevedo et al., 2002; Liu et al., 2002a,b). Barley and *N. benthamiana* SGT1 associate physically with one or several SCF ubiquitin E3 ligase complexes and the COP9 signalosome (Liu et al., 2002b).

The COP9 signalosome is a multiprotein complex that has homology with the "lid" subcomplex of the 26S proteosome that was shown to interact with SCF-type ubiquitin ligases in plants (Schwechheimer and Deng, 2001; Schwechheimer et al., 2001). The COP9 signalosome may be a central component of multiple processes that are mediated by E3 ubiquitin ligases belonging to different E3 families. It has been suggested that SGT1 may participate in a novel E3 complex that is also regulated by the COP9 signalosome (Dodds and Schwechheimer, 2002).

Gene silencing of the core SCF component, SKP1, or the COP9 signalosome compromised *R* gene-triggered resistance in *N. benthamiana*. It suggests that ubiquitin–protein conjugation pathways play an important role in plant disease resistance responses (Liu et al., 2002b). The SGT1–SKP1 interaction is conserved in planta, suggesting that ubiquitylation may be involved in regulation of disease resistance responses (Azevedo et al., 2002). SGT1 may participate in modification of protein activity or may have a dual role for activation and degradation of the target via ubiquitination (Azevedo et al., 2002). Such a dual role for activation and degradation has been reported in the regulation of transcriptional activation (Salghetti et al., 2001). The *R* gene product itself could be target of SGT1 for activation and degradation. The R protein RPM1 rapidly degrades during resistance response in *Arabidopsis* (Boyes et al., 1998) and this may lead to HR development by restricting cell death to a small number of host cells at sites of attempted pathogen invasion (Azevedo et al., 2002).

There may be some specificity in the action of SGT1. Barley or *Arabidopsis* SGT1 interacts with the LRR-CT of the barley R protein MLA1 but not with MLA6, indicating MLA isoform-dependent associations (Bieri et al., 2004). It has been suggested that subtle sequence-specific differences in the LRR are critical for associations with SGT1 (Bieri et al., 2004). This suggestion is based on a finding that a single amino acid substitution

in the LRR of yeast adenylate cyclase, Cyr1p, abolished *in vivo* interactions with yeast SGT1 (Dubacq et al., 2002). Activation of cell death in *N. benthamiana* by the interaction between tomato leaf mold resistance genes-encoded membrane-resident Cf4 or Cf9 proteins (containing extracellular LRRs) and *C. fulvum* avirulence peptides requires intracellular SGT1 (Peart et al., 2002b). It suggests that SGT1 may have a function in disease resistance, which may occur independently from a direct interaction with LRRs of the R protein.

3.7.4.3 RAR1/SGT1 Complex

In most cases, SGT1 may act in conjunction with RAR1 in the *R* gene-mediated signal transduction. In *Arabidopsis*, SGT1b and RAR1 contribute additively to RPP5-mediated pathogen recognition (Austin et al., 2002). In transiently transformed barley cells, double-stranded RNA inhibition of RAR1 or SGT1 resulted in the loss of Mla6-mediated powdery mildew disease resistance (Azevedo et al., 2002). It suggests that both RAR1 and SGT1 may act together in mediating the Mla6-induced defense response. In transiently transformed barley cells, RAR1 proteins interact physically with SGT1 through the C-terminal CHORD-II of RAR1 and the central domain of SGT1 (Kitagawa et al., 1999; Shirasu et al., 1999; Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002b). RAR1 and SGT1 form a complex *in vivo* (Azevedo et al., 2002). RAR1 could connect, via SGT1, the COP9 signalosome to SCF E3 ubiquitin ligases to promote ubiquitination of unknown targets in the *R* gene-mediated signaling system (Azevedo et al., 2002).

RAR1/SGT1 may have a function in protein degradation (Dodds and Schwechheimer, 2002). The RAR1/SGT1-mediated protein degradation may modulate the abundance of positive or negative regulators of pathogen-induced gene expression (Dodds and Schwechheimer, 2002). Expression of the *PR1* gene is constitutive in COP9 signalosome loss-of-function mutants and this can be due to an inability to degrade a transcriptional activator that is degraded normally in the absence of a stimulus (Mayer et al., 1996). Since RAR1/SGT1 complex acts along with the COP9 signalosome, RAR1/SGT1 may also have a function in protein degradation. Several studies have indicated that SGT1/RAR1 acts downstream of or coincident with the action of resistance protein-containing recognition complexes (Shen et al., 2003). It has also been reported that transcription of both *RAR1* and *SGT1* genes is enhanced in barley within 20 h after inoculation with incompatible isolates of *Blumeria graminis* f. sp. *hordei* (Figure 3.4; Halterman et al., 2003). This increase coincided with increased activities of the resistance genes *Mla6* and *Mla13* in barley (Figure 3.1). Similar increases were not observed in compatible interactions (Figure 3.1 and Figure 3.4). It suggests that both RAR1 and SGT1 may take part in the *R* gene-mediated signaling systems.

3.7.4.4 Interaction of RAR1/SGT1 with HSP90

Cytosolic HSP90 is another protein, which interacts with R proteins in the *R* gene-mediated signaling pathway. A central region of human SGT1, called the CS domain, is related to the cochaperone p23 and binds to HSP90 (Lee et al., 2004). Similar binding between SGT1 and cytosolic HSP90 has been found in plants (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004). Plant cytosolic HSP90 also interacts with RAR1 and plays an important role in the function of various NBS–LRR R proteins such as *N* gene of tobacco (Liu et al., 2004), *Rx* gene of potato (Lu et al., 2003), and *RPM1* and *RPS2* of *Arabidopsis* (Hubert et al., 2003; Takahashi et al., 2003). Cytosolic HSP90 interacts with the LRR-C-terminal (CT) part of all tested barley R proteins, MLA variants (Bieri et al., 2004). HSP90 proteins bind their target proteins in nearly mature conformations, retaining and releasing them in an activity cycle driven by ATP hydrolysis and regulated by binding of cochaperones (Shirasu et al., 1999).

Specific mutations in the ATP-binding domain of a single *Arabidopsis* cytosolic HSP90 isoform compromised the R protein RPM1 function (Hubert et al., 2003). It suggests that HSP90 is involved in the R protein-mediated disease resistance.

3.7.5 NPR1

NPR1 (nonexpressor of *PR* [pathogenesis-related] genes) gene also known as *NIM1* (non-immunity) and *SAI1* (salicylic acid insensitivity) is a key regulator of salicylic acid-signaling in *R* gene-mediated defense pathway in *Arabidopsis* (Zhang et al., 1999, 2003; McDowell et al., 2000; Liu et al., 2002a). The *NPR1*-mediated signal transduction has also been observed in rice (Chern et al., 2001). The rice NPR1 homolog (NH1) has been isolated and characterized (Chern et al., 2005). The *NPR1* gene encodes a novel protein with ankyrin repeats (Cao et al., 1997). Nuclear localization of NPR1 has been shown to be essential for its function in inducing PR gene expression (Kinkema et al., 2000).

NPR1 may function downstream of SA (Figure 3.5). NPR1 is stimulated by SA to translocate to the nucleus where it interacts with TGA transcription factors (Kinkema et al., 2000; Fan and Dong, 2002; Mou et al., 2003). NPR1 interacts with the *Arabidopsis* and rice TGA family members of basic-region leucine zipper (bZIP) transcription factors (Zhang et al.,

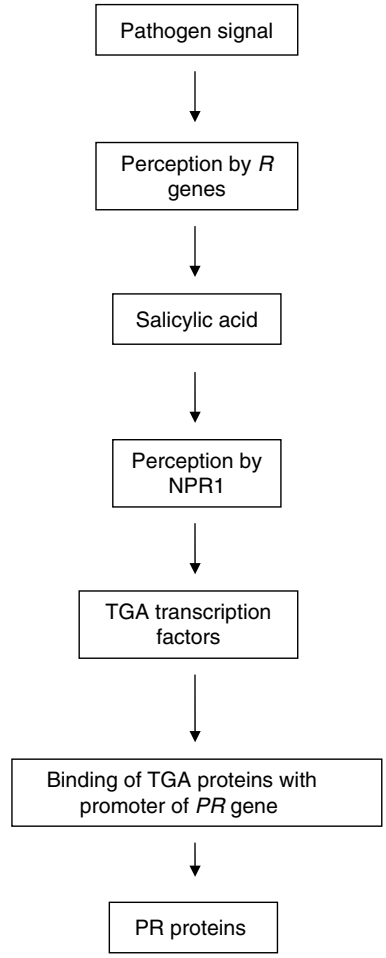

FIGURE 3.5 Function of NPR1 at downstream of salicylic acid signaling system.

1999; Després et al., 2000, 2003; Zhou et al., 2000; Fan and Dong, 2002; Chern et al., 2005). TGA2 has been demonstrated to be a salicylic acid-responsive and NPR1-dependent transcription activator (Fan and Dong, 2002). The TGA proteins may directly connect NPR1 with *PR* gene induction in the salicylic acid signal transduction pathway (Zhang et al., 1999). *Arabidopsis npr1* mutant leaves were susceptible to *Botrytis cinerea* infection and exogenous application of SA induced resistance in the *npr1* plants. It suggests that *NPR1* acts downstream of salicylic acid (Ferrari et al., 2003).

3.7.6 *Prf–Pto–Pti* Signaling System

Downstream of *R* gene signaling, several regulatory genes interact with the *R* gene and their interaction is necessary in downstream induction of defense genes. These regulatory genes include even another *R* gene. *Pto*, the *R* gene in tomato, confers resistance against *Pseudomonas syringae* pv. *tomato* strains carrying the *avr* gene *avrPto* (Martin et al., 1994). Another gene *Prf*, an LZ–NBS–LRR *R* gene, has been shown to be required, along with Pto kinase, for resistance to *P. syringae* expressing *avrPto* (Salmeron et al., 1994). The LRR protein Prf1 may act as receptor of the avr protein signal and the protein kinase R protein Pto may act in the signal transduction system. The LRR protein Prf may act upstream of the protein kinase R protein Pto (Bent, 1996).

Pti1 is the second serine/threonine protein kinase that participates in the Prf-Pto defense signaling system (Zhou et al., 1995). Pti1 was found to be a substrate for autophosphorylation and for phosphorylation by Pto. Pto was not a substrate for phosphorylation by Pti1. These observations suggest that Pti1 acts downstream of Pto in a protein kinase cascade (Bent, 1996; Bogdanove, 2002b; Ekengren et al., 2003). Pti4, Pti5, and Pti6 are the other Pto-interacting proteins and they have similarity to transcription factors (Zhou et al., 1997). They resemble ethylene-responsive element-binding proteins (EREBP) of tobacco. They specifically recognize and bind to a PR-box DNA sequence that is conserved within the promoters of the genes for many PR proteins (Zhou et al., 1997). This recognition process led to the expression of EREBP1 and PR proteins encoded by *PRP1* and *PR5* genes. The induction of *EREBP1* preceded that of PR protein genes (Zhou et al., 1997). These results suggest the probable sequence in the signaling system involved in the *R* gene-mediated disease resistance gene in tomato (Figure 3.6).

Overexpression of *Prf* gene induced accumulation of salicylic acid in transgenic tomato plants and conferred systemic resistance (SAR)-type of nonspecific resistance against several bacterial (*Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*, and *Ralstonia solanacearum*) and viral (TMV) pathogens (Oldroyd and Staskawicz, 1998). Similarly, overexpression of *Pto* gene in transgenic tomato lines resulted in expression of disease resistance not only against *P. syringae* pv. *tomato*, but also against *X. campestris* pv. *vesicatoria* and a fungal pathogen *Cladosporium fulvum* (Tang et al., 1999). These results suggest that overexpression of the cloned resistance genes *Pto* and *Prf* would have activated general defense mechanisms of the host. In transgenic tomato plants overexpressing the *Pto* gene, 223 genes were found to be induced (Mysore et al., 2003). In *Pto*-overexpressing plants, 40% of the genes induced have been shown to be differentially expressed during the human immune responses (Mysore et al., 2003). It suggests that the *R* gene would have activated general immune system of plants. Xiao et al. (2003) have suggested that Pti (Pto-interacting proteins) would have been involved in the general resistance induced by the *Pto* transgene.

3.7.7 Other Regulatory Genes

Crg is the regulatory (complementary) gene and it is required for the *R* gene *UR-3*-mediated rust (*Uromyces appendiculatus*) resistance in common bean (*Phaseolus vulgaris*) (Kalavacharla et al., 2000). Another regulatory protein, RIN4, modulates RPS2- (Mackey et al., 2003) and

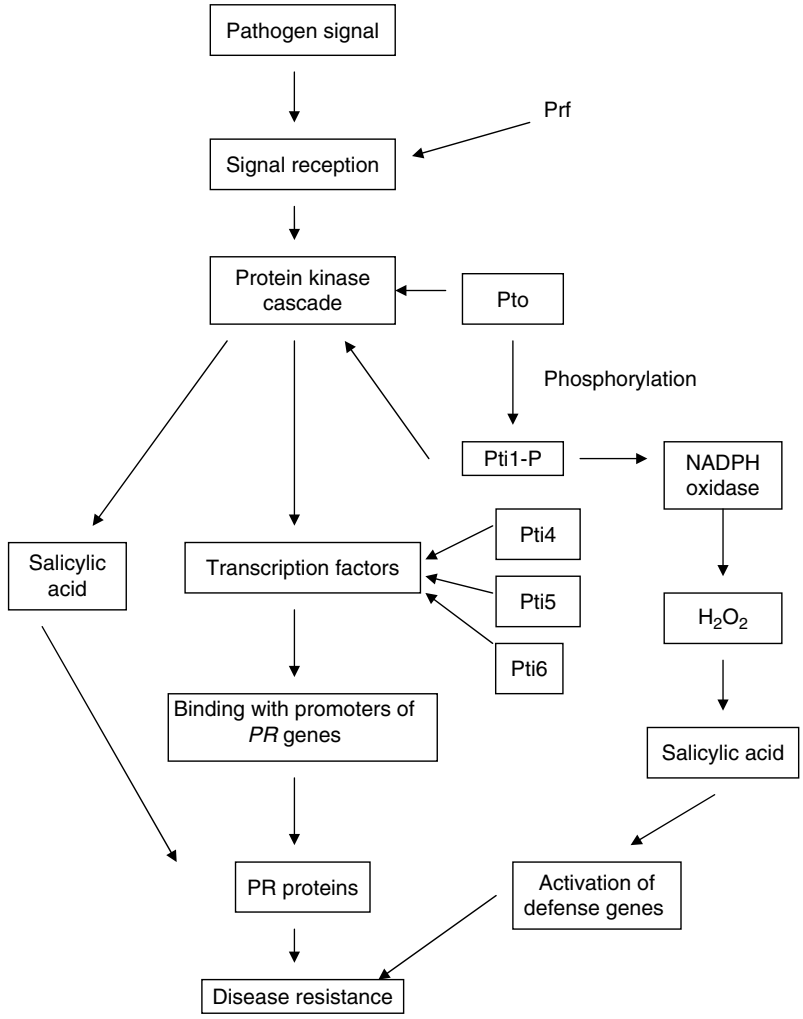

FIGURE 3.6 *R* gene-mediated signaling pathway in tomato.

RPM1-mediated resistance in *Arabidopsis* (Mackey et al., 2002). In tomato, *Rcr-1* and *Rcr-2* were found to be required for the function of *Cf9* (Hammond-Kosack et al., 1994). *Rcr3*, which encodes a secreted cystein protease, has been cloned and has been shown to be a positive regulator of *Cf*2-dependent resistance in tomato (Krüger et al., 2002). *CITRX*, which encodes a thioredoxin, interacts with the C-terminal cytoplasmic domain of Cf9 and increases the function of Cf9 in triggering defense responses in tomato (Rivas et al., 2004). Many *Avr9*/*Cf9* rapidly elicited (*ACRE*) genes are predicted to encode regulatory proteins, including protein kinases and transcription factors (Durrant et al., 2000). Three ACRE genes were found to be important for generating HR elicited by Cf9/Avr9 and Cf4/Avr4 in *Nicotiana benthamiana* (Rowland et al., 2005). These genes, when silenced compromised the Cf-mediated HR. One of these genes encodes a serine/threonine protein kinase called Avr9/Cf9 induced kinase1 (ACIK1). *ACIK1* mRNA was rapidly upregulated in tobacco and tomato upon elicitation by Avr9. Silencing of *ACIK1* in tomato decreased Cf9-mediated resistance to *Cladosporium fulvum*. It shows that ACIKI protein kinase is essential for full expression of Cf9-mediated disease resistance in tomato (Rowland et al., 2005).

3.8 DOWNSTREAM SIGNALING EVENTS IN *R* GENE-MEDIATED RESISTANCE

Both race-specific (R type) and general (basal) elicitor-mediated defense responses are basically similar in the downstream signal events (Dangl and Jones, 2001). The common induced events include ion influx, alkalinization of extracellular spaces, accumulation of reactive oxygen intermediates and reactive nitrogen intermediates, and transcriptional reprogramming. These responses lead to increased production of many antimicrobial substances (Veronese et al., 2003).

The downstream events in the tomato disease resistance *Cf* genes include production of active oxygen species (Lu and Higgins, 1998; Piedras et al., 1998), activation of calcium-dependent protein kinases (Romeis et al., 2000), and mitogen-activated protein kinases (MAPKs) (Romeis et al., 1999). The early responses in Cf9-transgenic tobacco plants to the AVR9 elicitor include changes in ion fluxes (Piedras et al., 1998; Blatt et al., 1999), production of reactive oxygen species (Piedras et al., 1998), and the activation of a calcium-dependent protein kinase and MAPKs (Romeis et al., 1999, 2000, 2001; Rivas and Thomas, 2002). An MPK6 was shown to be involved in *RPP4*-mediated resistance to *Hyaloperonospora parasitica* in *Arabidopsis* (Menke et al., 2004).

One important downstream component of *R* gene-mediated defense signal transduction is salicylic acid (SA) (Cao et al., 1994; Delaney et al., 1994; 1995; Ryals et al., 1996). Most *R* genes characterized so far require SA in induction of defense genes (Glazebrook, 1999). Salicylic acid appears to be a downstream signaling pathway in *RRS1-R* mediated disease resistance response in *Arabidopsis* (Deslandes et al., 2002). The bacterial wilt-resistant *Arabidopsis* plants expressing the bacterial salicylate hydroxylase gene, which converts salicylic acid into inactive catechol, became susceptible to the bacterial wilt disease, suggesting that salicylic acid is necessary in inducing defense response by *RRS1-R* gene in *Arabidopsis* (Deslandes et al., 2002). A recessive gene in *Arabidopsis*, *ssi4*, induces salicylic acid accumulation, which in turn induces expression of *PR* (pathogenesis-related) genes (Shirano et al., 2002).

In SA signaling, genes such as *EDS1* and *PAD4* have relatively early signaling functions downstream of *R* genes but upstream of SA (Zhou et al., 1998; Feys et al., 2001). *EDS5* and *SID2* may be involved in SA biosynthesis either directly or indirectly (Nawrath and Metraux, 1999; Nawrath et al., 2002). NPR1/NIM1 functions downstream of SA. NPR1 is stimulated by SA to translocate to the nucleus where it interacts with TGA transcription factors that bind the TGACG motif and that lead to expression of *PR* and other defense genes (Kinkema et al., 2000; Fan and Dong, 2002; Mou et al., 2003).

The downstream events in *R* gene-mediated signaling processes included activation of various defense genes (Durrant et al., 2000; Kruger et al., 2003). The downstream events in the barley–*Puccinia graminis* f. sp. *tritici* incompatible interactions included induction of the PR gene *HvPR-1a* and β-1,3-glucanase gene (Rostoks et al., 2004). The Avr9 peptide elicitor from *Cladosporium fulvum* and *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene *avrPto* induced resistance responses in tomato lines carrying the *Cf9* and *Pto* genes, respectively. The downstream responses in these interactions included activation of phenylpropanoid pathway and accumulation of soluble phenolics (von Roepenack-Lahaye et al., 2003). There was preferential induction of synthesis of two phenolic compounds such as *p*-coumaroyloctopamine (*p*-CO) and *p*-coumaroylnoradrenaline (*p*-CN) in these two *R* gene-mediated interactions in tomato. The elevated levels of these phenolics were accompanied by elevated mRNA levels of genes encoding phenylalanine ammonia lyase (PAL), *p*-coumarate:CoA ligase (4CL), and hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT) (von Roepenack-Lahaye et al., 2003).

3.9 SUSCEPTIBILITY GENES IN SIGNAL TRANSDUCTION

3.9.1 Susceptibility Alleles of Resistance Genes

Resistance and susceptibility may be opposite sides of the same coin (Bryan et al., 2000; Eckardt, 2002; Vogel et al., 2002; Christensen et al., 2004). Different forms of the same genes are called alleles (Vidhyasekaran, 2004) and resistance genes may have susceptibility (S) alleles (Bryan et al., 2000; Jia et al., 2000). Although the resistant varieties have resistance (R) allele, the susceptible varieties may have susceptibility (S) allele. The barley stem rust resistance gene *Rpg1* was detected in all resistant lines, whereas its susceptible allele *rpg1* could be detected in most of the susceptible lines (Brueggemann et al., 2002). *RAC1* is a dominant TIR–NBS–LRR resistance gene in *Arabidopsis* conferring resistance to the white rust pathogen *Albugo candida*. Strong identity of the TIR and NBS domains was observed between the predicted proteins encoded by the allele found in the resistant accession and the allele from a susceptible accession (99% and 98%, respectively). Major differences between the two predicted proteins occur within the LRR domain and mainly are confined to the β-strand/β-turn structure of the LRR (Borhan et al., 2004).

RPW8 confers resistance to powdery mildew pathogens in *Arabidopsis* and the gene was detected in the resistant accessions (Eckardt, 2002). In three moderately susceptible accessions, *RPW8* alleles were detected and these alleles encoded proteins with 90% to 95% similarity to the protein encoded by the resistance gene (Eckardt, 2002). Susceptibility alleles of several *R* genes, which encode proteins that differ from resistance (R) alleles by a variety of single amino acids, have been reported (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Salmeron et al., 1996; Scofield et al., 1996). Both the R and S alleles may participate in signal perception and emission, although the S allele may have a weak function in the process. Although resistance alleles perceive pathogen signals and trigger defense responses, susceptibility alleles either may not be able to perceive the pathogen signals or receive the signal only weakly, and hence unable to activate the defense genes, resulting in susceptibility. A single amino acid difference in the LRR domain distinguished susceptibility and resistance alleles of the rice blast resistance gene *Pi-ta* (Bryan et al., 2000). The LRR domain of the resistance allele directly interacted with the avirulence gene product (Avr-Pita), whereas the susceptibility allele displayed a much weaker interaction (Jia et al., 2000).

3.9.2 Susceptibility Genes

In some cases, susceptibility genes may be the dominant ones, and their recessive alleles may act as resistance genes. Resistance loci containing *R* gene clusters have been identified in many plants against various pathogens (Park et al., 2005; Smith and Hulbert, 2005). Similarly, susceptibility loci have been recently identified. Van Damme et al. (2005) have identified loci required for susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica* in *Arabidopsis*. They generated mutants in a highly susceptible line, which showed resistance to the pathogen. Six different susceptible gene loci have been identified in the susceptible line and mutations in these regions converted the susceptible line to resistant one.

Specific susceptibility genes have also been isolated. *RRS1-S* is the susceptibility gene isolated from *Arabidopsis*. RRS1-S contains TIR–NBS–LRR domains and a WRKY domain. Its allele *RRS1-R* is the recessive gene conferring resistance to *Ralstonia solanacearum* (Deslandes et al., 2002). Structurally, both the alleles are highly identical and a 98% identity between the nucleotide sequences of the two alleles has been reported. However, the two genes differ in the position of a stop codon that leads in *RRS1-S* to the formation of a protein truncated by 90 aa (Deslandes et al., 2002). It is suggested that the truncated RRS1-S protein may be a dominant negative regulator of the function of the full-length RRS1-R protein.

The RRS1-S and RRS1-R proteins may compete for some essential components involved in the pathogen perception or in signal transduction pathways (Deslandes et al., 2002).

Mlo is a susceptibility gene in barley. It was induced in response to the pathogen *Blumeria graminis* f. sp. *hordei*, as well as to the rice blast pathogen *Magnaporthe grisea* (Piffanelli et al., 2002). Induction of the wild-type allele (susceptible to powdery mildew) occurred 6 h after infection. At the same time, the amount of *Mlo* transcript also increased in isolated epidermis that was in direct contact with the pathogen (Piffanelli et al., 2002). The *Mlo* dampens the cell wall–restricted hydrogen peroxide burst at points of attempted fungal penetration of the epidermal cell wall, and in subtending mesophyll cells, it suppresses a second oxidative burst and cell death (Piffanelli et al., 2002). *MLO* gene induces susceptibility, probably by suppressing the defense signal transduction and thereby suppressing the induction of defense genes.

An *Arabidopsis* gene *PMR6* was cloned and found to encode a pectate lyase-like protein. It has been suggested that *PMR6* may be a susceptibility gene and the plants containing *PMR6* gene were susceptible to *Erysiphe cichoracearum* and *Golovinomyces orontii* (= *Erysiphe orontii*) (Eckardt, 2002). The allele of the *PMR6* gene, *pmr6*, was found to confer resistance against the powdery mildew fungi. *PMR6* may be a pectin-degrading enzyme (Eckardt, 2002) and pectin degradation may facilitate fungal penetration into host cells (Vidhyasekaran, 1997, 2002, 2004). Cell walls of the resistant *pmr6* mutant plants were enriched in pectin, suggesting that the susceptibility in *PMR6* plants may be due to less amount of pectin (Eckardt, 2002). However, Vogel et al. (2002) did not observe differences in fungal penetration efficiency on wild-type *PMR6* (susceptible plants) versus *pmr6* mutant (resistant) plants. It is known that pectic enzymes (Boudart et al., 2003) and pectic fragments (Movahedi and Heale, 1990) are involved in signaling defense genes and it is still to be proved experimentally the role of pectin metabolism in conferring susceptibility to powdery mildew pathogens in *Arabidopsis* plants containing the susceptibility gene *PMR6*.

Another *A. thaliana* susceptibility gene is *EDR1*. The *Arabidopsis EDR1* gene was isolated by positional cloning and was found to encode a Raf-like MAPKKK that may function in conferring susceptibility to powdery mildews (Frye et al., 2001). The *EDR1* gene may reduce salicylic acid production and also may block salicylic acid perception (Zhou et al., 1998; Falk et al., 1999) and by interfering with SA signaling system the *EDR1* would have contributed for susceptibility in plants (Zhou et al., 1998; Alonso et al., 1999; Falk et al., 1999). Orthologs of *EDR1* have been detected in rice, barley, and tomato (Frye et al., 2001) and also in wheat, sorghum, soybean, and potato (Tang and Innes, 2002). The mutant allele of the susceptible gene, *edr1*, confers high level of resistance against different bacterial and fungal pathogens such as *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *maculicola*, *Erysiphe cichoracearum*, and *E. cruciferarum* in *Arabidopsis* (Frye and Innes, 1998).

In Victoria blight of oats, susceptibility to the disease caused by *Cochliobolus victoriae*, is conferred by a single dominant susceptibility gene at the *Vb* locus. The pathogen produces a cyclized protein, victorin. A direct or indirect interaction with the product of the *Vb* gene appears to signal host disease susceptibility. The recessive alleles of the susceptibility gene, *vb*, conferred resistance to the pathogen (Navarre and Wolpert, 1999).

3.9.3 Resistance Gene May Act as Susceptibility Gene against Some Pathogens

A gene, which acts as a resistance gene against a pathogen, may act as a susceptibility gene against another pathogen. The barley recessive gene *mlo* confers resistance against the biotrophic pathogen *Blumeria graminis* f. sp. *hordei*, whereas the same *mlo* gene enhances susceptibility to the necrotrophic pathogen *Bipolaris sorokiniana* (Király et al., 2002). The gene activates reactive oxygen species-mediated signal transduction pathway resulting in necrotic cell death (Piffanelli et al., 2002). The necrotic cell death would favor development

of the necrotrophic pathogen *B. sorokiniana* (Király et al., 2002). The hypersensitive response has been shown to facilitate plant infection by the necrotrophic pathogen *Botrytis cinerea* (Govrin and Levine, 2000). Cell death may induce resistance against the biotrophic pathogen, since the biotrophic pathogens require living cells for their nutrition. By contrast, cell death may induce susceptibility to necrotrophic pathogen, since the necrotrophic pathogen can survive well on necrotic cells.

Arabidopsis ssi2 mutant plants showed susceptibility to *Botrytis cinerea*. In contrast, the ssi2 plants exhibited enhanced resistance to *Hyaloperonospora parasitica*, *Pseudomonas syringae*, and cucumber mosaic virus. The induction of specific signaling systems may determine susceptibility or resistance. Several *R* gene-signaling component genes such as *NDR1*, *EDS1*, and *PAD4* may function in both compatible and incompatible interactions. Increased transcript levels of *NDR1*, *EDS1*, and *PAD4* have been reported during both resistant and susceptible interactions (Century et al., 1997; Feys et al., 2001; Peart et al., 2002a). The *Arabidopsis* susceptibility gene *ssi2*-conferred susceptibility to *Botrytis cinerea* was dependent on *EDS5* and *PAD4* (Nandi et al., 2005). Mutations resulted in loss of function of the regulatory genes in *Arabidopsis* and the mutant plants showed enhanced susceptibility to bacterial and fungal pathogens (Nawrath and Metraux, 1999; Dewdney et al., 2000; Ton et al., 2002). It suggests that similar defense signaling pathways may exist in both *R* genes and susceptibility genes-mediated disease resistance or susceptibility. The expression of many defense-related genes has been reported in susceptible interactions (Huang et al., 2005). Both susceptible and resistant interactions may share a common signaling pathway. Different signaling pathways mediated by salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) operate in plants and depending on the type of expressed pathway, resistance or susceptibility may result against a pathogen. For example, salicylic acid-dependent pathway confers resistance to *Hyaloperonospora parasitica*, but susceptibility to *Alternaria brassicicola* in *Arabidopsis* (Delaney et al., 1994; Thomma et al., 1998).

3.9.4 Low Expression of Resistance Genes May Lead to Susceptibility

Some of the resistance genes may be expressed in both resistant and susceptible plants. However, the genes may be expressed at low levels in the susceptible plants. *eR* genes *At1* and *At2* were constitutively expressed in both downy mildew-resistant and -susceptible melon plants (Taler et al., 2004). In the susceptible plants, the genes were expressed at low levels and the low expression was regulated at the transcriptional level. Overexpression of these genes displayed enhanced activity of *eR* genes-encoded glyoxylate aminotransferases and conferred high resistance against the pathogen *Pseudoperonospora cubensis* (Taler et al., 2004).

3.9.5 Susceptibility Alleles of Resistance Genes May Negate the Function of Resistance Genes

The susceptibility allele may suppress the function of *R* genes. The *RPS5* gene confers resistance against *Pseudomonas syringae* strains in *Arabidopsis*. A mutant, which expressed the *rps5-1*, was developed. The *rps5-1* mutant showed susceptibility not only to the *P. syringae*, but also negatived the resistance conferred by another *R* gene, *RPP4*, against the downy mildew pathogen *Hyaloperonospora parasitica* in *Arabidopsis*. Sporulation of *P. parasitica* was enhanced in *RPP4-rps5-1* plants when compared with wild-type plants expressing both *RPP4* and *RPS5* (Warren et al., 1998). The mutant *rps5-1* also reduced the efficiency of other downy mildew resistance genes such as *RPP9*, *RPP2*, and *RPP6* (Warren et al., 1998). These results suggest that some susceptibility gene may interfere with the *R* gene-mediated signaling pathway and suppress the action of *R* genes.

3.9.6 Suppressor Genes

Specific genes, which suppress the action of resistance genes, have been detected in some plants. The resistance genes may be clustered with suppressor genes. In a cluster, both the resistance genes and suppressor genes may be present. A single dominant suppressor gene in maize called *Sht1* suppresses the expression of the *Ht* genes conferring resistance against the maize northern leaf blight caused by *Setosphaeria turcica* (Welz and Geiger, 2000). Eight dominant inhibitor genes that counteract the effect of certain oat crown rust (*Puccinia coronata*) resistance *Pc* genes in oat have been identified (Wise et al., 1996; Wilson and McMullen, 1997).

Various studies have indicated that resistance genes may be involved in signal perception and transduction triggering defense responses. The susceptible genes may suppress the action of the resistance genes and slow down or suppress the induction of defense genes, resulting in disease development.

3.10 CONCLUSION

Plants are endowed with various race-specific and nonspecific resistance genes conferring resistance to various types of plant pathogens. These resistance genes have been shown to be involved in pathogen signal perception and emission. Most of the major dominant genes show race-specific resistance following gene-for-gene hypothesis. According to this hypothesis, an *Avr* gene product of the pathogen interacts with an *R* gene product of the host plant and this type of protein–protein interaction results in disease resistance. In other words, specific signals from specific pathogens are recognized by different domains of R proteins and the perceived signals are transduced in a specific signal transduction pathway triggering plant defense responses.

Most of the disease resistance genes encode intracellular proteins with LRR and NBS domains. LRR domains of the R proteins may be involved in recognition of pathogen-derived ligands. LRR domain is an important determinant of pathogen specificity of the *R* gene. NBS domain may be important in inducing the activity of *R* gene. A change in structure of NBS domain converts race specificity of some *R* genes into non-race specificity, suggesting that NBS domain may also be important in the race specificity function of some *R* genes. NBS of R proteins forms a functional nucleotide-binding pocket and they are capable of binding ATP and/or act as ATPases. Hence, the NBS may be involved in signal transduction cascades through phosphorylation/dephosphorylation events.

N-terminus of NBS–LRR proteins is either a Toll and mammalian interleukin-1 (TIR) domain or a loosely predicted coiled-coil (CC) domain. TIR may be involved in conferring specificity to some *R* genes. CC domain may be involved in recognition of Avr gene product. C-terminal non-LRR region (CT) also may be involved in race specificity determination.

Some R proteins contain transmembrane (TM) proteins, besides LRR domains. Although the LRR domain may participate in recognition of pathogen signals, TM may pass down this signal to induce plant defense responses. An R protein contains a putative C-terminus WRKY transcriptional factor domain, besides TIR–NBS–LRR domains. WRKY factors play an important role in signal transduction systems. Another class of *R* genes encodes proteins with protein kinase domain. The protein kinase domain may play a role in protein phosphorylation, a key component in pathogen recognition. They may act as receptor sites for the pathogen signals.

Some R proteins are calmodulin-binding proteins, which are involved in Ca^{2+}-signaling cascades that trigger plant defense responses. An R protein is a lectin-type protein and lectins act as receptors for pathogen signals. Another R protein is an HSP-like protein, which may

play a regulatory role in *R* gene-mediated signal pathways. Some R proteins may act by suppressing the susceptibility inducing factors, such as toxins and pectic enzymes. For example, AAL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* inhibits the enzyme sphinganine *N*-acyltransferase involved in sphingolipid metabolism leading to programmed cell death, which is a key component in host defense responses. The R protein confers insensitivity to the toxin and allows the development of programmed cell death.

R proteins may directly bind with pathogen signal molecules or they may function indirectly in the recognition process, which involves the surveillance of a host protein or a complex that is targeted by the pathogen AVR products. A threshold for R protein-derived signal might exist to trigger effective resistance. *R* gene transcription may increase on perception of pathogen signals, resulting in abundance of R proteins. Activation of the *R* gene by the signals may facilitate to reach the active threshold level of R proteins necessary to confer disease resistance.

Several regulatory genes appear to act downstream of *R* gene signaling. The regulatory genes *EDS1* and *PAD4* are involved in *R* gene-mediated production of reactive oxygen species, accumulation of salicylic acid, and induction of hypersensitive cell death. The regulatory gene *RAR1* controls steady-state levels of R proteins in plants and aids in increasing the R protein levels that are needed to trigger effective resistance responses. Another regulatory gene *SGT1* may be involved in folding of R proteins into a form competent for effector recognition and activation of the resistance response. SGT1 may act in conjunction with RAR1 in the *R* gene-mediated signal transduction. The RAR1/SGT1 may induce protein degradation and may modulate the abundance of positive or negative regulators of pathogen-induced gene expression. Cytosolic HSP90 is another protein, which interacts with R proteins in the *R* gene-mediated signaling pathway. It binds with SGT1 and ATP-binding domain of the HSP90 activates function of R proteins.

The regulatory protein *NPR1* may function downstream of salicylic acid in *R* gene-signaling system. NPR1 is stimulated by salicylic acid to translocate to the nucleus where it interacts with TGA transcription factors. Several other regulatory genes acting downstream of *R* gene-mediated signaling in various plants have been identified.

Downstream events in both race-specific *R* gene-mediated and general disease resistance responses are basically similar. By modifying certain domains in race-specific proteins, the race-specific *R* genes can be converted into nonspecific genes. A change in structure of NBS domain of the flax *L6* gene-encoded R protein conferring resistance to specific races of *Melampsora lini* converted race specificity of *L6* gene into nonspecificity (Howles et al., 2005). Tomato *Pto* gene is a race-specific resistance gene conferring resistance to *Pseudomonas syringae* pv. *tomato* races carrying the avirulence gene *avrPto. Pto* gene was constructed by using cauliflower mosaic virus 35S promoter instead of native promoter. Transgenic tomato lines containing the 35S::*Pto* transgene showed resistance not only against *P. syringae* pv. *tomato*, but also against other bacterial and fungal pathogens of tomato, such as *Xanthomonas campestris* pv. *vesicatoria* and *Cladosporium fulvum* (Tang et al., 1999). These experiments suggest that modified *R* gene can confer general resistance and specificity of the *R* genes reside in only certain domains of R proteins.

Perception of pathogen signals by host plants may sometimes lead to susceptibility, instead of resistance. In fact, resistance and susceptibility are similar to opposite sides of a coin. By tossing the coin, we can get resistance or susceptibility. However, the phenomenon is not so simple; susceptibility or resistance results due to complex molecular signaling events occurring in those plants on perception of pathogen signals. Alleles of *R* genes may be present in both resistant and susceptible plants. The *R* gene allele in resistant plants may be more effective in triggering defense response than that present in the susceptible plants. In some plants, the same type of *R* gene may be present in both resistant and susceptible plants. Appropriate pathogen signal may activate transcription of the *R* gene in the resistant

interaction and increase abundance of R proteins to reach the threshold level needed to activate defense responses. In the compatible interactions, such an increase in R protein abundance may be absent or less pronounced. In most cases, *R* gene allele detected in susceptible plants is nonfunctional, mostly because of slight variation, sometimes only a mutation in a single amino acid. This type of alteration in molecular structure would have altered the function of the allele from conferring resistance to that conferring susceptibility. The susceptibility allele may suppress the function of not only related *R* genes, but also other *R* genes.

Some susceptibility genes have been identified in plants and these genes induce susceptibility by suppressing the defense signal transduction. Some resistance genes, which confer resistance against biotrophic pathogens, confer susceptibility against necrotrophic pathogens. *R* genes may induce cell death, which may be involved in conferring resistance against biotrophs and susceptibility against necrotrophs. Thus, *R* genes may behave as susceptibility genes and both susceptible and resistant interactions may share a common signaling pathway. Depending upon the type of signal transduced, resistance or susceptibility may result. Both resistance and susceptibility genes may be involved in perception and emission of pathogen signals.

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4 Cell Death Programs during Fungal Pathogenesis

4.1 INTRODUCTION

Response of plants to fungal infection is accompanied by different types of cell death in various plant–pathogen interactions. The types of cell death observed in plants during fungal pathogenesis include programmed cell death (PCD) (Wang et al., 2005; Zuppini et al., 2005), hypersensitive (= hypersensitive response (HR)-related) cell death (Dangl et al., 1996; Peterhänsel et al., 1997; Hückelhoven et al., 1999; Sasabe et al., 2000; Moeder et al., 2005; Montillet et al., 2005), runaway cell death (RCD) (Jabs et al., 1996; Rustérucci et al., 2001), spontaneous cell death (Peterhänsel et al., 1997; Dong, 2004; Brodersen et al., 2005), and normosensitive or susceptibility-related cell death (= cell necrosis) (Király et al., 2002; Eckardt, 2005).

Cell death and disease development are intimately connected. Cell death may be phenotypically associated with both susceptible and resistance interactions (Gilchrist, 1998; Dickman et al., 2001; Greenberg and Yao, 2004; Eckardt, 2005; Takemoto et al., 2005). Whereas hypersensitive cell death (HR cell death) is associated with disease resistance, normosensitive cell death or cell death by necrosis is associated with susceptibility. Regulation of cell death may be crucial to determine the outcome of plant–pathogen interactions, susceptibility, or resistance. In either case, pathogen challenge can trigger groups of plant cells to die (Dickman et al., 2001). Cell death in plants accompanies both susceptible and resistant reactions, suggesting that overlapping biochemical pathways are operative in these two contrasting outcomes. This chapter describes the cell death programs operative in both susceptible and resistance interactions and their role in disease or resistance development.

4.2 CELL DEATH IN RESISTANT INTERACTIONS

4.2.1 Programmed Cell Death

PCD is a functional concept that refers to cell death that is part of a normal life of a multicellular organism; it involves controlled disassembly of the cell (Woltering et al., 2002). Cells are programmed to die if their continued existence is detrimental (Richael and Gilchrist, 1999). PCD is genetically controlled cellular suicide (Sanmartin et al., 2005). PCD in plants resembles apoptosis in mammals. Kerr et al. (1972) coined the term apoptosis to signify a form of PCD in certain animal cells with specific morphological features. Apoptosis is recognized as the common cellular default mechanism of PCD, and cells appear to possess the machinery necessary to carry out the terminal steps of death, even if all steps are not executed (Richael and Gilchrist, 1999).

Specific features of apoptosis in animal cells include cell shrinkage, plasma membrane blebbing, condensation and fragmentation of the nucleus, internucleosomal cleavage of DNA, nuclear deformations, and fragmentation of the cell into cellular debris-containing

vesicles called apoptotic bodies (Gilchrist, 1998; Woltering et al., 2002). The affected cells show typical cell corpse morphology (Vidhyasekaran, 2002). Similar apoptotic symptoms including compaction and shrinkage of the cytoplasm and nucleus, and the formation of DNA-containing apoptotic-like bodies have been reported in dying plant cells during PCD (Wang et al., 1996; De Jong et al., 2000). The features of plant PCD include DNA fragmentation (Mittler et al., 1997; Asai et al., 2000; Sasabe et al., 2000), loss of plasma membrane integrity (Fath et al., 2000), and vacuolization of the cells (Mittler et al., 1997).

PCD program in plant cells appears to be similar to that in animal cells. Proteins that activate or inhibit PCD in animals show a similar effect on PCD when expressed in plants (Lacomme and Santa Cruz, 1999; Danon et al., 2000, 2004; Sanchez et al., 2000; Lincoln et al., 2002; Sanmartin et al., 2005). The Bcl2 family of pro- and antiapoptotic proteins activates or inhibits PCD in animals. Similarly when they are expressed in plants, they activate or inhibit PCD in plants (Sanmartin et al., 2005). The animal proapoptotic protein Bax induced cell death in tobacco (Lacomme and Santa Cruz, 1999). Overexpression of Bax inhibitor suppresses the fungal elicitor-induced cell death in rice cells (Matsumura et al., 2003). AtBI-1, a plant homolog of Bax inhibitor-1 (BI-1) suppresses Bax-induced cell death in yeast and is rapidly upregulated during pathogen challenge in plants (Sanchez et al., 2000). Homologs of genes that control apoptosis in animals, such as *DAD-1* (Defender against Apoptotic Death-1) and *Bax-inhibitor-1*, are encoded in plant genomes and they control PCD in plants (Matsumura et al., 2003; Danon et al., 2004). These observations suggest that PCD in animals and plants may be similar in their function.

PCD is associated with active metabolic changes (Tsunezuka et al., 2005). PCD is commonly expressed during different plant development stages. The developmentally triggered PCD takes place during seed germination, formation of root caps, death of petals after fertilization, and leaf senescence (Gilchrist, 1998). PCD has been detected in plants infected by pathogens also (Greenberg et al., 1994; Asai et al., 2000; Wang et al., 2005; Zuppini et al., 2005).

4.2.2 Hypersensitive Cell Death

In some plant–pathogen interactions, the plant's response to attempted infection by fungal pathogens is accompanied by rapid cell death in and around the initial infection sites (Morel and Dangl, 1997). Visually, this reaction appears as a region of dead cells at the point of pathogen recognition by the plant (Dickman et al., 2001). This reaction is known as HR (Moffett et al., 2002; Xiao et al., 2003; Jurkowski et al., 2004; Moeder et al., 2005). The cell death reaction of the pathogen-attacked host plant cell showing HR is called hypersensitive cell death (= HR-related cell death) (Peterhänsel et al., 1997). This cell death is induced by host signals and hence it is also called host-induced cell death. PCD forms part of the HR. The HR occurs at the site of pathogen entry and involves PCD in and around the infection site. It is also accompanied by the induction of plant defense responses that serve to confine the pathogen and protect the plant (Lam et al., 2001).

HR is a form of PCD in plants, which differs from the developmentally triggered PCD by associated induced defense responses. Hypersensitive cell death, as exemplified by incompatible isolate of *Uromyces vignae*–cowpea interaction, is accompanied by degradation of host DNA into oligonucleosomal fragments as well as terminal deoxynucleotidyltransferase-mediated uridine triphosphate (UTP) end labeling-positive cells. Both the events are hallmark characteristics of PCD (Ryerson and Heath, 1996).

4.2.3 Spontaneous Cell Death

Spontaneous cell death resembles hypersensitive cell death, except that it is induced without pathogen attack. It is induced in some mutant plants (Ishikawa et al., 2003; Dong, 2004;

Park et al., 2004; Brodersen et al., 2005) and in transgenic plants (Lacomme and Santa Cruz, 1999; Xiao et al., 2003). Some of the mutants, called lesion mimics, spontaneously exhibit cell death in the absence of pathogen attack (Morel and Dangl, 1999). Such mutants have been found in maize (Walbot et al., 1983; Gray et al., 1997), rice (Takahashi et al., 1999), barley (Wolter et al., 1993), and Arabidopsis (Brodersen et al., 2002, 2005; Dong, 2004; Park et al., 2004). The Arabidopsis genes *RPW8.1* and *RPW8.2* induced resistance to powdery mildew and developed spontaneous cell death, when expressed in tomato line Tm-7 (Xiao et al., 2003). Tobacco plants expressing Bax protein developed spontaneous lesions (Lacomme and Santa Cruz, 1999). The spontaneous cell death may constitutively activate defenses in the absence of pathogen infection (Mach et al., 2001).

Spontaneous cell death instead of HR cell death may play an important role in fungal pathogenesis in some plant–pathogen interactions. The barley powdery mildew resistance recessive gene *mlo* confers resistance against almost all tested isolates of *Blumeria graminis* f. sp. *hordei*. In *mlo*-controlled interactions, no HR-related cell death occurs, and the attacked host cell survives the fungal penetration attempt. However, *mlo*-resistant plants exhibit a spontaneous leaf cell death phenotype in the absence of the pathogen and the spontaneous cell death induces defense responses in barley cells (Wolter et al., 1993). Similar to the hypersensitive cell death, spontaneous cell death can also be separated from disease resistance. Unlike the various cell death mutants, the Arabidopsis *acd5* mutant does not show increased resistance to the oomycete *Peronospora parasitica* and bacterial pathogens (Greenberg et al., 2000).

4.2.4 Runaway Cell Death

RCD (spreading lesions) is also reported in some plant–pathogen interactions. This type of cell death is seen as cell death beyond HR. RCD was detected in an Arabidopsis mutant. Arabidopsis plants carrying the recessive null *lsd1* (lesion stimulating disease resistance gene1) allele exhibited normal HR after infection by various incompatible pathogens, but RCD was initiated subsequently at the margins of these sites (Dietrich et al., 1994).

4.2.5 Cell Death-Inducing Systemic Acquired Resistance

Resistance responses that occur locally can induce long-term resistance at the whole plant level in a process that requires salicylic acid (SA) and this process is called systemic acquired resistance (SAR) (Ryals et al., 1996). The primary host defense reaction is accompanied by plant cell death at the site of infection. This type of host cell death is called HR cell death. The resulting lesions are correlated with the establishment of SAR. Thus, the cell death may activate SAR (Hunt et al., 1997). In Arabidopsis, *lsd* mutants spontaneously develop lesions in the absence of pathogen infection. The *lsd* mutants express various SAR marker defense genes including genes encoding PR proteins and genes involved in phenolics and callose synthesis when lesions are present. These mutants were resistant to the same spectrum of pathogens as plants activated for SAR by necrogenic pathogens (Hunt et al., 1997).

4.3 MOLECULAR MECHANISM OF INDUCTION OF HYPERSENSITIVE CELL DEATH

4.3.1 Mediators, Regulators, and Executioners of Cell Death

The mechanism of induction of cell death is complicated. Several factors may contribute to the cell death process. These may include several mediators, regulators, and executioners of cell death. Mediators themselves cannot cause cell death; but they mediate the induction of

cell death (Epple et al., 2003). The mediators of cell death include resistance gene-encoded proteins (Moffett et al., 2002; Rivas et al., 2004), elicitors (Takemoto et al., 2005), reactive oxygen species (ROS) (Levine et al., 1996; Overmyer et al., 2003; Apel and Hirt, 2004; Gechev and Hille, 2005), nitric oxide (NO) (Klessig et al., 2000), calcium (Li et al., 2004; Zuppini et al., 2005), guanosine-3′,5′-cyclic monophosphate (cGMP) (Clarke et al., 2000), G proteins (Joo et al., 2005; Moeder et al., 2005), cytochrome *c* (Hückelhoven et al., 2003; Zuppini et al., 2005), SA (Greenberg, 2000; Greenberg et al., 2000; Li et al., 2002; Lu et al., 2003), and ethylene (Moussatos et al., 1994).

The regulators of cell death regulate the function of various mediators of cell death. These include caspases (Rojo et al., 2004), Bax proteins (Danon et al., 2004; Sanmartin et al., 2005), mitochondrial ion channels (O'Rourke et al., 2005), alternative oxidase (AOX) (Lam et al., 2001), EDS1, PAD4, and NDR1 (Rustérucci et al., 2001).

The executioners of cell death may directly kill the cells and these may include executioner caspases (Woltering et al., 2002), ROS (Levine et al., 1996; Lu and Higgins, 1999), lipid hydroperoxides (Montillet et al., 2005), and NO (Klessig et al., 2000; Epple et al., 2003; Polverari et al., 2003).

4.3.2 *R* Gene Signals Involved in Triggering Cell Death

Several resistance gene-encoded proteins have been shown to signal the induction of cell death. Cell death is a common phenotype in HR induced by various resistance gene-encoded proteins (Moffett et al., 2002; Muskett et al., 2002). Several *R* genes-encoded leucine-rich repeat (LRR)–Nucleotide-binding site (NBS) proteins have been shown to be involved in triggering cell death (Moffett et al., 2002). The C-terminal part of NBS is called the ARC (Apoptosis, *R* gene product, and CED-4) subdomain. It is structurally related to regulators of animal apoptosis, including human Apaf-1 and nematode CED-4, and is conserved in plant NBS–LRR proteins (van der Biezen and Jones, 1998). The N-terminus of several NBS–LRR proteins is a Toll and interleukin receptor (TIR) domain. The flanking regions of many of these plant receptor-like proteins contain a nucleotide-binding domain and conserved amino acids, termed the Apoptosis-ATPase (Ap-ATPase) domain (van der Biezen and Jones, 1998; Aravind et al., 1999). This motif is similar to that present in the mammalian mediator Apaf-1 (Lam et al., 2001). The molecular structure of *R* genes suggests that the *R* genes may be involved in signaling cell death. The tomato *R* gene *Cf9* protein regulates cell death (Rivas et al., 2004). Tobacco *N* gene (Dinesh-Kumar et al., 2000), flax rust resistance genes *L*, *M*, *N*, and *P* (Lawrence et al., 1995; Ellis et al., 1999; Gassmann et al., 1999; Dodds et al., 2001), and Arabidopsis genes *RPP1*, *RPP4*, *RPP5*, *RAC1*, and *RPS4* (Lawrence et al., 1995; Gassman et al., 1999; Meyers et al., 2003; Borhan et al., 2004; Yang and Hua, 2004) trigger cell death.

4.3.3 Reactive Oxygen Species in Cell Death

Cell death occurs concomitant with the marked generation of ROS, otherwise called reactive oxygen intermediates (ROI). The ROS appear to be involved in PCD in many plant systems (Jabs et al., 1996; Desikan et al., 1998; Mittler et al., 1998, Hückelhoven et al., 1999). H_2O_2-induced PCD appears to be essential for the HR to pathogens (Apel and Hirt, 2004). In tomato, leaves treated with the fungal elicitor AVR9 showed necrosis indicating cell death. When the leaves were injected with the antioxidants, ascorbic acid or glutathione, the necrosis was reduced by more than 50% (Figure 4.1; Lu and Higgins, 1998). Overexpression of the H_2O_2-degrading enzyme ascorbate peroxidase suppresses cell death induced by H_2O_2 (Murgia et al., 2004). The scavengers of ROS such as catalase also reduced the cell necrosis (Lu and Higgins, 1998). These results suggest that ROS are involved in cell death.

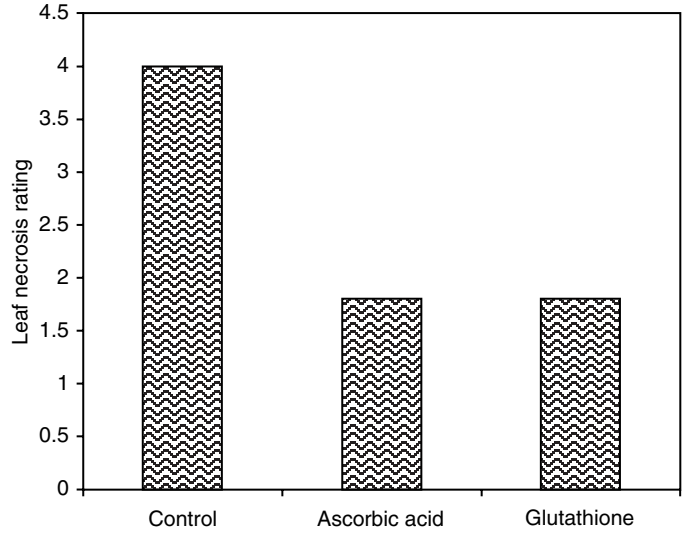

FIGURE 4.1 Effect of antioxidants in suppression of leaf necrosis in tomato induced by *Cladosporium fulvum* elicitor AVR9. (Adapted from Lu, H. and Higgins, V.J., *Physiol. Mol. Plant Pathol.*, 52, 35, 1998.)

ROS result from the activation of a plasma membrane NADPH oxidase in a manner similar to that of phagocytic neutrophils in mammals (Deleo and Quinn, 1996). The plasma membrane-localized NADPH oxidase induces ROS generation (Keller et al., 1998). ROS are derived from electron-transfer intermediates in the inner mitochondrial membrane (Lam et al., 1999b). Heterotrimeric GTP-binding or G proteins were shown to promote generation of ROS in soybean cell cultures (Legendre et al., 1992). The GTP-binding proteins (G proteins) may be involved in cell death (Joo et al., 2005; Moeder et al., 2005). GTP-binding proteins are required for ROS production in Arabidopsis (Joo et al., 2005) and ROS trigger a cell death (Overmyer et al., 2003). Kawasaki et al. (1999) introduced the constitutively active and dominant negative forms of the small GTP-binding protein OsRac1, a rice homolog of human Rac, into the wild-type rice plants. The transformed rice cells showed increased production of H_2O_2 as well as cell death (Kawasaki et al., 1999). It suggests that GTP-binding protein may be an important mediator in inducing H_2O_2 and cell death.

AOX, an inner mitochondrial enzyme, regulates production of H_2O_2 and hypersensitive cell death in plants. AOX catalyzes electron flow directly from ubiquinol to oxygen, thereby creating an electron shunt that bypasses complexes III and IV of the inner mitochondrial membrane and results in a cyanide-insensitive electron-transfer pathway (Lam et al., 2001). The AOX lowers mitochondrial ROS in plant cells (Maxwell et al., 1999). AOX may act as a safety valve for the control of ROS generation from the mitochondrion and its suppression results in induction of ROS and cell death (Lam et al., 1999b).

Some ROS are extremely reactive and kill invading pathogens as well as plant cells (Levine et al., 1994; Keller et al., 1998). ROS produced by the plant cell on perception of pathogen signals cause host cell death in tomato (Figure 4.2; Lu and Higgins, 1999). Levine et al. (1994, 1996) reported that PCD of soybean cells occurred on exposure to 8–10 mM H_2O_2. Cell death may result probably due to direct deleterious effects on cellular membranes and organelles by ROS (Wojtaszek, 1997).

H_2O_2 may have dual function in induction of cell death. H_2O_2 at sublethal doses may act as a signal molecule and at high doses it may act as a toxic molecule directly killing plant cells

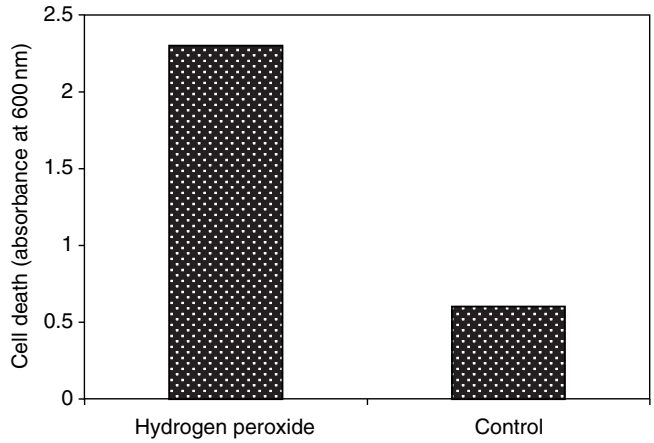

FIGURE 4.2 Induction of cell death by hydrogen peroxide in tomato (cell death was measured by recording absorbance of extracts of cells at 600 nm). (Adapted from Lu, H. and Higgins, V.J., *Physiol. Mol. Plant Pathol*., 54, 131, 1999.)

(Gechev and Hille, 2005). H_2O_2 signals induction of cell death (Torres et al., 2002), probably through mitogen-activated protein kinase (MAPK) cascade (Kovtun et al., 2000).

MAPKs may be involved in elicitor signal transduction system triggering cell death. Elicitins are the elicitors produced by *Phytophthora* spp. and induce hypersensitive cell death in most *Nicotiana* spp. and in some cultivars of *Brassica rapa* and *Raphanus sativus*. Transgenic *Nicotiana* spp. and *R. sativus* expressing *Arabidopsis* MAPK kinase 4 ($AtMEK_4^{DD}$) were developed (Takemoto et al., 2005). Elicitin-responsive *Nicotiana* species and *R. sativus* cultivars showed significantly stronger cell death responses following expression of $AtMEK_4^{DD}$ compared with nonresponsive species and cultivars (Takemoto et al., 2005). These results suggest that MAPKs may have a role in induction of cell death.

The MAPKs may induce cell death acting through H_2O_2 signaling system. The probable H_2O_2 signaling pathway acting through MAPKs in inducing cell death in Arabidopsis is presented in Figure 4.3.

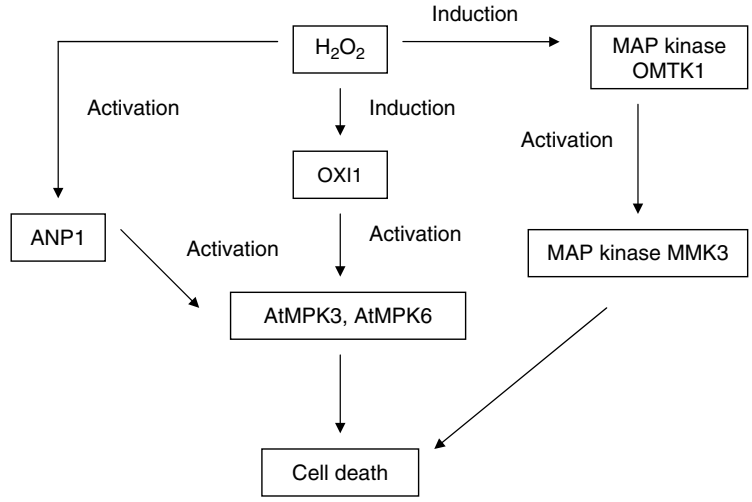

FIGURE 4.3 Role of MAPK cascade in H_2O_2-mediated cell death programs in Arabidopsis.

Serine/threonine kinase, oxidative signal-inducible1 (OXI1), is an active component in H_2O_2 signaling system inducing cell death in Arabidopsis (Rentel et al., 2004). The Arabidopsis oxi1-null mutant showed enhanced susceptibility and suppressed H_2O_2-mediated signaling process (Rentel et al., 2004). OXI1 is needed for full activation of two MAPKs, AtMPK3 and AtMPK6 (Rentel et al., 2004). These two MAPKs are also activated by the H_2O_2-regulated MAPK kinase kinase ANP1 (Kovtun et al., 2000; Menke et al., 2004). Oxidative stress-activated MAP triple-kinase 1 (OMTK1) is a more specific MAPKKK that can be activated only by H_2O_2 (Nakagami et al., 2004). OMTK1 specifically activates the downstream MAP kinase MMK3, which results in cell death (Gechev and Hille, 2005). MMK3 is activated also by ethylene and elicitors and may serve as a convergence point of the cell death program (Nakagami et al., 2004; Gechev and Hille, 2005).

In addition to the MAPK cascade network, the H_2O_2 signal can also be transmitted through alterations in calcium ion fluxes and cellular redox state (Gechev and Hille, 2005). Both these events are very early events following the rises in H_2O_2 levels (Rentel and Knight, 2004). Calcium ion increases lead to cell death through the numerous Ca^{2+}-interacting proteins such as calmodulins and calcium-dependent protein kinases (Harper et al., 2004). Ca^{2+}/calmodulin-regulated NAD kinase is involved in the production of H_2O_2 and cell death (Harding et al., 1997). Ca^{2+}/calmodulin may modulate catalase activity and catalase deficiency leads to elevation of H_2O_2 levels, which trigger cell death (Yang and Poovaiah, 2002; Gechev et al., 2004; Vandenabeele et al., 2004).

Besides H_2O_2, 9-lipoxygenase (LOX)-dependent lipid peroxidation products (lipid hydroperoxides) have been shown to act as executioner of hypersensitive cell death in tobacco leaves (Montillet et al., 2005).

4.3.4 Nitric Oxide in Cell Death

NO, a gaseous free radical, has been reported to be a cellular mediator of cell death in mammals and its involvement in plant cell death has been documented (Durner et al., 1998; Delledonne et al., 2001; Wendehenne et al., 2001; Gould et al., 2003). Clarke et al. (2000) found that Arabidopsis suspension cultures generated elevated levels of NO in response to challenge by avirulent bacteria, and that these levels of NO were sufficient to induce cell death in Arabidopsis cells independently of ROS. They concluded that NO-induced cell death is a form of PCD, requiring gene expression. NO induced chromatin condensation and caspase-like activity in Arabidopsis cells. NO-induced death could be blocked by a caspase-1 inhibitor (Clarke et al., 2000).

NO may act through a cGMP-dependent pathway in inducing cell death (Minorsky, 2003). In this pathway, NO posttranslationally activates guanylate cyclase (GC), which leads to a transient increase in cyclic GMP (Klessig et al., 2000). A specific inhibitor of GC also blocked NO-induced cell death in Arabidopsis cells. This inhibition was reversed by a cell-permeable cGMP analog. The cGMP analog alone did not potentiate NO-induced cell death. It suggests that cGMP synthesis is required but not sufficient for NO-induced cell death in Arabidopsis (Clarke et al., 2000). cGMP activates ADP-ribosyl cyclase (ADPRC) to elevate levels of cyclic ADP ribose (cADPR). NO may induce cell death through signaling pathways that may involve both cGMP and cADPR (Durner et al., 1998).

NO inhibits cytosolic and mitochondrial aconitase activities in tobacco (Navarre et al., 2000). NO converts the cytosolic aconitase into an mRNA binding protein known as IRP-1 (iron regulatory protein-1) in mammals (Klessig et al., 2000). IRP-1 regulates intracellular free iron concentrations. In the presence of ROS, free iron promotes oxidative damage via the Fenton reaction. Thus, NO-mediated increases in iron may contribute for induction of hypersensitive cell death in plants (Navarre et al., 2000).

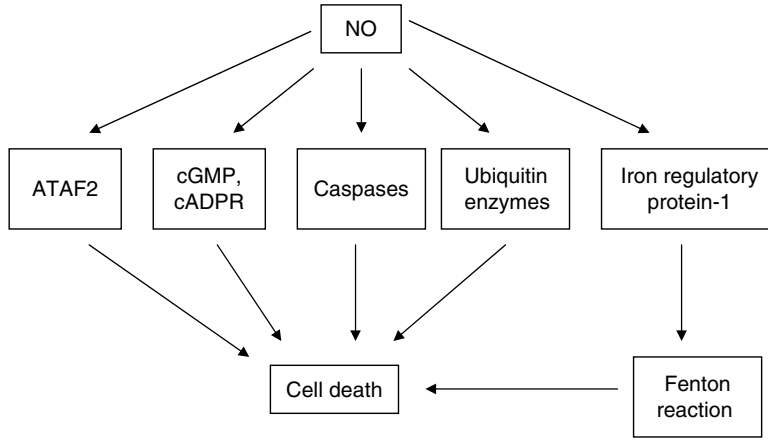

FIGURE 4.4 Role of NO in induction of cell death in Arabidopsis.

NO appears to be involved in the pathway leading to the accumulation of transcripts encoding the ethylene-forming enzyme (Moussatos et al., 1994; Lamotte et al., 2004). NO activates *ein3*, a gene involved in ethylene perception and transduction in Arabidopsis (Leshem et al., 1998). NO is known to influence several ethylene-dependent processes in the plant life cycle (Polverari et al., 2003). Ethylene is involved in induction of cell death (Lamotte et al., 2004).

NO induces caspase-like activity, and caspases have been shown to execute cell death (Clarke et al., 2000). A gene encoding cysteine protease RD21A was induced by NO in Arabidopsis (Polverari et al., 2003). NO also triggered the activation of two ubiquitin-conjugating enzymes, namely UBC10 and Ahus5 (Polverari et al., 2003). Connections between ubiquitylation and PCD have been well demonstrated (Bachmair et al., 1990, 2001; Karrer et al., 1998). NO also activates ATAF2, a protein belonging to the NAC domain protein family involved in cell death during fungal infection (Collinge and Boller, 2001). Thus several factors including caspases, ubiquitin conjugating enzymes, and ATAF2 may be involved in NO-mediated cell death in Arabidopsis (Figure 4.4).

NO cooperates with ROS in the activity of inducing hypersensitive cell death (Delledonne et al., 2001; Polverari et al., 2003). NO accumulation coincides with that of H_2O_2 in soybean suspension cells (Delledonne et al., 1998). NO production precedes the accumulation of H_2O_2 in elicitor-induced NO burst in tobacco (Foissner et al., 2000). NO inhibited the H_2O_2-scavenging enzymes catalase and ascorbate peroxidase activities in tobacco (Clarke et al., 2000) and this would have resulted in increase in H_2O_2 levels. Accumulation of ROS and NO directs cells to initiate cell death (Shirasu and Schulze-Lefert, 2000). The balance of ROS, most particularly O_2^- and H_2O_2, and NO, is crucial for the establishment of the hypersensitive cell death (Delledonne et al., 1998, 2001; Klessig et al., 2000).

4.3.5 Bax Family of Proteins

In animal cells, mitochondria-mediated PCD acts through the Bax (for B-cell leukemia/lymphoma 2 (BCL2)-associated X protein) family of proteins. The Bax proteins are associated with the outer mitochondrial membrane. They are involved in the permeability of the mitochondrial membrane. These proteins form channels through which macromolecules and other metabolites can pass or interact with other proteins that form ion-conducting channels, by promoting or inhibiting oligomerization of channel subunits (Lam et al., 2001). Bcl-2 and Bax are the most important components of mitochondrion-mediated PCD

in mammals (Lam et al., 2001). The hallmarks of PCD in animal cells are Bcl-2 and Bax and both of them are absent in plants (Aravind et al., 1999). However, there are indirect evidences to show that similar mechanisms may exist in plants. The hypersensitive cell death could be activated in plant cells in which Bax was expressed from a viral vector (Lam et al., 1999a). Expression of Bax in plants causes cell death (Lacomme and Santa Cruz, 1999) and the animal cell-death suppressors Bcl-x1 and Ced-9 inhibit cell death in tobacco plants (Mitsuhara et al., 1999). These results suggest that Bax proteins may be involved in cell death program.

BI-1 proteins are suppressors of PCD in mammals. The homolog of BI-1 has been detected in plants (Matsumura et al., 2003; Danon et al., 2004). The barley BI-1 has been shown to be a suppressor of nonspecific background resistance and *mlo*-mediated penetration resistance to the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* when overexpressed in epidermal cells of barley (Eichmann et al., 2004). Overexpression of *BI-1* rendered barley cells susceptible to penetration by incompatible wheat powdery mildew pathogen *Blumeria graminis* f. sp. *tritici* (Eichmann et al., 2004). These studies suggest that Bax proteins-mediated cell death plays an important role in conferring resistance against pathogens.

4.3.6 Ion-Conducting Channels

Several membrane channel-related proteins have been found to mediate hypersensitive cell death. The Arabidopsis gene *dnd1* (defense, no death1) is required for the activation of hypersensitive cell death. This gene encodes a cyclic nucleotide-dependent calcium channel (Clough et al., 2000). Elevation of cytosolic Ca^{2+} is involved in triggering PCD (Li et al., 2004). Another cyclic nucleotide-gated ion channel gene *DND2* is also involved in hypersensitive cell death in Arabidopsis (Jurkowski et al., 2004). The mutation of the gene results in loss of HR cell death, indicating the importance of ion channel in cell death (Jurkowski et al., 2004).

4.3.7 Function of Mitochondrion in Induction of Cell Death

The mitochondrion may respond to the pathogen signals transducted through second messengers such as calcium ion, ROS, and changes in cellular pH (Vander Heiden et al., 2000; O'Rourke et al., 2005). This may increase permeability of the mitochondrion by forming a pore constituted by Bax proteins. Through this pore a number of cell-death activators, including cytochrome *c*, apoptosis-inducing factor (AIF), and Smac/DIABLO, may be released in animal cells (Lam et al., 2001). Cytochrome *c* regulates the activity of caspases responsible for ordered disassembly of the cell (Green, 2000). AIF induces nuclear DNA cleavage, whereas Smac/DIABLO activates intracellular caspases (Chai et al., 2000). Similar mechanisms may exist in plants also (Lam et al., 1999b).

4.3.8 Proteolytic Enzymes

4.3.8.1 Plant Caspases

In mammals, caspases (cysteinyl Asp-specific proteinases) function as molecular switches to activate the apoptotic cell death program (Shi, 2002). Caspases belong to a class of specific cysteine proteases that show a high degree of specificity with an absolute requirement for cleavage of an Asp residue and a recognition sequence of at least four amino acids N-terminal to this cleavage site (Woltering et al., 2002). Caspases are synthesized as inactive pro-enzymes and are activated by directed proteolysis that removes the N-terminal peptide. Apoptotic cell death involves a sequence of caspase activation events in which initiator caspases activate downstream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype (Woltering et al., 2002).

Inhibitors to mammalian caspases markedly suppressed cell death induced by plant pathogens (Lam and del Pozo, 2000) or chemicals (De Jong et al., 2000, 2002; Woltering et al., 2002). Caspase-specific peptide inhibitors, Ac-YVAD-CMK and Ac-DEVD-CHO, could abolish bacteria-induced plant PCD (del Pozo and Lam, 1998). Caspase-like proteolytic activity was detected in tobacco tissues that were developing HR following infection with tobacco mosaic virus (TMV) (del Pozo and Lam, 1998). These results indicate that caspases may play an important role in plant cell death.

Caspase-like protease activity has been demonstrated in plants expressing the hypersensitive cell death and in PCD associated with other responses (del Pozo and Lam, 1998; Korthout et al., 2000; Watanabe and Lam, 2004). Vacuolar processing enzymes (VPEs) and the metacaspases are the two groups of proteases that encode caspase-like activities in plants (Woltering et al., 2002). They are related in sequence and in tertiary structure to animal caspases (Sanmartin et al., 2005). VPEs and metacaspases have caspase activity and regulate PCD in plants (Hoeberichts et al., 2003; Rojo et al., 2003, 2004; Hatsugai et al., 2004; Suarez et al., 2004).

4.3.8.2 Vacuolar Processing Enzymes (VPEs)

Plant VPEs show caspase-like activities. Rojo et al. (2003) characterized a VPE of Arabidopsis (VPEγ) and modeling of the three-dimensional structure of VPEγ predicted a close alignment of its catalytic residues with those of the animal caspase-8. Rojo et al. (2004) provided direct *in vivo* evidence to show that the Arabidopsis VPEγ gene encodes a protein with caspase-like activity and it is involved in cell death progression. VPEγ binds to caspase inhibitors that block self-maturation of this enzyme and all its downstream *in vivo* activities. An increase in caspase activity was observed in Arabidopsis plants infected with incompatible strain of *Pseudomonas syringae* pv. *tomato* DC3000. Concurrent with increase in caspase activity, a rapid PCD response was initiated. In *vpeγ* mutants, the increase in caspase activity was not observed and the *vpeγ* mutants did not induce PCD (Rojo et al., 2004). These results suggest that increase in caspase activities may result in cell death.

Tobacco (*Nicotiana tabacum*) plants carrying the *N* resistance gene show characteristic PCD when infected by TMV. When the plants were treated with VPE inhibitors or caspase1-inhibitors, the development of PCD was blocked. In *VPE*-silenced *Nicotiana benthamiana* plants, the induction of caspase activity in response to TMV was suppressed and PCD was blocked (Hatsugai et al., 2004). These results suggest that the VPEs have caspase-like activity and regulate cell death in *Nicotiana* spp.

VPEs may be transferred to the vacuole where they are activated and promote vacuolar collapse (Sanmartin et al., 2005). Arabidopsis VPEγ has been shown to process a vacuolar protease and to regulate the degradation of vacuolar proteins, including a cystatin that may inhibit PCD progression (Rojo et al., 2004). It suggests that proteolytic events downstream of VPE activation may be important for PCD initiation.

4.3.8.3 Metacaspases

Metacaspases are another caspase-related family of proteases, which are involved in PCD (Bozhkov et al., 2004; Suarez et al., 2004). A tomato type II metacaspase (LeMCA1) is induced during infection with *Botrytis cinerea*, and it has been shown that it plays a role in the PCD induced by this pathogen (Hoeberichts et al., 2003). Infection with *Phytophthora infestans* or treatment with NPP1, a peptide PAMP from *Phytophthora*, induces the expression of *AtMCP1b*, *AtMCPIc*, and *AtMCP2e*, the genes encoding metacaspases in Arabidopsis (Sanmartin et al., 2005).

4.3.8.4 Other Types of Proteolytic Enzymes

Besides caspases, other proteolytic enzymes have also been shown to be involved in regulation of cell death. Members of the cathepsin family of proteases are important in disease-related PCD (Johnson, 2000). In soybean cells, PCD-activating oxidative stress induces a set of Cys proteases. Inhibition of the induced Cys protease activity by ectopic expression of the Cys protease inhibitor, cystatin, blocks PCD triggered by an avirulent pathogen (Solomon et al., 1999). It shows that cystatin-sensitive Cys proteases are critical regulators of hypersensitive cell death in soybean (Solomon et al., 1999).

A Ser protease from tomato has been shown to cleave an extracellular LRR protein and induce PCD during bacterial infection (Tornero et al., 1996). A Ser protease displaying caspase-like activity regulates PCD in oat (Coffeen and Wolpert, 2004). Ser protease triggers cell death in zinnia (Groover and Jones, 1999). Trypsin inhibitor inhibits the protease activity and the cell death process in zinnia cell cultures (Groover and Jones, 1999). These results suggest that the interplay between proteases and endogenous protease inhibitors may play a regulatory role in cell death.

4.3.9 Probable Sequence in Induction of Hypersensitive Cell Death

Although several factors have been identified to cause cell death, the exact sequence of molecular events leading to cell death is not yet known (Apel and Hirt, 2004; Gechev and Hille, 2005). The possible pathway in induction of hypersensitive cell death is presented in Figure 4.5 and it probably includes Ca^{2+}, protein kinases, SA, ethylene, ROS, NO, Bax proteins, cytochrome *c*, and proteases, and evidences are accumulating to prove that this pathway may operate in plant–pathogen interactions (De Jong et al., 2002; Li et al., 2002; Matsumura et al., 2003; Danon et al., 2004; Rojo et al., 2004; Watanabe and Lam, 2004).

4.4 MOLECULAR MECHANISM OF INDUCTION OF SPONTANEOUS CELL DEATH

4.4.1 Spontaneous Cell Death-Regulating Genes

Several genes responsible for cell death phenotypes in the mutations have been identified. Some of these genes may function as regulators of cell death, whereas others may perturb cellular metabolism in a way that leads to cell death (Ishikawa et al., 2003). The lesion initiation1 (*len1*) Arabidopsis mutant develops lesions on its leaves and expresses SAR. *LEN1* was identified to encode a chloroplast chaperonin 60β (Cpn60β). *LEN1* functions as a molecular chaperone in chloroplasts and its deletion leads to cell death in Arabidopsis (Ishikawa et al., 2003). In Arabidopsis, *acd* (accelerated cell death) and *lsd* genes confer spontaneous cell death. The *LSD1* gene encodes a novel class of zinc finger protein that could act as a negative regulator of signals involved in the propagation of cell death (Dietrich et al., 1997). The Arabidopsis LSD1 and LOL1 (LSD-one-like 1) proteins both contain three conserved zinc finger domains and have antagonistic effects on plant PCD (Wang et al., 2005). LSD1 and LOL1 proteins (Epple et al., 2003; Wang et al., 2005) may negatively or positively regulate the cell death process in plants (Lam et al., 1999b; Wang et al., 2005; Sanmartin et al., 2005). LSD1 is a negative regulator (Aviv et al., 2002), whereas LOL1 is a positive regulator of cell death (Epple et al., 2003). A rice functional homolog of LSD1, designated OsLSD1, was identified. Antisense transgenic rice plants expressing OsLSD1 exhibited lesion mimic phenotype. It suggests that OsLSD1 plays a negative role in regulating plant PCD (Wang et al., 2005).

The *acd2* mutants of Arabidopsis have spontaneous spreading cell death lesions. The *ACD2* gene has been cloned (Mach et al., 2001). Its predicted product shows significant

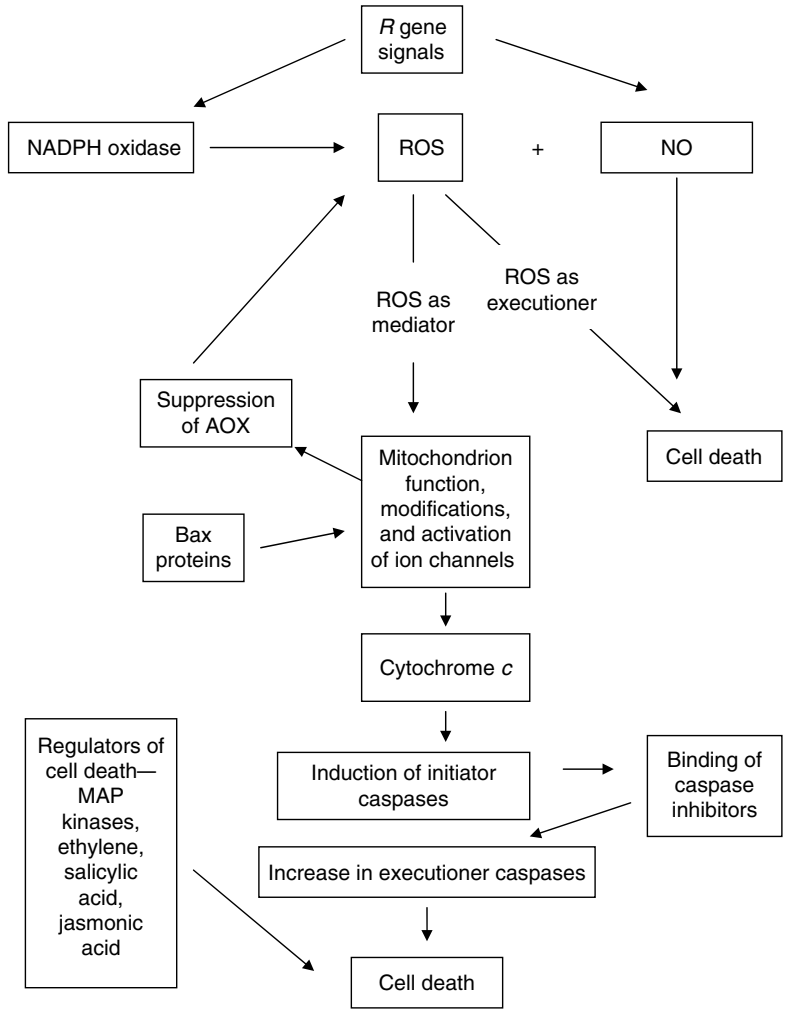

FIGURE 4.5 Suggested pathways in induction of hypersensitive cell death.

and extensive similarity to red chlorophyll catabolite reductase, which catalyzes one step in the breakdown of the porphyrin component of chlorophyll. It suggests that cell death in *acd2* plants is caused by the accumulation of chlorophyll breakdown products (Mach et al., 2001).

Arabidopsis *acd5* and *acd6* mutants show spontaneous cell death (Greenberg et al., 2000). Arabidopsis *ACD6* has been cloned. The cloned *ACD6* gene was identified in an Arabidopsis mutant, *acd6*, which undergoes PCD in the absence of a pathogen challenge (Dong, 2004). It encodes a novel protein with putative ankyrin and transmembrane regions (Lu et al., 2003). Cell death in the recessive *acd11* Arabidopsis mutant exhibits characteristics of animal apoptosis (Brodersen et al., 2002). Several other mutants of Arabidopsis such as *lsd7*, *ssi1*, and *cpr20cpr21* also show spontaneous cell death (Rate et al., 1999; Shah et al., 1999; Silva et al., 1999).

The *LLS1* (lethal leaf spot1) gene from maize has been cloned (Gray et al., 1997). It encodes a novel protein, and two consensus-binding motifs of aromatic ring-hydroxylating dioxygenases are present in the predicted LLS1 protein. It may function to degrade a phenolic mediator of cell death and it could be responsible for the detoxification of signals generated during cell death. The *LLS1* gene may be required to limit the spread of cell death in leaves

(Gray et al., 1997). The *MLO* resistance gene, which is involved in spontaneous cell death in barley, encodes a putative transmembrane protein (Büschges et al., 1997).

4.4.2 Salicylic Acid

The spontaneous cell death appears to be activated by SA. In Arabidopsis, cell death activated in *acd11* was SA-dependent (Brodersen et al., 2005). The SA-dependent pathway required two regulators of SA-mediated resistance responses, PAD4 and EDS1 (Brodersen et al., 2002, 2005). In Arabidopsis *acd5* plants, cell death was strictly dependent on SA (Greenberg et al., 2000). The phenotype of the *acd5–nahG* plants (expressing salicylate hydroxylase gene from *Pseudomonas putida* which degrades SA) did not accumulate SA and did not exhibit the spontaneous cell death. The suppressed phenotype could be reversed by application of the synthetic SA analog benzothiazole (BTH). *acd5* plants also accumulated high levels of SA relative to wild-type plants (Greenberg et al., 2000). These results suggest that spontaneous cell death in *acd5* is dependent on SA. Basal expression of *ACD6* mRNA in Arabidopsis required SA and an intact SA signaling pathway. *ACD6* mRNA levels were increased in plants treated with SA agonist BTH (Lu et al., 2003). These results suggest that SA may be involved in *ACD6*-mediated cell death. SA mediates *ACD6* forming a feedback signal amplification loop (Dong, 2004).

It has been demonstrated that the SA signal transducer *NPR1* (nonexpressor of PR1) plays a key role in controlling cell death (Rate et al., 1999). *npr1* suppressed much of the spontaneous cell death of *acd5* mutant and completely blocked cell death induced by SA (Rate et al., 1999). Double mutants with *acd5* and *NPR1*, in which SA signaling is blocked, show greatly attenuated cell death, indicating a role for the SA signal transducer *NPR1* in controlling the cell death (Greenberg et al., 2000).

Isochorismate-derived compounds other than SA may also be involved in induction of PCD. The *acd11* mutant was earlier shown to depend on SA in inducing cell death (Brodersen et al., 2002). A double mutant, *acd11/sid2-2*, was developed in Arabidopsis. The *sid2-2* mutant is deficient in isochorismate synthase required for SA biosynthesis (Brodersen et al., 2005). *sid2-2* fully suppressed SA accumulation and cell death in *acd11*. Application of exogenous SA to *acd11/sid2-2* was insufficient to restore cell death (Brodersen et al., 2005). It suggests that isochorismate-derived compounds other than SA are required for induction of PCD in *acd11*.

4.4.3 Ethylene

Ethylene may be involved in spontaneous cell death. Arabidopsis *acd5* mutant plants produce more ethylene than wild-type plants. The *ein2* mutation, which blocks ethylene signal transduction (Alonso et al., 1999), suppresses the *acd5*-conferred phenotypes. The presence of ethylene-insensitive *ein2* mutation reduces cell death in the *acd5* Arabidopsis mutant (Greenberg et al., 2000). These results suggest the importance of ethylene in cell death program.

4.4.4 Phosphatidic Acid

Phosphatidic acid (PA) induced spontaneous cell death in Arabidopsis. This treatment elevated levels of ROS in the whole leaf in Arabidopsis (Park et al., 2004). Rho-related small G protein (ROP) 2 also enhanced ROS production. In response to PA, transgenic leaves of Arabidopsis, expressing a constitutively active *rop2* mutant, exhibited earlier cell death and high levels of ROS than the wild type. In the absence of exogenous PA, no spontaneous cell death or elevated ROS were observed in constitutively active *rop2* plants

(Park et al., 2004). These results suggest that PA modulates an additional factor required for the active ROP-mediated ROS generation pathway, which contributes for cell death (Park et al., 2004).

4.5 MOLECULAR MECHANISM OF INDUCTION OF RUNAWAY CELL DEATH

RCD was detected in an Arabidopsis mutant carrying the recessive null *lsd1* allele. This mutant plant exhibited normal HR after infection by various incompatible pathogens and RCD was initiated subsequently at the margins of these sites resulting in cell death beyond HR (Dietrich et al., 1994). The molecular mechanism of induction of RCD in Arabidopsis mutant plants is presented in Figure 4.6.

Superoxide anion (O_2^-) and SA are the key components triggering RCD in Arabidopsis *lsd1* mutant. In the *lsd1* plants, accumulation of O_2^- preceded the RCD and the spreading lesions in RCD could be induced by providing O_2^- in uninfected tissues (Jabs et al., 1996).

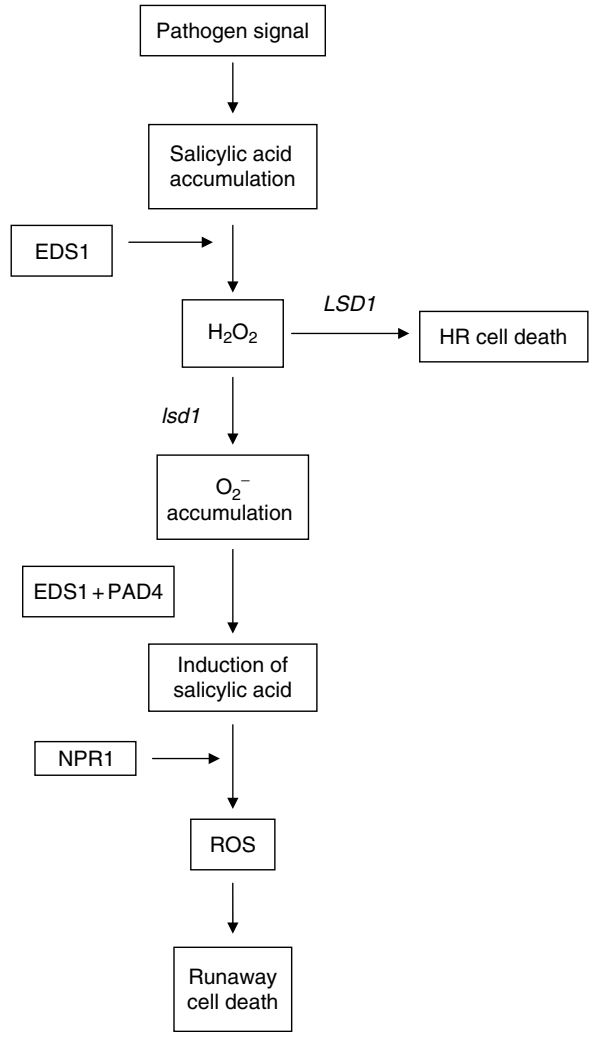

FIGURE 4.6 Molecular mechanism of induction of RCD.

These plants also responded to SA treatment in inducing the lesion development (Jabs et al., 1996). The regulatory proteins EDS1, PAD4, and NPR1, which regulate the accumulation of SA, are involved in RCD development (Rustérucci et al., 2001).

The Arabidopsis *LSD1* gene is a negative regulator of HR in Arabidopsis plants (Aviv et al., 2002). The LSD1 protein may regulate both the normal hypersensitive cell death and RCD in Arabidopsis (Epple et al., 2003). LSD1 regulates production of H_2O_2 from superoxide (O_2^-) in the wild-type Arabidopsis plants. SA accumulates in the wild-type HR plants and it influences ROS levels locally and leads to cell death (Shirasu et al., 1997). In the wild-type plants, superoxide is rapidly converted by superoxide dismutase (SOD) to H_2O_2. By contrast, in the *lsd1* mutant, RCD was activated by a superoxide-dependent signal (Jabs et al., 1996). The upregulation of cytosolic copper–zinc SOD after SA application to wild-type plants was lacking in *lsd1* mutant plants and this would have resulted in accumulation of superoxide in *lsd1* mutants (Kliebenstein et al., 1999). It is suggested that the balance of H_2O_2 in the wild-type plants may ultimately control hypersensitive cell death (Delledonne et al., 1998, 2001; Wendehenne et al., 2001) and the LSD1 may be required for correct interpretation of ROS or ROS-dependent signals emanating from an HR site to induce RCD (Jabs et al., 1996).

The regulatory proteins EDS1, PAD4, and NDR1 have been shown to be required for the development of RCD. EDS1 and PAD4 function upstream of SA accumulation, whereas NPR1 is an important regulator of responses downstream of SA (Cao et al., 1994; Delaney et al., 1995). Requirements for *EDS1* and *PAD4* in runaway lesion formation were separable from their roles in localized HR cell death. Neither *EDS1* nor *PAD4* function in the *R* gene *RPM1* resistance, yet both have been shown to be required for RCD after *RPM1* stimulation in *lsd1* plants. *EDS1*, but not *PAD4*, was necessary for ROS production and HR after local *RPS4*- or *RPP1*-mediated pathogen recognition, yet both *EDS1* and *PAD4* were required for the RCD in these responses (Rustérucci et al., 2001). These results suggest that the activities of *EDS1* and *PAD4* leading to RCD formation are in defense signal potentiation, downstream or independent of the HR.

RCD in *lsd1* plants could be triggered by superoxide furnished by local applications of xanthine and xanthine oxidase (Jabs et al., 1996). Superoxide accumulation preceded lesion formation in *lsd1* tissue and was detectable in cells bordering the developing lesion (Jabs et al., 1996). The mutants lacking EDS1 (*eds1*/*lsd1*), PAD4 (*pad4*/*lsd1*), and NDR1 (*ndr1*/*lsd1*) failed to initiate spreading RCD lesions after local provision of superoxide, supplied either by Rosebengal or xanthine/xanthine oxidase applications (Rustérucci et al., 2001). It suggests that the activities of *EDS1*, *PAD4*, and *NDR1* in ROS signaling leading to RCD are genetically distinct from their roles during the oxidative burst associated with a pathogen-induced HR. It also suggests that *EDS1*, *PAD4*, and *NDR1* may have a second function operating downstream or independently of the HR and this second function may help to establish the signal required to initiate RCD (Rustérucci et al., 2001).

4.6 ROLE OF CELL DEATH IN INDUCTION OF SYSTEMIC ACQUIRED RESISTANCE

The cell death may activate systemic signaling system. The cell death is known to activate SA accumulation and SA is involved in SAR development (Hunt et al., 1997). SAR was correlated with an increase in SA levels in both infected and noninfected leaves. Expression of salicylate hydroxylase (*nahG*) transgene in Arabidopsis and tobacco inhibited SA accumulation in response to pathogen infection and prevented the establishment of SAR (Lawton et al., 1995). These results suggest that SA may be involved in SAR development. To assess the relationship between SA accumulation and cell death, transgenic Arabidopsis unable to accumulate SA because of the *nahG* gene were used in crosses with the dominant mutants

lsd2 or *lsd4*. Progeny from the crosses were inhibited for SAR gene expression and disease resistance. However, these progeny retained the spontaneous cell death phenotype similar to siblings not expressing the *nahG* (Hunt et al., 1997). It suggests that SA may act downstream of the cell death.

SAR, but not the HR, requires the *NPR1* gene (Cao et al., 1994; Delaney et al., 1995). *npr1* mutants of Arabidopsis show extra susceptibility to multiple pathogens (Cao et al., 1994; Delaney et al., 1995).

4.7 SUSCEPTIBILITY-RELATED CELL DEATH

Cell death may also be induced in susceptible interactions. This cell death is called susceptibility-related cell death or normosensitive cell death (Király et al., 2002), and may be involved in conferring susceptibility to necrotrophic pathogens. Susceptibility-related cell death also shows characteristic apoptosis, similar to that observed in resistance-related cell death. Certain features of apoptosis have been observed in plants infected with *Cochliobolus victoriae* (Navarre and Wolpert, 1999), *Alternaria alternata* (Wang et al., 1996), and *Fusarium moniliforme* (Wang et al., 1996). AAL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* is the primary determinant of Alternaria stem canker disease of tomato. It induces a death process in plant cells that shows apoptotic morphology (Lincoln et al., 2002). Tomato protoplasts treated with the fumonisin B1 (FB1) produced by *F. moniliforme* underwent PCD-like response as indicated by DNA ladders and terminal deoxynucleotidyltransferase-mediated UTP end labeling-positive reacting cells (Wang et al., 1996). FB1 treatment of Arabidopsis protoplasts resulted in terminal deoxynucleotidyltransferase-mediated UTP end labeling-positive reacting cells (Asai et al., 2000; Stone et al., 2000). Infiltration of FB1 into Arabidopsis plants resulted in cell death (Stone et al., 2000). FB1-induced cell death in Arabidopsis shared some of the features of hypersensitive cell death (Stone et al., 2000). FB1-induced susceptibility-related cell death was accompanied by deposition of phenolic compounds and callose, production of ROS, accumulation of the phytoalexin camalexin, and expression of *PR* genes; all of them are hallmarks of hypersensitive cell death (Stone et al., 2000).

Transgenic tobacco plants expressing different antiapoptotic genes Bcl-2, CED-9, or Op-IAP were developed. All these transgenic plants were highly resistant or tolerant to *Sclerotinia sclerotiorum*, a necrotrophic fungal pathogen, whereas the nontransformed tobacco plants were highly susceptible to the pathogen (Dickman et al., 2001). The fungus grew vegetatively along the leaf surfaces of plants expressing the antiapoptotic genes, without infecting host tissue (Dickman et al., 2001). Transgenic tobacco plants expressing various antiapoptotic genes also showed resistance to *Botrytis cinerea* and *Cercospora nicotianae* (Dickman et al., 2001). The biochemical method for demonstrating apoptosis is the presence of oligonucleosome-sized fragments of DNA that, when electrophoresed on agarose gels, produce a characteristic ladder. In the transgenic tobacco plants expressing the antiapoptotic gene Bcl-2, the DNA laddering did not occur and these transgenic plants showed resistance to *S. sclerotiorum* (Dickman et al., 2001). These results suggest that cell death is essential for pathogenesis of necrotrophic pathogens.

4.8 MOLECULAR MECHANISMS IN INDUCTION OF CELL DEATH IN SUSCEPTIBLE INTERACTIONS

4.8.1 Mediators, Regulators, and Executioners of Susceptibility-Related Plant Cell Death

Necrotrophic pathogens produce toxins (Hockenbery et al., 1993; Lincoln et al., 2002; Coffeen and Wolpert, 2004; Sarma et al., 2005) and cell wall degrading enzymes (Zuppini et al., 2005) to

cause diseases. These pathogenicity/virulence factors may be involved in induction of plant cell death (Asai et al., 2000; Stone et al., 2000; Chivasa et al., 2005; Eckardt, 2005; Manning and Ciuffetti, 2005; Zuppini et al., 2005). The toxins are capable of inducing cell death in most susceptible plants (Markham and Hille, 2001). Toxins produced by *Alternaria alternata* f. sp. *lycopersici* (Spassieva et al., 2002), *Fusarium moniliforme* (Asai et al., 2000; Chivasa et al., 2005), *Pyrenophora tritici-repentis* (Manning and Ciuffetti, 2005; Sarma et al., 2005), *Cercospora nicotianae* (Hockenbery et al., 1993), *Helminthosporium oryzae* (Vidhyasekaran et al., 1986), *Rhizoctonia solani* (Vidhyasekaran et al., 1997), and *Cochliobolus victoriae* (Navarre and Wolpert, 1999; Coffeen and Wolpert, 2004) have been shown to induce cell death in susceptible interactions.

Necrotic cells may provide nutrition for the necrotrophic pathogens. The cell death may also be caused by the same type of mechanisms as seen in the resistance interactions. PCD was also observed in some susceptible reactions (Coffeen and Wolpert, 2004).

4.8.2 Reactive Oxygen Species

Necrotrophic pathogens often promote susceptibility-related host cell death through the generation of ROS (Mengiste et al., 2003). The necrotrophic pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* induce toxic levels of ROS to cause cell death, which promote infection (Govrin and Levine, 2000). A positive correlation between the pathogenicity and the intensity of ROS has been reported (Edlich et al., 1989). The inhibition of ROS formation in the host reduces susceptibility to *B. cinerea* (Govrin and Levine, 2000). Cell death resulting from HRs by incompatible pathogens has been shown to facilitate *B. cinerea* infection (Govrin and Levine, 2000). The expression of animal antiapoptotic genes in plants increases resistance to the necrotrophic pathogens *B. cinerea*, *Sclerotinia sclerotiorum*, and *Cercospora nicotianae* (Dickman et al., 2001). *C. nicotianae* produces a polyketide toxin, cercosporin. Cercosporin generates ROS such as singlet oxygen and the ROS induce cell death. The antiapoptotic gene *Bcl-2* interfered with cell death by promoting the scavenging of free radicals (Hockenbery et al., 1993). These results suggest that ROS may be involved in inducing susceptibility-related cell death.

4.8.3 Proteolytic Enzymes

Caspases, which regulate the hypersensitive cell death, may also be involved in regulation of susceptibility-related cell death. The baculovirus protein p35 is known to inhibit caspases during viral replication in insect cells. This inhibition prevents caspase-mediated cleavage of several substrates that are important for homeostasis, and thereby blocks death of the infected cells until viral replication is complete. The specificity of the p35 protein is resident in a tetrapeptide sequence, DQMD, which binds to the active site of the target caspase. It appears that the p35 protein is involved in a direct interaction with the active site of the target caspases and is a potent caspase-specific inhibitor (Zhou et al., 1998). The baculovirus p35 protein was exploited to prove that caspases are involved in the development of susceptibility-related cell death (Lincoln et al., 2002). Transgenic tomato plants bearing the *p35* gene were developed and these transgenic plants did not develop cell death during the pathogenesis of the fungal pathogen *A. alternata* f. sp. *lycopersici* and also showed resistance to the disease caused by the pathogen. The transgenic tomato plants also showed resistance to *Colletotrichum coccodes* and *Pseudomonas syringae* pv. *tomato*, besides *A. alternata* f. sp. *lycopersici* (Lincoln et al., 2002). Transgenic tomato plants expressing p35-binding site mutant (DQMD to DRIL), which is inactive against animal caspases, showed cell death and hence did not protect against AAL-toxin and the pathogen (Lincoln et al., 2002). The tetrapeptide caspase inhibitors, DEVD and YVAD, blocked cell death and completely

blocked compatible necrotrophic pathogens in tobacco (Richael et al., 2001). These results demonstrate that caspases are involved in the development of susceptibility-related cell death.

VPE exhibiting a caspase-1 activity appears to be a key molecule in FB1 toxin-induced cell death in Arabidopsis (Kuroyanagi et al., 2005). FB1-induced cell death was accompanied with disruption of vacuolar membrane followed by lesion formation. The features of FB1-induced cell death were completely abolished in the Arabidopsis VPE-null mutant, which lacks all four VPE genes of the genome (Kuroyanagi et al., 2005). An inhibitor of caspase-l abolished FB1-induced lesion formation, as did a VPE inhibitor. The VPE-null mutant had no detectable activities of caspase-1 or VPE in FB1-treated leaves (Kuroyanagi et al., 2005). Similar VPE-mediated cell death has been reported also in hypersensitive cell death (Hatsugai et al., 2004). It seems that VPE-mediated vacuolar mechanism is common to both susceptibility-related and resistance-related cell death in plants.

The oat Victoria blight pathogen *Cochliobolus victoriae* produces the toxin victorin. The toxin activates a PCD response in susceptible cultivars (Navarre and Wolpert, 1999). Victorin-induced PCD was shown to be associated with two caspase-like activities (Coffeen and Wolpert, 2004). One of these caspase-like enzymes was translocated to the apoplasm in response to victorin treatment, correlating with the induction of PCD by the victorin. This caspase was homologous to subtilin-like Ser proteases and hence it was named saspase (Coffeen and Wolpert, 2004).

The oat saspase is expressed constitutively. This saspase may be maintained inactive by its retention in the secretory pathway and may be activated by secretion to the apoplasm, where it may gain access to effector substrates that activate PCD. It is suggested that the oat saspase may cleave an LRR protein and activate a signaling cascade inducing PCD (Coffeen and Wolpert, 2004).

4.8.4 Calcium Ion

The susceptibility-related PCD may be mediated through cytosolic calcium. A basic endo-polygalacturonase (PG) isoform produced early by *Sclerotinia sclerotiorum* when infecting soybean seedlings induced calcium-mediated PCD in soybean cells (Figure 4.7; Zuppini et al., 2005). Within 1 h of PG treatment, a remarkable level of cell death was observed. Ca^{2+} level in cytosol increased rapidly. Cytochrome *c* release in the cytoplasm and activation of both caspase9-like and caspase3-like proteases were found in the soybean cells treated with the PG. When a polygalacturonase-inhibiting protein (PGIP) and the PG were simultaneously applied to cells, both the Ca^{2+} increase and cell death were annulled. The results suggested that Ca^{2+} might mediate the fungal PG-induced cell death (Zuppini et al., 2005).

4.8.5 Salicylate, Ethylene, and Jasmonate

Under some circumstances, cell death has been shown to be dependent on SA accumulation (Rate et al., 1999; Shah et al., 1999; Asai et al., 2000). The Arabidopsis mutant *acd6* showed elevated levels of dead cells. The *acd6*-conferred phenotypes were suppressed by removing SA using the nahG transgene (Rate et al., 1999). The fungal toxin FB1 induces PCD in wild-type Arabidopsis protoplasts. FB1, however, only marginally affected the viability of protoplasts isolated from the transgenic NahG plants, in which SA is metabolically degraded, and from *pad4-1* mutant plants, in which an SA amplication is impaired (Asai et al., 2000). FB1-induced PCD does not appear to require the SA signal transmitter NPR1, since protoplasts from *npr1-1* mutant showed PCD in response to FB1 (Asai et al., 2000).

Ethylene may be involved in susceptibility-related cell death. AAL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* causes interveinal cell death in tomato susceptible lines with morphological characteristics of apoptosis. The earliest changes in gene expression in

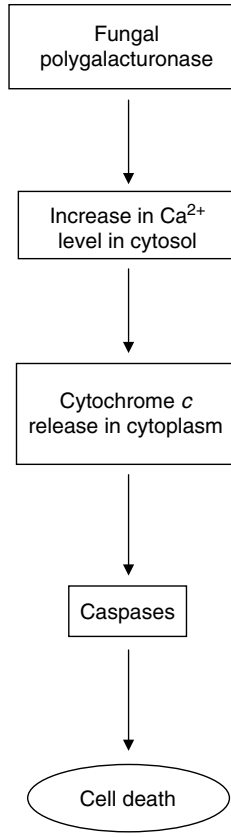

FIGURE 4.7 Induction of cell death by polygalacturonase (PG) of *Sclerotinia sclerotiorum*.

the cell death system in tomato were the changes in expression of genes encoding ACC (1-aminocyclopropane-1-carboxylic acid) synthase and ACC oxidase (Moore et al., 1999). The ACC synthase catalyzes the first committed step of ethylene biosynthesis, whereas ACC oxidase catalyzes the final step of ethylene biosynthesis. ACC synthase is encoded by nine genes whereas ACC oxidase is encoded by at least three in tomato. At 12 h of toxin treatment, the ACC synthase gene *LEACS2* mRNA was detected; no LEACS2 transcripts were detected in untreated tissue. The ACC oxidase mRNA was almost undetectable in untreated leaf tissue; but increased significantly after 6 h in AAL-toxin-treated tissue (Moore et al., 1999). By 24 h of toxin treatment, cell death was observed in the tomato leaflet (Moore et al., 1999). It suggests that induction of expression of genes encoding ACC synthase and ACC oxidase precedes the cell death in tomato.

An increase in the precursor of ethylene and the increased ethylene evolution were observed to precede the development of interveinal cell death in AAL-toxin-treated susceptible tomato isolines (Moussatos et al., 1994). The addition of inhibitors of ethylene biosynthesis or action significantly reduced the amount of dead tissue in susceptible tomato tissues inoculated with the pathogen *A. alternata* f. sp. *lycopersici* (Moussatos et al., 1994). *Never ripe* locus of the tomato mutant affects ethylene perception. The *Never ripe* mutant showed significantly less cell death in response to AAL-toxin (Moore et al., 1999). The fungal toxin FB1 induced cell death in Arabidopsis protoplasts. The protoplasts isolated from the *etr1-1* mutant plants, which are insensitive to ethylene, displayed reduced cell death in response to treatment with the fungal toxin FB1 (Asai et al., 2000). These results suggest that ethylene is involved in inducing cell death in susceptible interactions.

Besides ethylene, jasmonate may also be involved in signaling cell death. The protoplasts isolated from Arabidopsis mutant *jar1-1*, which is insensitive to jasmonate, showed reduced cell death in response to the fungal toxin FB1 treatment (Asai et al., 2000).

4.8.6 Sphingolipid Metabolism

Disruptions in sphingolipid metabolism have been suggested to be involved in susceptibility-related cell death in some plant–pathogen interactions (Abbas et al., 1994; Yoo et al., 1996; Brodersen et al., 2002; Spassieva et al., 2002; Liang et al., 2003). Ceramides and their phosphorylated derivatives are signaling molecules associated with apoptosis in animals (Hannun and Obeid, 2000). Sphingolipids are important signal molecules triggering cell death (Gechev and Hille, 2005). AAL-toxin produced by *A. alternata* f. sp. *lycopersici* and FB1 produced by *F. moniliforme* inhibit the central enzyme of ceramide biosynthesis, sphinganine *N*-acyltransferase (acyl-CoA-dependent ceramide synthase) (Wang et al., 1991; Spassieva et al., 2002). These toxins inhibit sphingolipid biosynthesis by competitive inhibition of ceramide synthase, which can lead to cell death (Eckardt, 2005). Several observations suggest that AAL-toxin inhibits ceramide synthase. Alternaria stem canker (*Asc1*) resistance gene, which confers resistance to AAL-toxin in tomato, has been suggested to be related to sphingoid metabolism (Brandwagt et al., 2000). Asc1 is a homolog of *Saccharomyces cerevisiae* LAG1, which has been associated with yeast life span. LAG1 functions to facilitate transport of glycosyl-phosphatidylinositol (GPI)-anchored proteins from the endoplasmic reticulum (ER) to the Golgi apparatus. Sphingolipids and GPI-anchored proteins are both major components of lipid rafts, which are involved in membrane trafficking and endocytosis (Eckardt, 2005). The function of Asc1 was similar to LAG1 and prevented cell death by restoring ER-to-Golgi transport of GPI-anchored proteins (Brandwagt et al., 2000).

A fine balance of sphingolipids determines the induction of cell death; both depletion and accumulation of ceramides trigger cell death (Liang et al., 2003). In Arabidopsis, C2 ceramide treatment induced PCD and ceramide was found to be sufficient to induce cell death. The phosphorylated derivative of ceramide, ceramide-1-phosphate, was able to partially abrogate the cell death-inducing effects of ceramide (Liang et al., 2003). These results suggest that the balance between ceramide and its phosphorylated derivative modulates the amount of PCD in plants.

4.8.7 Extracellular ATP Levels

It appears that maintenance of extracellular adenosine triphosphate (ATP) levels is essential for cell viability, and depletion of extracellular ATP induces cell death in a number of plant species (Chivasa et al., 2005). Treatment of Arabidopsis cell cultures with the fungal FB1 toxin resulted in rapid depletion of extracellular ATP (Chivasa et al., 2005). Exogenous application of ATP significantly attenuated the FB1-induced cell death response. Intact ATP has been shown to be necessary for this effect, as ADP, AMP, or inorganic phosphate had no effect (Chivasa et al., 2005).

4.9 WHAT IS THE FUNCTION OF CELL DEATH IN FUNGAL PATHOGENESIS?

Cell death is a common phenomenon observed during pathogenesis in both susceptible and resistance reactions. Hypersensitive cell death is a common feature of many resistance reactions. It is well demonstrated that HR cell death is seen in the region where growth of the pathogen is halted. It is generally agreed that cell death assists in the retardation of

pathogen proliferation within the host (Lam et al., 2001). Cell death in resistance-related reactions may restrict invasion of fungal pathogen beyond the point where cell death occurs (Morel and Dangl, 1997; Heath, 1998). It is suggested that HR-related cell death removes the substrate for growth of biotrophic pathogens. Cell death may deprive pathogens of water and nutrients. Biotrophic pathogens that require living hosts may be very much affected by rapid cell death (Richael and Gilchrist, 1999). Dying cells may release toxic compounds upon self-destruction (Richael and Gilchrist, 1999) and inhibit growth of pathogen beyond the HR region.

However, in many cases, resistance could be separated from cell death. There are many resistant plant–pathogen interactions, without exhibiting any visible hypersensitive cell death (Richael and Gilchrist, 1999). The resistance-related hypersensitive cell death is generally observed in race-specific qualitative resistance reactions; again it is not without exceptions. Under high humidity conditions, the race-specific resistance genes of tomato, *Cf* genes, confer resistance to *Cladosporium fulvum* strains without invoking an HR cell death (Hammond-Kosack et al., 1996).

It is still not clear whether the hypersensitive cell death is an important defense response of plants to infection (Lam et al., 2001). In some plant–pathogen interactions, hypersensitive cell death may not be necessary to trigger disease resistance. In these cases, resistance is maintained and pathogen proliferation is checked even when HR cell death is blocked (Cawly et al., 2005). The *dnd1* (defense with no death1) mutation in Arabidopsis results in loss of HR without loss of disease resistance (Yu et al., 1998). The *dnd1* mutant expresses resistance to pathogens without invoking an HR (Clough et al., 2000). The *dnd2* mutation in Arabidopsis results in loss of HR cell death without loss of gene-for-gene resistance (Jurkowski et al., 2004). In some mutant plants, resistance may be lost, although the mutant plants express HR cell death. In Arabidopsis, *ndr1* mutant plants retained an HR initiated by two *R* genes, *RPM1* and *RPS5*, even though they failed to prevent the pathogen growth, suggesting that resistance and HR cell death are separable (Century et al., 1995). Cell death could be uncoupled from resistance in the HR of many plant–pathogen interactions (Cawly et al., 2005).

It is still not clear whether the HR cell death is important defense response of plants to infection or whether it is simply invoked as part of a more generalized response to pathogens, regardless of its role in disease response (Lam et al., 2001). It is not yet known whether the failure of a pathogen to extend invasion beyond the point where cell death is observed is due to host cell death or whether the spread of pathogen is arrested before the affected host cells die and death is an anticlimax (Morel and Dangl, 1997; Heath, 1998, 2000; Richael and Gilchrist, 1999).

It appears that dying cells may be able to release signals to trigger local and systemic resistance (Lamb and Dixon, 1997). Diffusible signals eliciting *PR* gene expression in cells neighboring HR cell death have been reported in many cases (Samac and Shah, 1991; Levine et al., 1994; Chappell et al., 1997). ROS accumulation occurred at the margins of lesions in Arabidopsis (Stone et al., 2000). It is possible that the cell death response may reinforce or stimulate the induction of defenses by activating local and SAR (Staskawicz et al., 1995; Ryals et al., 1996; Alvarez et al., 1998). HR cell death may provide signals to induce resistance beyond the infection zone. The localized necrosis can induce a SAR, which heightens defenses in uninoculated tissues against a broad spectrum of pathogens (Rustérucci et al., 2001).

Cell death may cause opposite effects on the outlines of biotrophic versus necrotrophic plant–pathogen interactions (Takemoto et al., 2005). An elicitor-induced (elicitin from *Phytophthora* sp.) cell death in *Brassica rapa* conferred susceptibility to *Alternaria brassicicola* (a necrotroph), whereas the elicitor-induced cell death in *Nicotiana* sp. showed less susceptibility to *Phytophthora nicotianae* (a hemibiotroph) (Takemoto et al., 2005). Cell death may

provide nutrients for the necrotrophic pathogens (Govrin and Levine, 2000), whereas it may prevent the supply of nutrients from living cells to the biotrophic/hemibiotrophic pathogens (Takemoto et al., 2005).

4.10 CONCLUSION

PCD is the common phenomenon observed in plants during fungal pathogenesis. The dying cells show typical cell corpse morphology, and similar apoptosis has been found in mammals also. The PCD has been detected in plants infected with both compatible and incompatible pathogens. Several mediators, regulators, and executioners of cell death have been identified. *R* gene products, elicitors, calcium ions, ion channels, calmodulins, Ca^{2+}/calmodulin-regulated NAD kinase, G proteins, alterations in cellular redox state, protein kinases, MAPKs, ROS, NO, cGMP, cADPR, and ubiquitin conjugating enzymes have been suggested to mediate cell death.

Bax family of proteins regulates the cell death programs. They may form channels in the mitochondrion, through which cytochrome *c* and other AIFs may be released in the cytoplasm. Caspases may function as molecular switches to activate the cell death program. Both initiator caspases and executioner caspases may take part in the death programs; the initiator caspases may activate downstream executioner caspases in this process. Various other proteolytic enzymes also regulate cell death.

Ethylene, jasmonate, SA, SA regulatory proteins EDS1 and PAD4, and SA signal transducer NPR1 are other key regulators of cell death. Sphingolipids may be the signaling molecules triggering cell death. Depletion of extracellular ATP induces cell death in some plants. Both ROS (superoxide anion, H_2O_2, singlet oxygen) and reactive nitrogen species (NO) are directly toxic to plant cells. Some caspases may directly execute cell death. Thus several factors have been reported to be involved in both resistance-related and susceptibility-related PCD. However, the exact sequence of mechanism of induction of cell death under different conditions is not yet known.

The cell death programs appear to be very important in disease or resistance development. The function of cell death in susceptible and resistant interactions may be distinctly different. Although the hypersensitive cell death triggers disease resistance by suppressing invasion of fungal growth, the susceptibility-related cell death enhances the disease development. The same type of cell death may induce resistance against some pathogens and susceptibility against another group of pathogens. Cell death may confer resistance against biotrophic pathogens, which require living hosts for their nutrition. By contrast, cell death may confer susceptibility to necrotrophic pathogens, which can produce toxins and cell wall degrading enzymes to obtain nutrients even from dying and dead cells. Although dying cells in the hypersensitive cell death program may signal induction of defense genes and accumulation of toxic chemicals, the toxins and enzymes produced by the necrotrophic pathogens during the cell death program may degrade and suppress the accumulation of defense gene products. It appears that regulation of cell death is crucial to determine the outcome of plant–fungal pathogen interactions, susceptibility, or disease resistance.

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5 Cell Wall Degradation and Fortification

5.1 INTRODUCTION

Plant cell wall is the first barrier and penetration of the cell wall appears to be the first requirement for the pathogenesis of fungal pathogens (Thordal-Christensen, 2003; D'Ovidio et al., 2004). The cell walls are complex amalgams of carbohydrates (cellulose, hemicellulose, and pectic polysaccharides), proteins, lignin, and incrusting substances such as cutin, suberin, and certain inorganic compounds (Showalter, 1993; Chacón-Martínez et al., 2004). Cuticle is the incrusting substance and it forms the first layer covering the outer walls of epidermal cells (Gevens and Nicholson, 2000). Fungal pathogens are able to produce a variety of enzymes degrading the plant cell wall, and these enzymes help the pathogens in penetration and colonization of their host plants (Chacón-Martínez et al., 2004; Herbert et al., 2004; Espino et al., 2005; Yang et al., 2005). The degraded wall components may signal the bulk production of these cell wall–degrading enzymes and aggravate the disease incidence (Kemp et al., 2004; Chassot and Metraux, 2005).

When the pathogen attempts to penetrate the host cell wall by producing a series of enzymes, the host plant tries to fortify its cell wall with papilla formation (Gjetting et al., 2004) and incrustation with callose (Soylu et al., 2004; He and Wolyn, 2005; Hamiduzzaman et al., 2005), lignin (Zheng et al., 2005), wall-bound phenolics (de Ascensao and Dubery, 2003), suberin (Chen et al., 2004b), and minerals (Ghanmi et al., 2004). The cell wall degradation products also trigger host defenses (D'Ovidio et al., 2004; Hammerschmidt, 2004). The successful pathogens overcome all these defense barriers and cause infection. A delicate balance exists between susceptibility and resistance, and modulation of production of cell wall–degrading enzymes by the pathogens determines disease or disease resistance development (Kemp et al., 2004; Wegener and Olsen, 2004). Plant cell walls may participate in the molecular dialogue between plants and pathogens (Hammerschmidt, 2004; Kemp et al., 2004; Zuppini et al., 2005). This chapter describes cell wall degradation and fortification events during fungal pathogenesis.

5.2 STRUCTURE OF CUTICLE

Cuticle is a hydrophobic layer of material that covers much of the aerial plant surface and is composed most notably of a polymer of cutin interspersed with waxes (Gevens and Nicholson, 2000). The waxes are complex mixtures of materials composed of hydrocarbons, alcohols, ketones, C_{16} and C_{18} fatty acids, mono- and dicarboxylic acids of both saturated and unsaturated types (the alcohols and acids occur free or as long-chain esters), acid estolides (interesterification of ω-hydroxy fatty acids and α- and ω-diols), terpenoids, and a variety of phenols including various flavonoids and sometimes phenylpropanoids

(Köller, 1991; Walton, 1994; Gevens and Nicholson, 2000; Jenks et al., 2002; Broun et al., 2004). Hentriacontane, hexacosanol, and octacosanol are predominant in pea leaf cuticular waxes (Gniwotta et al., 2005).

Cutin is composed of mainly two families of monomers namely a C_{16} family and a C_{18} family. The most predominant component of the C_{16} family is 10,16-dihydroxypalmitic acid and its positional isomers in which the mid-chain hydroxyl group is at C-9, C-8, or C-7; in most cases one isomer predominates. Smaller quantities of 16-hydoxypalmitic acid and palmitic acid are also found in most cases. Major components of the C_{18} family of monomers are 18-hydroxyoleic acid, hydroxy-9,10-epoxystearic acid, and threo-9,10,18-trihydroxystearic acid together with their analogs, containing an additional double bond at C-12 (Kolattukudy, 1981, 2001; Kolattukudy et al., 1987).

Suberin is another polyester that has many of the same components as cutin, including hydroxy fatty acids, alcohols, C_{16} and C_{18} fatty acids, and mono- and dicarboxylic acids. In addition to these components, suberin consists of phenylpropanoid phenols and sometimes, phenolic polymers such as lignin (Gevens and Nicholson, 2000).

5.3 PENETRATION OF EPICUTICULAR WAXY LAYER BY PATHOGENS

Epicuticular wax is the first barrier in the cell wall. Rust fungi (*Puccinia graminis* f. sp. *hordei* and *P. recondita*) failed to form appressoria on certain genotypes of *Hordeum chilense* because of the presence of a prominent waxy layer. Removal of this layer increased appressorium formation on *H. chilense* accessions studied (Rubiales and Niks, 1996). It has been reported that surface wax of nonhost plants suppressed the development of the avocado pathogen *Colletotrichum gloeosporioides* (Podila et al., 1993). Virulent pathogens may be able to penetrate the waxy layer. Although the mechanism of degradation of the waxes by the pathogens has not been studied in detail (Kolattukudy, 1985), utilization of these waxes by the fungal pathogens for their growth and sporulation has been reported. The major components of the lipid extract from urediniospores of wheat stripe rust, *Puccinia striiformis*, are β-diketones, *n*-alcohols, and hydrocarbons (Jackson et al., 1973). The surface lipid extract of the host plant wheat had a composition that was qualitatively similar to that found in the fungal urediniospores. Comparison of the β-diketone and free alcohol compositions of both the host plant and the pathogen indicated that these were produced by the host and transferred to urediniospore (Jackson et al., 1973). The presence of the same wax components in the host and the pathogen indicates that the pathogen would have used the host's wax for its growth. Surface wax of avocado plants induced germination and appressorium formation of *C. gloeosporioides* (Podila et al., 1993). The pea cuticular waxes trigger germination and appressorium development of spores of *Erysiphe pisi* (Gniwotta et al., 2005).

5.4 PRODUCTION OF CUTINASES TO BREACH CUTICLE BARRIER

Several fungal pathogens have been shown to penetrate the cuticle barrier without producing any specialized infection structures (Kolattukudy, 1985; Davies et al., 2000), and they penetrate by producing enzymes capable of degrading the cuticle (Tanabe et al., 1988a; Trail and Köller, 1990; Köller et al., 1991; Kolattukudy et al., 1995; Van Kan et al., 1997; Davies et al., 2000). Several esterases and lipases show cutinolytic activity (Comménil et al., 1999; Kolattukudy, 2001). Penetration of brassica hosts by the light leaf spot pathogen *Pyrenopeziza brassicae* occurs as a result of the production of an esterase that exhibits cutinase activity (Davies et al., 2000). An inducible lipase isolated from *Botrytis cinerea* was shown to have cutinolytic activity (Comménil et al., 1998, 1999).

The cutinase produced by *C. gloeosporioides* catalyzed hydrolysis of *p*-nitrophenyl esters of C_4, C_6, C_8, C_{10}, C_{12}, C_{14}, and C_{16} fatty acids at comparable rates (Dickman et al., 1982),

but cutinase from *Fusarium solani* f. sp. *pisi* could not hydrolyze the longer-chain esters (Lin and Kolattukudy, 1980). Cutinases from *Magnaporthe grisea*, *Colletotrichum capsici*, *C. gloeosporioides*, and *F. solani* f. sp. *pisi* contain a conserved region (–leu–glu–ala/thr–arg–gln–leu/ser–) in close vicinity to the putative signal cleavage site (Ettinger et al., 1987; Sweigard et al., 1992b). The conserved sequence found in the cutinases from these fungi was lacking in *Alternaria brassicicola* cutinase (Yao and Köller, 1994). The N-terminus of *A. brassicicola* cutinase contained a short hydrophobic core, followed by more polar residues and several short and neutral amino acids (–ala–ser–thr–thr–) as putative signal cleavage sites (Yao and Köller, 1994).

There was variation in cutinase produced by a single fungal species also. Two RFLP-defined distinct populations of *C. gloeosporioides* isolated from citrus have been reported. Both populations of the strains produced esterases with cutinolytic activity. One population of the strains secreted 26 and 19 kDa esterases, whereas the second population of strains secreted 24 and 21 kDa esterases (Liyanage et al., 1993).

Optimal pH for hydrolysis of the polymer cutin varied remarkably between cutinases isolated from the fungal pathogens infecting leaf or stem or both. Alkaline pH optima have been reported for the cutinases of pathogens such as *F. solani* f. sp. *pisi* (Purdy and Kolattukudy, 1975), *Fusarium roseum* f. sp. *culmorum* (Soliday and Kolattukudy, 1976), *F. solani* f. sp. *phaseoli* (Baker and Bateman, 1978), and *Pythium ultimum* (Baker and Bateman, 1978), which infect stems and roots only. *Rhizoctonia solani*, which infects stem base of bean, has been shown to produce a cutinase with a pH optimum of 8.5 (Trail and Köller, 1990).

Acidic pH optima have been reported for pathogens infecting leaves. Cutinase from *Botrytis cinerea* had a pH optimum of 6.5 (Salinas et al., 1986). Cutin hydrolysis by cutinase of the corn leaf pathogen, *Cochliobolus heterostrophus*, was optimal at pH 6.5 (Trail and Köller, 1990). The pathogens infecting both stem and leaf (besides other organs) produce cutinases with both acidic and basic pH optima. *A. brassicicola*, the pathogen of *Brassica olearaceae*, infects both leaves and stems, and its cutinase shows two distinct pH optima, namely 7.0 and 9.0 (Trail and Köller, 1990). In fact, two distinct forms of cutinase have been detected in culture filtrates of *A. brassicicola* (Trail and Köller, 1990). The two cutinases A_c and B_a were purified to apparent homogeneity. Cutinase A_c had a cutinolytic pH optimum of 6.5 and cutinase B_a had a cutinolytic pH optimum of 8.5. Their molecular weights were 23.0 and 21.0 kDa, respectively. Amino acid compositions of them were similar but not identical (Trail and Köller, 1993). *M. grisea*, the rice blast pathogen, produces two different cutinases with pH optima of 6.0 and 9.0, and this pathogen is known to cause leaf blast and neck rot (Sweigard et al., 1992a).

Inoculum from the stem-specific isolate of *R. solani* nonpathogenic on leaves was amended with cutinase purified either from a leaf pathogen *Venturia inaequalis* or from a stem pathogen *F. solani* f. sp. *pisi* (Trail and Köller, 1990). Inoculum amended with cutinase from the leaf-specific pathogen penetrated the bean cuticle and produced disease symptoms, whereas inoculum amended with cutinase from the stem-base pathogen remained nonpathogenic on bean leaves (Trail and Köller, 1990). These studies revealed that several types of cutinases might be involved in fungal pathogenesis.

5.5 GENES ENCODING CUTINASES

Several genes encoding cutinases of fungal pathogens have been cloned (Yao and Köller, 1994; Van der Vlugt-Bergmans et al., 1997; Davies et al., 2000). Cutinase cDNA has been cloned from *Fusarium solani* f. sp. *pisi*. The cutinase mRNA was 1,050 nucleotides in length. An initiation codon and a termination codon were found 702 nucleotides apart in the sequence. This reading frame translated into a protein with a molecular weight of 23,951 Da

(Soliday et al., 1984). Cutinase genes from *Colletotrichum capsici* and *C. gloeosporioides* have also been cloned and sequenced. Sequence analysis of 5′-flanking region showed that all three cutinase genes from *C. capsici*, *C. gloeosporioides*, and *F. solani* f. sp. *pisi* lacked consensus TATAA box, whereas they have a TAAATA box. Although *C. capsici* and *C. gloeosporioides* had a CAAT box in the 5′-flanking region, *F. solani* f. sp. *pisi* had no CAAT box. In *F. solani* f. sp. *pisi* there was a CAAG sequence. All three genes from *C. capsici*, *C. gloeosporioides*, and *F. solani* f. sp. *pisi* share consensus core sequence CAGAC associated with transcriptional start site (Ettinger et al., 1987). The promoter for cutinase gene from *F. solani* f. sp. *pisi* was present within 360 bp in the 5′-flanking region (Soliday et al., 1989). An inducible promoter has been identified for the cutinase gene of *F. solani* f. sp. *pisi* (Bajar et al., 1991). Two distinct populations of *Colletotrichum gloeosporioides* have been isolated from citrus. A DNA probe containing the cutinase gene from an isolate of *C. gloeosporioides* hybridized strongly to DNA from the second population of strains and not at all to the first population. The results suggest that distinct cutinase genes may be present in the two types of *C. gloeosporioides* populations (Liyanage et al., 1993).

A cutinase gene (*CUT1*) from *Magnaporthe grisea* has also been cloned (Sweigard et al., 1992b). This gene showed strong sequence similarity to other cutinase genes (Sweigard et al., 1992b). The gene encoding cutinase has been cloned from *Pyrenopeziza brassicae* (Davies et al., 2000). A cutinase gene from *Alternaria brassicicola* has been cloned and characterized (Yao and Köller, 1994). The cutinase gene was designated *CUTAB1*. The predicted size of the *Alternaria* cutinase was 22.4 kDa of the full-length protein and 20.7 kDa without the putative signal peptide. The total nucleotide sequence was 984 bp long with an open reading frame encoding a protein of 209 amino acids (Yao and Köller, 1994). The cutinase gene *cutA* has been cloned from *Botrytis cinerea*. Expression of the gene was detected from the onset of conidial germination and during penetration into epidermal cells of gerbera and tomato (Van Kan et al., 1997).

A cDNA clone of the cutinase gene from a strain of *Colletotrichum capsici* hybridized with genomic DNA from representative strains of *C. graminicola*, *C. gloeosporioides*, and *C. lindemuthianum* but not with DNA from strains of *C. lagenarium* or *C. coccodes* (Ettinger et al., 1987). Comparison of the DNA sequences for cutinase gene of *C. gloeosporioides* and a gene isolated from a strain of *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*) revealed considerable dissimilarity (Ettinger et al., 1987). These studies indicated that different genes may encode various cutinases produced by pathogens.

5.6 PLANT SIGNALS TRIGGERING FUNGAL CUTINASES

Fungal pathogens constitutively produce small amount of cutinase, but bulk of cutinase produced is only inducible by cutin monomers. A constitutive cytoplasmic cutinase has been reported in ungerminated conidia of *Botrytis cinerea* (Gindro and Pezet, 1997). However, spores of *Fusarium solani* f. sp. *pisi* generated cutinase only when cutin was present in the medium (Woloshuk and Kolattukudy, 1986). Cutinase could not be detected in the fungal cultures grown in cutin monomer-free medium (Flurkey and Kolattukudy, 1981). Host cutin is insoluble polymer (Lin and Kolattukudy, 1978). If cutin is used to induce fungal cutinase, the insoluble polymer should penetrate the fungal cell. Unless the insoluble polymer is converted into soluble monomer, it is not possible. Hence, the fungal pathogen should secrete some basal level of cutinase even in the absence of cutin monomer. Köller et al. (1982a) have demonstrated that the germinating spores of *F. solani* f. sp. *pisi* released a small quantity of cutinase. This small amount of cutinase may generate soluble cutin monomers, which may signal the bulk production of cutinase (Kolattukudy et al., 1989).

The genes encoding constitutive cutinase and inducible cutinase have been cloned from *F. solani* f. sp. *pisi*. The *cut2* gene encodes the constitutive cutinase, whereas *cut1* encodes the inducible enzyme (Li et al., 2002). It is suggested that the constitutive expression of *cut2* that causes production of low levels of cutin monomers may strongly induce *cut1* (Li et al., 2002). A palindrome binding protein (PBP) binds to palindrome 1 of *cut1* promoter. The PBP may interfere with the binding of CTF1α, the transcription factor involved in induction, to *cut1* promoter and thus keep *cut1* gene repressed until induced by cutin monomers (Li et al., 2002). PBP is not able to bind palindrome 1 of *cut2*, and hence this gene is not repressed. A Cys_6Zn_2 motif-containing transcription factor, CTF1β, that binds palindrome 2 transactivates *cut2* promoter. The CTF1β is involved in constitutive expression of *cut2*, which causes production of low levels of cutin monomers that strongly induce *cut1* using CTF1α as the transcription factor (Li et al., 2002).

Expression of cutinase gene was observed in conidial postgermination stage in the tomato powdery mildew pathogen *Oidium neolycopersici*, when the pathogen came in contact with the tomato cuticle (Matsuda et al., 2005). It suggests that the plant might have signaled induction of fungal cutinases. The most common host cutin monomers, which may act as signals, are 10,16-dihydroxyhexadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid (Woloshuk and Kolattukudy, 1986). Messenger RNA for cutinase could be detectable within 15 min after spores of *F. solani* f. sp. *pisi* were exposed to cutin (Woloshuk and Kolattukudy, 1986). When nuclei isolated from *F. solani* f. sp. *pisi* culture after different periods of induction with 10,16-dihydroxyhexadecanoic acid were incubated with (^{32}P) UTP, the amount of label incorporated into cutinase transcripts increased with increasing periods of induction. When nuclei isolated from uninduced (glucose-grown) culture of *F. solani* f. sp. *pisi* were incubated with (^{32}P) UTP, little label was incorporated into cutinase transcripts (Kolattukudy et al., 1989). A protein factor also could be isolated from *F. solani* f. sp. *pisi* mycelium, which triggered events that are required for the selective activation of transcription of cutinase gene (Podila et al., 1988). When both the host cutin monomer and the fungal protein factor were added together with the nuclei a major increase in the incorporation of label into cutinase transcript was observed. Neither the protein extract nor the cutin monomer alone had any effect on transcription of cutinase gene (Kolattukudy et al., 1989). These results suggest that the pathogen would have evolved an effective mechanism to sense its contact with the plant surface via the cutin monomers and consequently turn on the genes necessary to penetrate the defensive barrier of the plant.

5.7 IMPORTANCE OF CUTINASES IN PENETRATION OF CUTICLE

The importance of cutinases in penetration of cuticle has been demonstrated in many plant–pathogen interactions. The tomato pathogen *Botrytis cinerea* produces a lipase enzyme with cutinolytic activity. An antilipase antibody was able to prevent penetration of *B. cinerea* on tomato plants, suggesting the importance of this enzyme in penetration of cuticle barrier (Comménil et al., 1998).

The importance of cutinases in cuticle penetration has been demonstrated by using cutinase inhibitors. When spores of *Venturia inaequalis* were applied to apple leaves in the presence of the cutinase inhibitor *O*-methyl-*O*-butyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphate, subcuticular growth of the fungus was prevented (Köller et al., 1991). Ebelactone B is an inhibitor of cutinase and it was shown to have a preventive effect on rice sheath blight caused by *Rhizoctonia solani*. This effect was shown to be a direct result of inhibition of cutinase rather than a toxic effect of the ebelactone, since spores sprayed onto wounded surfaces in the presence of the ebelactone caused infection (Chun et al., 1995). When ebelactones, the cutinase inhibitors, were added to spore suspensions of *Pyrenopeziza brassicae* before inoculation of oilseed rape (*Brassica napus*) leaf discs, the proportion of leaf discs showing no

symptoms was increased in the ebelactone-treated samples in comparison with the controls. There were fewer penetrating germ tubes in ebelactone-treated samples compared with that in untreated leaf disc samples (Davies et al., 2000). These results show that cutinases are important to facilitate cuticle penetration.

Diisopropylfluorophosphate (DFP), a serine hydrolase inhibitor, completely inhibited *C. gloeosporioides* cutinase. Rabbit antibodies prepared against *C. gloeosporioides* cutinase (anticutinase) also inhibited the cutinase enzyme activity. Both the anticutinase and DFP showed marked suppression of lesion formation in *C. gloeosporioides*-inoculated papaya fruits (Dickman et al., 1982). DFP and a variety of organophosphates inhibit cutinase produced by *F. solani* f. sp. *pisi*. These organophosphates prevented pathogenic ingress in pea tissues without affecting fungal development (Köller et al., 1982b). These results indicate that cutinases are important in pathogenesis of various fungal pathogens.

The importance of cutinases in degradation of cuticle has been demonstrated by developing transgenic plants. Transgenic *Arabidopsis* plants expressing a cutinase from the fungal pathogen *Fusarium solani* f. sp. *pisi* were developed. These plants expressing the cutinase in the extracellular space showed an altered ultrastructure of the cuticle and enhanced permeability. These results suggest that the fungal cutinase is involved in degradation of cuticle (Sieber et al., 2000).

5.8 CUTINASES AS VIRULENCE/PATHOGENICITY FACTORS

Virulence of some of the pathogens has been found to be related to their ability to produce cutinases. Virulence of the isolates of the pea pathogen *F. solani* f. sp. *pisi* was related to their ability to produce cutinase (Köller et al., 1982a). Isolate T-8 was highly virulent whether or not the stem was wounded. On the other hand, isolate T-30 expressed high virulence only when the stem surface was wounded; but very less infection occurred when the stem surface was intact. The isolate T-30 was as virulent as T-8 when the cuticle–cell wall barrier was absent, but it was almost avirulent when the intact barrier layer was present. T-30 produced less cutinase than T-8 during germination (Table 5.1; Köller et al., 1982a), suggesting a role for cutinase in virulence of the isolates of *F. solani* f. sp. *pisi*.

Cutinase-deficient mutants of the pea pathogen *F. solani* f. sp. *pisi* (Dantzig et al., 1986), the papaya pathogen *C. gloeosporioides* (Dickman and Patil, 1986), and the pear pathogen *Alternaria alternata* (Tanabe et al., 1988a,b) showed reduced pathogenicity. Introduction of the cutinase gene into a cutinase-less mutant of *F. solani* restored pathogenicity (Kolattukudy et al., 1989). *Mycosphaerella* sp. can infect papaya fruits only when the cuticular barrier is

TABLE 5.1
Relationship between Cutinase Activity and Virulence of the Pea Pathogen *Fusarium solani* f. sp. *pisi*

		Degrees of Virulence	
***F. solani* f. sp. *pisi* Isolate**	**Cutinase Activity in Units**	**Intact Stem Surface**	**Wounded Surface**
T-8	2,610,000	+++	+++
T-30	3,100	+	+++

Source: Adapted from Köller, W., Allan, C.R., and Kolattukudy, P.E., *Physiol. Plant Pathol.*, 20, 47, 1982a.

Note: + to +++ indicate increased degrees of virulence.

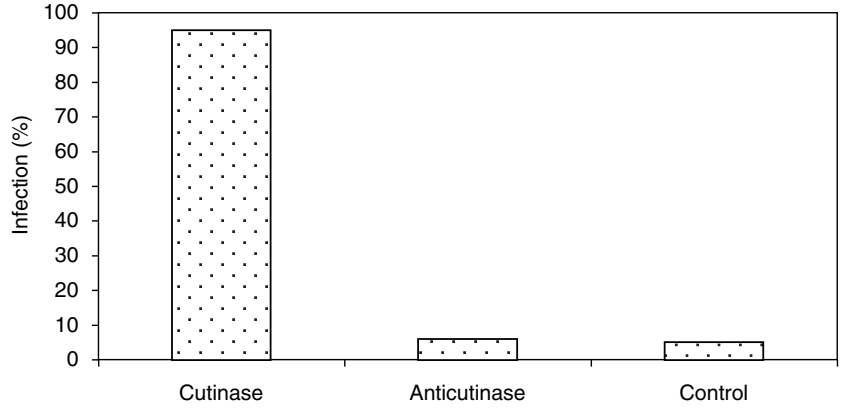

FIGURE 5.1 Effect of cutinase on infection by *Mycosphaerella* sp. (Adapted from Dickman, M.B., Patil, S.S., and Kolattukudy, P.E., *Phytopathology*, 73, 1209, 1983.)

mechanically breached. Introduction of the cutinase gene from *F. solani* into the wound pathogen *Mycosphaerella* sp. rendered transformants pathogenic on intact surfaces (Dickman et al., 1989).

When the conidia of avirulent isolate T-30 of *F. solani* f. sp. *pisi* were supplemented with cutinase isolated from virulent isolate of *F. solani* before inoculation of intact pea-stem surfaces, the conidia caused severe infection (Köller et al., 1982a). *Mycosphaerella* sp. is a fungal pathogen of papaya, which can enter the fruit only through wounds. However, a very high frequency of infection was observed when the surface of fruit was pretreated for 6 h with purified *C. gloeosporioides* cutinase before inoculation. When the cutinase was inactivated with anticutinase before treating the fruit, *Mycosphaerella* sp. could not cause infection (Figure 5.1; Dickman et al., 1983). These results showed that cutinase plays an important role in pathogenesis of some pathogens. Some pathogens do not produce cutinase but still they penetrate the cuticle efficiently. Other mechanisms such as mechanical pressure may also be involved in cuticle penetration.

5.9 MELANINS IN FUNGAL PENETRATION OF CUTICLE BARRIER

5.9.1 Biosynthesis of Melanins

Some fungal pathogens may penetrate the cuticle barrier by mechanical means, through the formation of specialized infection structures without any necessity for enzymatic digestion (Howard et al., 1991). High turgor pressure of appressoria generated by melanization has been shown to be involved in coffee cuticle penetration by the pathogen *Colletotrichum kahawae* (Chen et al., 2004b). Unmelanized appressoria induced by tricyclazole treatment showed turgor pressures as low as one-quarter of melanized ones and, as a consequence, the percentage of infection on leaves and green berries by the pathogen was much lower. Cutinase was present in conidial mucilage and in extracellular fluids of germinated conidia of the fungus *in vitro* and in planta. Cutinase was induced by growing the fungus in Czapek-Dox medium if cutin was used as the sole carbon source. DFPs, a cutinase inhibitor, totally abolished cutinase activity of culture filtrates and extracellular fluids but did not prevent infection (Chen et al., 2004a). These results suggest that the turgor pressure induced by melanins in appressoria of *C. kahawae* might play a major role in coffee cuticle penetration.

Several other fungal pathogens penetrate cuticle through mechanical force generated by the appressorium, and melanin provides the necessary strength and rigidity in the appressorial

wall to confine and focus this force (Howard et al., 1991; Talbot, 2003). Several reports have indicated that melanin biosynthesis is required for the penetration of host plant cell walls by appressoria (Kubo and Furusawa, 1986; Kubo et al., 1987). 1,8-Dihydroxynaphthalene (DHN) is a fungal melanin precursor. DHN melanin is produced at detectable levels in a variety of fungal cell types but the pigment is produced most abundantly just before penetration when a thick melanin layer is deposited in the inner appressorial cell wall (Kumura et al., 1990; Butler et al., 2005). DHN melanin mediates the build up of hydrostatic pressure in the appressorium, and this high pressure provides the essential driving force for a mechanical penetration component (Howard and Ferrari, 1989).

The melanins contain protein, carbohydrate, and lipid moieties as well as polymeric nucleus containing quinone, hydroquinone, and semiquinone moieties. The latter polymer is the chromophore (Bell and Wheeler, 1986). Fungal melanins are classified into four types: DHN melanin derived from pentaketide, β-3,4-dihydroxyphenylalanine (DOPA) melanin derived from tyrosine, catechol melanin derived from catechol, and γ-glutaminyl-3,4-dihydroxybenzene (GDHB) melanin (Bell and Wheeler, 1986). In all cases, these phenolic compounds are oxidized enzymatically to quinones, which polymerize by nonenzymatic means to form melanin pigments. Oxidation of these phenolic compounds is commonly catalyzed by tyrosinase (Bell and Wheeler, 1986).

In the Ascomycota and Mitosporic Fungi, the melanins in cell walls are synthesized from the pentaketide pathway. For example, the DHN melanin in *Alternaria alternata* is synthesized through the pentaketide pathway, which involves the condensation and cyclization of acetate to form 1,3,6,8-tetrahydroxynaphthalene (THN), followed by alternating reduction and dehydration reactions to form 1,8-DHN, which finally undergoes oxidative polymerization to form melanin (Figure 5.2; Carzaniga et al., 2002).

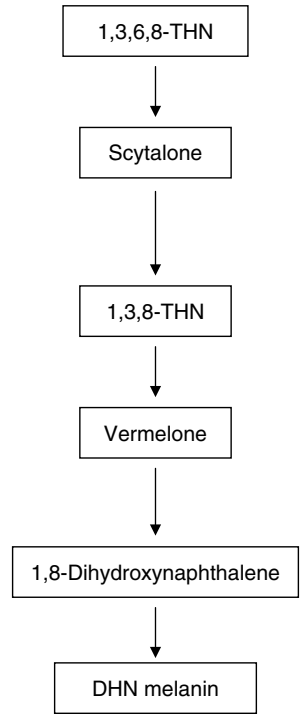

FIGURE 5.2 Melanin biosynthesis pathway in *Alternaria alternata*. (Adapted from Carzaniga, R., Fiocco, D., Boroyer, P., and O'Connell, R.J., *Mol. Plant Microbe Interact.*, 15, 216, 2002.)

1,3,6,8-THN, (+)Scytalone, 1,3,8-THN, vermelone, and 1,8-DHN are main intermediates in the melanin pathway commonly found in a wide range of economically important plant pathogens such as species of *Verticillium*, *Alternaria*, *Cochliobolus*, *Colletotrichum*, *Magnaporthe*, and *Gaeumannomyces* (Wheeler, 1982; Kubo and Furusawa, 1991; Tanabe et al., 1995; Henson et al., 1999; Carzaniga et al., 2002; Riźner and Wheeler, 2003; Takagaki et al., 2004). 1,3,8-Trihydroxynaphthalene reductase gene (*THR1*) has been cloned from *Bipolaris oryzae*, and this gene has been shown to be essential for melanin biosynthesis (Kihara et al., 2004). Two trihydroxynaphthalene reductase genes (*THN1* and *THN2*) have been cloned from the sap-staining fungus *Ophiostoma floccosum* (Wang and Breuil, 2002). A THN reductase has been shown to be involved in melanin biosynthesis in *Magnaporthe grisea* (Thompson et al., 2000). *Sclerotinia sclerotiorum* produced copious amounts of the melanin precursor 1,8-DHN. Much reduced quantities of this product were produced in the presence of tricyclazole, an inhibitor of pentaketide melanin biosynthesis (Carzaniga et al., 2002), suggesting the importance of pentaketide pathway in synthesis of DHN melanins. Melanins in cell walls of Basidiomycota are derived from GDHB or catechol, as immediate phenolic precursor of the melanin polymer (Bell and Wheeler, 1986).

5.9.2 Melanins Aid in Penetration of Cuticle Barrier by Fungal Pathogens

The importance of melanins in fungal penetration of cuticle layer has been demonstrated by developing various melanin-deficient mutants of fungal pathogens. Several melanin-deficient mutants of *Colletotrichum* and *Magnaporthe* species have been isolated, and these melanin-deficient mutants were nonpathogenic suggesting that melanin biosynthesis is a metabolism essential for pathogenicity in *Colletotrichum* species and *Magnaporthe grisea* (Kubo and Furusawa, 1991; Kubo et al., 1991).

Albino mutants of *Colletotrichum lagenarium* form colorless appressoria and have little penetrating ability by germinating laterally from appressoria. A cosmid vector pkVB was constructed for isolating genes by complementation of mutations in *C. lagenarium* (Kubo et al., 1991). A genomic DNA library of wild-type *C. lagenarium* was constructed in pKVB. An albino mutant strain was transformed with DNA from this library. Seven melanin-restored transformants were obtained. Although albino mutants of *C. lagenarium* formed nonmelanized appressoria and possessed little penetrating ability, the transformants formed melanized appressoria with the ability to penetrate as efficiently as the wild strain (Figure 5.3; Kubo et al., 1991). These transformants also had pathogenicity to the host cucumber plants. The extent of the pathogenicity was the same as that in the wild-type strain (Kubo et al., 1991). These results suggest that melanization is important in host cell wall penetration by pathogens.

Albino mutants of *C. lagenarium* that produced appressoria without melanin pigmentation were obtained by ultraviolet irradiation or *N*-methyl-*N*′′-nitro-*N*-nitrosoguanidine treatment (Kubo et al., 1982a). About 70% of appressoria of the parent strain penetrated nitrocellulose membranes after incubation for 72 h. In albino mutants, most appressoria germinated laterally on the surface of membranes, and the percentage of penetration into membranes from appressoria was below 10%. However, when spores of mutants were incubated in the presence of 3,4-DOPA for 72 h, appressoria with pigment were formed and about 60% of these pigmented appressoria penetrated the membranes (Kubo et al., 1982a). The results suggest that the decrease in penetration into nitrocellulose membranes in albino mutants is due to the lack of pigmentation of appressoria.

When cucumber leaves were inoculated with a spore suspension of the parent strain of *C. lagenarium*, lesions were observed within 5 days. Pigmented appressoria and elongation of penetration hyphae were also observed. In contrast, when the leaves were inoculated with spores of the albino mutant, lesions were rarely observed. The appressoria were

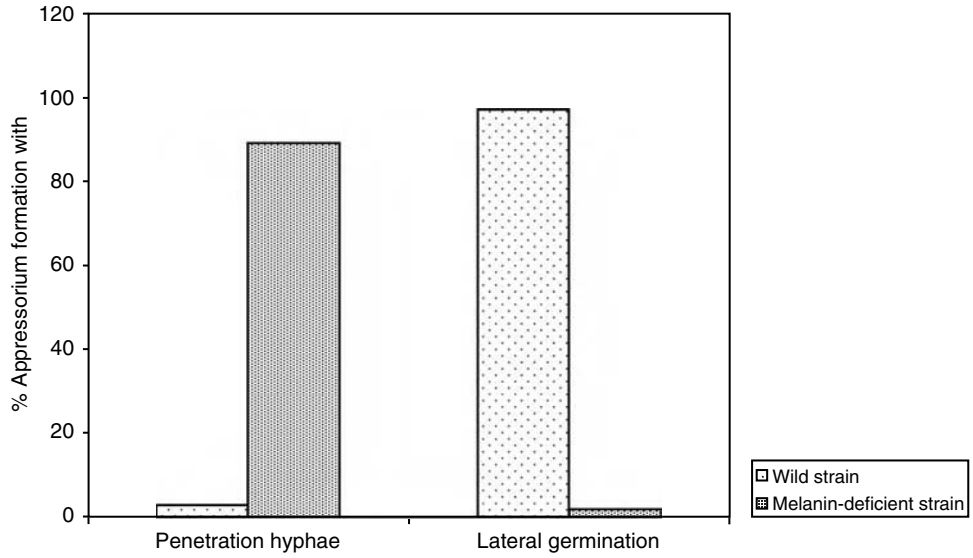

FIGURE 5.3 Effect of melanization on penetration hyphae formation in *Colletotrichum lagenarium*. (Adapted from Kubo, Y., Nakamura, H., Kobayashi, K., Okuno, T., and Furusawa, I., *Mol. Plant Microbe Interact*., 4, 440, 1991.)

nonpigmented and germinated laterally on the surface of the leaves (Kubo et al., 1982b). These results suggest that pigmentation of appressoria is essential for host penetration by *C. lagenarium*.

Magnaporthe grisea variants with buff pigmentation lacked pathogenicity. The variants with buff pigmentation, which lacked pathogenicity, were deficient in the melanin biosynthetic activity (Woloshuk et al., 1980b). Three distinctive phenotypic classes of pigment mutants, namely albino (Alb^-), rosy (Rsy^-), and buff (Buf^-) have been identified by Chumley and Valent (1990). Some of these mutants appeared spontaneously, whereas others were obtained by UV mutagenesis. All of them were melanin-deficient. Alb^-, Rsy^-, and Buf^- mutants were crossed to strains with wild-type pigmentation, and segregation of the melanin-deficient phenotypes was scored among the progeny. In all cases, mutant and wild-type phenotypes segregated 1:1 among the progeny, indicating the presence of single gene mutations in the pigment mutants. Pigment mutants caused no visible symptoms when inoculated on rice. Progeny from several crosses between pigment mutants and wild-type strains were tested for pathogenicity. All the pigment-deficient progeny were nonpathogenic, whereas the progeny that were gray were pathogenic. It suggests that nonpathogenicity and lack of melanin production were due to the same single gene defect (Chumley and Valent, 1990).

Wheeler and Greenblatt (1988) obtained buff-colored mutants of *M. grisea*. Extracts from melanin-deficient buff-colored mutants were tested for both reductase and dehydratase activities. They dehydrated scytalone to quantities of 1,3,8-THN similar to those produced by a wild-type control, but the extracts did not have much reductase activity, and conversion of 1,3,8-THN to DHN was very much less. Scytalone and vermelone did not remain in the mixture containing extracts of the melanin-deficient mutants, since they were completely dehydrated to 1,3,8-THN and DHN, respectively (Wheeler and Greenblatt, 1988). The results suggest that the melanin-deficient buff mutants have a genetic defect that prevents reductase activity and blocks melanin biosynthesis in their hyphae and appressoria. The lack of melanin in appressorial cell walls in the buff strains prevents penetration of the host cell wall (Wheeler and Greenblatt, 1988).

Chida and Sisler (1987) showed that exogenously supplied DHN restored the ability of appressoria of buff mutants of *M. grisea* with blocked 1,3,8-THN reductase activity to penetrate a membrane *in vitro*. DOPA restored melanization of melanin-deficient mutants of *M. grisea* and led to penetration of host epidermal cell walls (Woloshuk et al., 1980a; Okuno et al., 1983). DOPA and scytalone restored melanization and penetration of cucumber cell walls and cellulose membranes by the melanin-deficient mutant of *C. lagenarium* (Kubo et al., 1983). These results suggest that melanin is essential for penetration. Wounding allows infection of rice by melanin-deficient mutants suggesting that penetration is the important factor for pathogenesis of *M. grisea* (Woloshuk et al., 1983).

Several chemicals inhibit biosynthesis of melanins, and these chemicals prevented penetration of host plant cell wall by pathogens. Tricyclazole at concentrations that inhibited melanization but not mycelial growth, conidial germination, and appressorial formation prevented penetration of rice epidermis by *M. grisea* (Inoue et al., 1984). Tricyclazole-treated appressoria of *Colletotrichum lindemuthianum* could not penetrate plant epidermal walls, and it has been shown to be due to lack of wall rigidity in the unmelanized appressoria of the fungus (Kubo et al., 1982a). Thus, several studies have clearly shown the importance of melanin biosynthesis in appressoria of pathogens in penetration of host cell wall.

Although several pathogens penetrate the host cell wall by melanization, there are also reports that some pathogens do not require melanin for penetration. Melanin-deficient mutants of *Helminthosporium oryzae* were found to be fully pathogenic (Kubo et al., 1989). *Verticillium dahliae* has no DHN melanin requirement for pathogenicity (Bell and Wheeler, 1986). It is possible that fungi such as *Fusarium solani* f. sp. *pisi* and *Colletotrichum gloeosporioides*, which penetrate the cuticle via hyphae, may rely primarily on cutinase when fungi such as *M. grisea* and *C. lindemuthianum*, which penetrate the cuticle via an appressorium, may rely primarily on mechanical force provided by melanin (Sweigard et al., 1992a).

5.10 DEGRADATION OF PECTIC POLYSACCHARIDES

5.10.1 Types of Pectic Polysaccharides

The pectic polysaccharides are a complex mixture of acidic and neutral polymers, which are characterized by chains of α-1,4-linked galacturonosyl residues in which 2-linked rhamnosyl residues are interspersed. Pectins are the group of polysaccharides that are associated with D-galactosyluronic acid residues (Bolwell, 1993). Of these, homogalacturonan, rhamnogalacturonan-I, and a substituted galacturonan, rhamnogalacturonan-II, are commonly present. Homogalacturonan is a chain of 1,4-linked α-D-galactosyluronic acid residues. The carboxyl groups of the galacturonosyl residues of the cell wall pectic polysaccharides are known to be highly methyl esterified. The degree of esterification of the carboxyl groups varies depending on the source of the pectic polymers in different plants. There are regions that are highly methyl esterified as well as regions that are relatively free of methyl esters. Pectins are regarded as α-1,4-galacturonans with various degrees of methyl esterification, and the terms pectic acid and pectinic acid refer to the nonesterified and partially esterified forms, respectively (Vidhyasekaran, 1993, 2002, 2004).

5.10.2 Types of Pectic Enzymes

Plant pathogens produce different types of pectic enzymes (Collmer and Keen, 1986). The activity of these pectic enzymes varies in their specificity to the substrate (pectin or pectic acid), in their mechanism to split α-1,4-glycosidic bond (hydrolytic or lytic), and in the type of cleavage (random or terminal). The pectic enzymes are classified as pectin methylesterase (PME), which converts pectin into polygalacturonic acid, exo-polygalacturonase (exo-PG),

which cleaves polygalacturonic acids in a terminal manner releasing monomeric products (galacturonic acids), and endo-polygalacturonase (endo-PG), which cleaves polygalacturonic acids in a random manner releasing oligogalacturonic acid. Exo- and endo-pectin methyl galacturonases (PMG) act similar to polygalacturonases (PGs) but the preferred substrate is pectin instead of polygalacturonic acid. Both PGs and polymethylgalacturonases act by hydrolytic action. Contrastingly, polygalacturonate *trans*-eliminase [PGTE; pectate lyase (PL)] and pectin *trans*-eliminase (PTE; pectin lyase) induce lytic degradation of the glycosidic linkage resulting in an unsaturated bond between carbons 4 and 5 of a uronide moiety in the reaction product. The *trans*-eliminases break the glycoside linkage at carbon number 4, simultaneously eliminating the H from carbon number 4. It results in oligouronides that contain an unsaturated galacturonyl unit. Both exo (terminal) and endo (random) types of reactions are seen in the *trans*-eliminases and they are called exo-PGTE/exo-PL, endo-PGTE/endo-PL, exo-PTE/exo-pectin lyase, and endo-PTE/endo-pectin lyase (Vidhyasekaran, 1993). These enzymes show not only preference to substrates but also confer an added advantage to pathogens, because they might be more adaptable to changing environmental conditions and because some isoenzymes might be more active or stable within the host plant than others (Forster and Rasched, 1985; Peres-Artes and Tena, 1990).

5.10.3 Fungal Pathogens Produce Multiple Pectic Enzymes

Pathogens produce several types of pectic enzymes. *Botrytis cinerea* produces a pectin lyase (Kaile et al., 1991), at least six endo-PGs (Johnston et al., 1993; Sharrock and Labavitch, 1994; Wubben et al., 1999; Manfredini et al., 2005), and endo-PLs (Dilenna et al., 1981; Movahedi and Heale, 1990b). *Rhizoctonia solani* strain AG 2–2 causing crown and root rot of sugarbeet (*Beta vulgaris*) produced an exo-PG and a pectin lyase (Bugbee, 1990). An exo-PG and three endo-PGs are produced by the two pear scab pathogens, *Venturia pirina* and *Venturia nashicola* (Isshiki et al., 2000). *Magnaporthe grisea*, the rice blast pathogen, produced PME, pectin lyase, and PG (Bucheli et al., 1990). Three PGs (PG-I, PG-II, and PG-III) were detected in the culture filtrate of *Penicillium italicum*, the causal organism of blue mold of citrus fruits. PG-I was an exo-enzyme, whereas the other two were endo-enzymes. Exo-PG-I showed higher molecular weight than PG-II and PG-III (Hershenhorn et al., 1990). The tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* produces two different endo-PGs, PG1 and PG5. The PG1 has only 43% identity with PG5 and falls into a very distant class of endo-PGs (García-Maceira et al., 2000). The bean pathogen *Colletotrichum lindemuthianum* produces two endo-PGs, CLPG1 and CLPG2. CLPG1 is the major extracellular endo-PG produced both during culture and in planta, whereas CLPG2 is transiently produced only during early stages of growth (Centis et al., 1997). *Mycocentrospora acerina*, the fungus responsible for liquorice rot on carrot, produced PME, PG, and PL (Lecam et al., 1994b). *Cochliobolus carbonum* produces endo-PG, exo-PG, and PME (Scott-Craig et al., 1998). *Helminthosporium nodulosum*, the fingermillet pathogen, produces PME, endo-PG, exo-PG, PGTE, and pectin lyase (Vidhyasekaran, 1972, 1974a,b,c, 1975, 1977, 1978). *Fusarium solani* produced at least 14 PG isozymes with pIs of 4.5–9.5 in culture (Zhang et al., 1999).

In some cases, even the types of pectic enzymes produced by different races of a single pathogen may vary. For example, the race 0 and race 5 of *Fusarium oxysporum* f. sp. *ciceri*, the chickpea wilt pathogen, produce extracellular PG and PL *in vitro* (Peres-Artes and Tena, 1989). Both the enzymes were purified. The race 0 produces three PG forms (endo-PGI_0, endo-$PGPII_0$, and exo-$PGIII_0$) and only one PL form (endo-PL_0), whereas the race 5 produces one PG form (exo-PG_5) and two PL forms (endo-PLI_5 and endo-$PLII_5$). The characteristics of these enzymes are given in Table 5.2 (Peres-Artes and Tena, 1990). The exo-PGs from both races ($PGIII_0$ and PG_5) were apparently the same enzyme. All other enzymes were different from each other (Peres-Artes and Tena, 1990).

TABLE 5.2
Properties of PGs and PLs Produced by Race 0 and Race 5 of *Fusarium oxysporum* f. sp. *ciceri*

	Enzymes of Race 0				Enzymes of Race 5		
Property	**PGI_0**	**$PGII_0$**	**$PGIII_0$**	**PL_0**	**PG_5**	**PLI_5**	**$PLII_5$**
Mode of action	Endo	Endo	Exo	Endo	Exo	Endo	Endo
Molecular weight in kDa	44	44	76	25	76	37	37
Optimum pH	4.5	4.5	4.5	9.5	4.5	10.5	10.5
Isoelectric point	8.0	6.4	4.2	>9.0	4.2	>9.0	—
Optimum temperature in °C	45	45	60	45	60	45	45

Source: Adapted from Peres-Artes, E. and Tena, M., *Physiol. Mol. Plant Pathol.*, 37, 107, 1990.

5.10.4 Genes Encoding Pectic Enzymes

The genes encoding endo-PGs of several fungi, including those of *Aspergillus flavus*, *Aspergillus niger*, *Botrytis cinerea*, *Cochliobolus carbonum*, *Colletotrichum lindemuthianum*, *Cryphonectria parasitica*, *Fusarium moniliforme*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*, have been cloned and characterized (Bussink et al., 1991, 1992; Caprari et al., 1993, 1996; Gao et al., 1996; Reymond-Cotton et al., 1996; Centis et al., 1997; Shieh et al., 1997; Di Pietro and Roncero, 1998; Scott-Craig et al., 1998; ten Have et al., 1998; Wubben et al., 1999; García-Maceira et al., 2000; Herbert et al., 2004). A gene (*PGN1*) encoding endo-PG was isolated from *Cochliobolus carbonum* race 1. Genomic and cDNA copies of the gene were isolated and sequenced. The DNA sequence of PGN1 predicted a polypetide of 33,992 Da. The *PGN1* gene was required for endo-PG production (Scott-Craig et al., 1990). Two endo-PG genes, *CLPG1* and *CLPG2*, have been cloned from the bean pathogen *Colletotrichum lindemuthianum*, and these genes show different expression patterns (Centis et al., 1997; Boudart et al., 2003; Herbert et al., 2004). CLPG2 is transcriptionally induced by pectin, and a *cis*-acting sequence regulates expression of the pectinase gene (Herbert et al., 2002). A gene encoding endo-PG, Bcpg1, has been cloned from *B. cinerea* (ten Have et al., 1998). Two pectin lyase genes, *pnl-1* and *pnl-2*, have been isolated from *Colletotrichum gloeosporioides* f. sp. *malvae*, the pathogen of *Malva pusilla* (Wei et al., 2002). Four PL (*pelA*, *pelB*, *pelC*, and *pelD*) genes have been isolated from the pea pathogen *N. haematococca* (González-Candelas and Kolattukudy, 1992; Guo et al., 1996; Rogers et al., 2000).

Regulation of endo-PG gene expression generally depends on the carbon source available, with the exception of a constitutively expressed gene in *B. cinerea* (Van der Cruyssen et al., 1994). These genes are mainly induced by pectin and subjected to glucose repression (Di Pietro and Roncero, 1998; Scott-Craig et al., 1998; Tonukari et al., 2000; Wubben et al., 2000; Herbert et al., 2002), although complete regulation of endo-PG gene expression is not fully understood. The gene encoding an exo-PG, *pgx4*, was isolated from *Fusarium oxysporum* f. sp. *lycopersici*, and it encodes a 454 amino acid polypeptide with 9 potential N-glycosylation sites and a putative 21 amino acid N-terminal signal peptide (García-Maceira et al., 2000). An endo-PG gene has also been isolated from *F. oxysporum* f. sp. *lycopersici* (Di Pietro and Roncero, 1998). Both the endo- and exo-PG genes were expressed by the fungus during infection (Di Pietro and Roncero, 1998b; García-Maceira et al., 2000). *pg5*, encoding a novel endo-PG, from *F. oxysporum* has been cloned (García-Maceira et al., 2001). The *pg5* is expressed during saprophytic growth on citrus pectin and in planta during the initial stages of infection (García-Maceira et al., 2001).

5.10.5 Evidences to Show That Pectic Enzymes Aid Pathogens to Penetrate Cell Wall

5.10.5.1 Immunocytochemical Evidences

Immunocytological evidences have been provided to show the importance of pectic enzymes in cell wall penetration by fungal pathogens. The gold-complexed *Aplysia depilans* gonad lectin (AGL), an agglutinin with polygalaturonic acid-binding specificity, was used to localize pectin in bean leaf tissues infected with *Colletotrichum lindemuthianum* (Benhamou et al., 1991). At 5 days after inoculation with the pathogen, an intense degradation of cell wall components was observed. The intense host cell wall disintegration in infected area was always accompanied by the release of tiny fragments specifically labeled by gold-complexed particles (Benhamou et al., 1991). The results suggest degradation of pectin during cell wall penetration by the pathogen.

The localization of the fungal endo-PGs in *C. lindemuthianum*-infected bean cell walls was studied by using polygalacturonase inhibiting protein (PGIP)–gold complex (Benhamou et al., 1991). Large amounts of gold particles were found over walls of invading hyphae as well as over host cell walls closely neighboring fungal cells. The qualitative evaluation of the label patterns obtained over cells of *C. lindemuthianum* following the PGIP–gold complex revealed that the PG synthesis increased severalfold when the fungus developed in bean leaf tissues. The results demonstrate that *C. lindemuthianum* is capable of producing large amounts of endo-PG, causing extensive pectin breakdown (Benhamou et al., 1991).

When tobacco roots were inoculated with zoospores of *Phytophthora parasitica* var. *nicotianae*, penetration of the root epidermis was seen 24 h after inoculation. By 72 h after inoculation, the fungus had developed through much of the cortex and it was always associated with considerable host wall alterations, ranging from swelling and shredding to complete disruption of the middle lamellar matrices (Benhamou and Cote, 1992). AGL was applied to infected root tissues for studying the pattern of pectin distribution in host cell walls. Examination of infected tissues from 48 to 120 h after inoculation showed that labeling with the gold-complexed lectin was markedly reduced in wall areas exhibiting signs of obvious damage (Figure 5.4; Benhamou and Cote, 1992). It indicates that pectin would have been

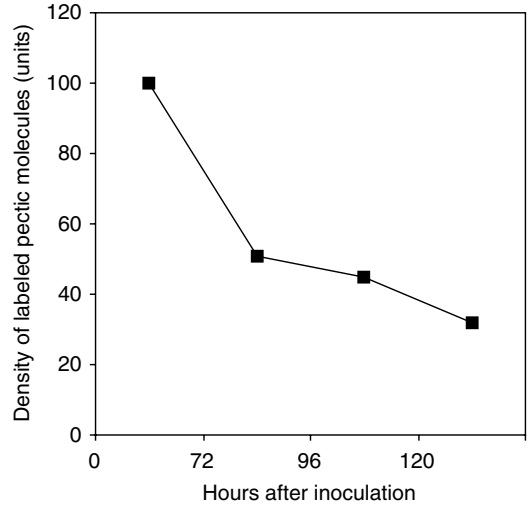

FIGURE 5.4 Density of labeled pectic molecules in tobacco root cells inoculated with *Phytophthora parasitica* var. *nicotianae*. (Adapted from Benhamou, N. and Cote, F., *Phytopathology*, 82, 468, 1992.)

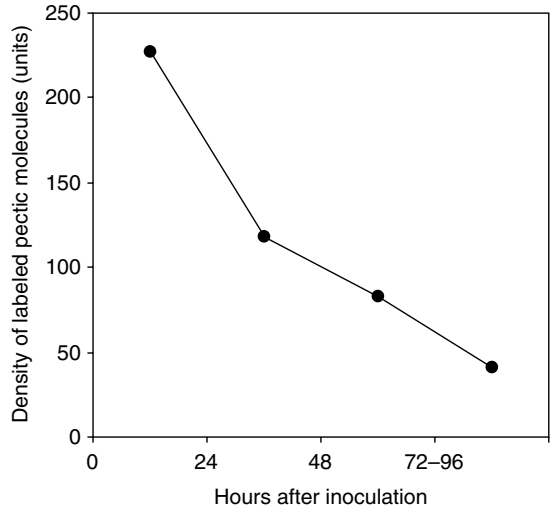

FIGURE 5.5 Density of labeled pectic molecules in cucumber root tissues inoculated with *Pythium ultimum*. (Adapted from Cherif, M., Benhamou, N., and Belanger, R.R., *Physiol. Mol. Plant Pathol.*, 39, 353, 1991.)

degraded in the infected tissues. The AGL failed to label pectic molecules in primary wall and middle lamella areas near invading hyphae indicating the release of pectic enzymes by *P. parasitica* var. *nicotianae* (Benhamou and Cote, 1992). Alteration of pectic molecules occurred in wall areas closely adjacent to fungal cells and at a distance from the point of pathogen penetration (Benhamou and Cote, 1992). It suggests that pectic enzymes may have freely diffused extracellularly to facilitate pathogen ingress through loosened middle lamella matrices and host cell walls.

AGL was applied to *Pythium ultimum*-infected cucumber tissues for studying the pattern of pectin distribution during the early stages of colonization by the pathogen. Alteration of pectic macromolecules was seen even at 24 h after inoculation with the pathogen (Cherif et al., 1991; Figure 5.5). These observations suggest that degradation of pectic substances occurs during pathogenesis of fungal pathogens.

5.10.5.2 Evidences by Showing Protection of the Host by Inhibition of Pectic Enzymes with Specific Antibodies

Strong evidences to demonstrate the role of pectic enzymes in pathogenesis have been presented by protecting the host plants against fungal infection by inhibiting the fungal pectic enzymes by using specific antibodies. Antibodies prepared against PL showed protection of the host against infection (Rogers et al., 1994; Guo et al., 1996; Wattad et al., 1997). *Fusarium solani* f. sp. *pisi*, the pea pathogen, produces PL. Antibodies against the enzyme were developed. When suspension of the conidia of the fungus was prepared in these antibodies, and inoculated on pea stem, no infection of the pathogen was observed. Penetration of the fungus into the host was very much suppressed by the antibodies (Figure 5.6; Crawford and Kolattukudy, 1987). The results suggest the importance of the PL aiding the pathogen to penetrate the host tissues and cause disease.

The PL produced by *Colletotrichum gloeosporioides* (the causal organism of anthracnose of avocado fruits) macerates avocado fruit tissues (Wattad et al., 1994). Antibodies against the enzyme were developed and these antibodies inhibited the enzymatic activity. These antibodies suppressed maceration of the fruit tissue by the enzyme (Wattad et al., 1994).

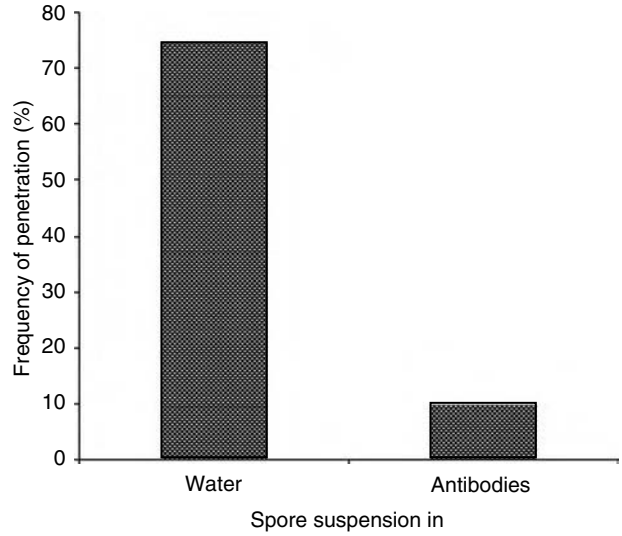

FIGURE 5.6 Effect of PL antibodies on the penetration of *Fusarium solani* f. sp. *pisi* into pea stems. (Adapted from Crawford, M.S. and Kolattukudy, P.E., *Arch. Biochem. Biophys*., 258, 196, 1987.)

Development of decay in avocado fruits could be inhibited when *C. gloeosporioides* spores were coinoculated with PL antibodies (Wattad et al., 1994). These results suggest the importance of the enzyme in the anthracnose disease development in avocado.

5.10.5.3 Evidences Showing Protection of Host Plants by Inhibition of Pectic Enzymes with Selective Inhibitors

The role of pectic enzymes in fungal pathogenesis has been demonstrated by inhibiting the pectic enzymes by specific inhibitors of these enzymes. Pear fruit polygalacturonase inhibitor (pPGIP) specifically inhibits endo-PG of fungal pathogens. Transgenic tomato plants expressing the pPGIP inhibited the endo-PG produced by *Botrytis cinerea* and showed resistance to the pathogen. The PGIP inhibition of fungal PG slowed the expansion of disease lesions and the associated tissue maceration in tomato (Powell et al., 2000). Pectolytic enzymes of the avocado pathogen *Colletotrichum gloeosporioides* could be inhibited by the host epicatechin and it correlated with the inhibition of symptom development in avocado (Wattad et al., 1994). The avocado fruit decay was suppressed under conditions that were not permissive to PL secretion by the pathogen (Yakoby et al., 2000b). These results suggest that PG is important in the fungal disease development.

5.10.5.4 Evidences Using Pectic Enzyme-Deficient Fungal Isolates

The importance of pectic enzymes in fungal pathogenesis has been shown by using fungal isolates deficient in some pectic enzymes. The fungal isolates, which were deficient in those pectic enzymes, have been shown to be less virulent. The highly virulent isolate of *Fusarium oxysporum* f. sp. *ciceri* produced two forms of endo-PL, whereas the low virulent isolate produced only one form of endo-PL (Peres-Artes and Tena, 1990). Virulent isolates of *Rhizoctonia solani* produced endo-PL, whereas hypovirulent isolates did not produce the enzyme (Marcus et al., 1986). *Aspergillus flavus* isolates that did not produce the PG P2c caused less damage to cotton bolls than those isolates that produced the enzyme (Shieh et al., 1997).

These results provide evidences to show that the pectic enzymes are virulence factors in many fungal pathogens.

5.10.5.5 Evidences Showing Correlation between the Level of Pectic Enzymes and Virulence

A correlation between the amount of pectic enzymes produced by fungal isolates and their virulence has been reported by several workers. Hypoaggressive isolate of *Mycocentrospora acerina* produces less amount of PME, PG, and PL *in vitro* (Lecam et al., 1994b). A hypovirulent strain of *Cryphonectria parasitica* produced less PG compared with the virulent strain (Gao and Shain, 1995). A mutant of *Colletotrichum magna* (Path-1) with limited secretion of PL induced only reduced symptom development in cucurbits (Yakoby et al., 2000a). This type of correlation between virulence and the level of enzymes suggests that pectic enzymes may be involved in fungal pathogenesis.

5.10.5.6 Evidences Showing Enhancement of Virulence by Gene Transfer

Some of the genes encoding pectic enzymes have been cloned, and when the cloned genes were transferred to avirulent isolates, the avirulent isolates became virulent. *Aspergillus flavus*, the causal organism of boll rot of cotton, produces the PG P2c, and the gene encoding this enzyme, *pecA*, has been cloned (Shieh et al., 1997). Adding the *pecA* gene to an *A. flavus* strain previously lacking the gene resulted in the ability to cause significantly more damage to the cotton boll compared with the abilities of a control transformant (Shieh et al., 1997). A PL gene (*pel*) from the avocado pathogen *C. gloeosporioides* was expressed in a pathogen of cucurbits, *C. magna*, which causes only minor symptoms in avocado fruits. The transformed isolates showed more enzyme activity than the wild-type isolates and caused severe disease (Yakoby et al., 2000a). These results provide strong evidences for the role of the pectic enzyme in disease development.

5.10.5.7 Evidences Showing Decrease in Virulence by Gene Disruption

Genes encoding various pectic enzymes have been cloned from various pathogens (Rogers et al., 2000; Wei et al., 2002). Virulence of some fungal pathogens has been reduced by disrupting some of these genes. The *Bcpg1* gene encoding endo-PG was eliminated by partial gene replacement in *Botrytis cinerea*. The resulting mutants showed reduced virulence, indicating that *Bcpg1* gene is required for full virulence (ten Have et al., 1998). Eliminating the expression of *pecA* gene encoding a PG in the cotton boll rot pathogen, *A. flavus*, by targeted disruption caused a significant reduction in aggressiveness compared with that of a nondisrupted control transformant (Shieh et al., 1997). These results provide direct evidences to show that pectic enzymes are involved in the invasion and spread of fungal pathogens.

5.10.6 Plant Signals to Induce Pectic Enzymes

Pectic enzymes are inducible by pectin *in vitro* and by specific plant signals *in vivo*. The pea pathogen *N. haematococca* produces several endo-PLs, encoded by at least four PL (*pel*) genes (Guo et al., 1996). Among them, *pelA* is inducible by pectin (González-Candelas and Kolattukudy, 1992) and *pelD* only by the host (Guo et al., 1996). It suggests that some specific plant signals may be necessary for induction of *pelD*. Homoserine and asparagine, two free amino acids found in uniquely high levels in pea seedlings, have been found to be the plant signals that trigger the *pelD* induction in planta (Yang et al., 2005). It has been suggested that *N. haematococca* has evolved a mechanism to sense the host tissue environment by using the high levels of two free amino acids in this plant, thereby triggering the expression of *pelD* to assist the pathogenic process (Yang et al., 2005).

Pectin oligomers released from breakdown of plant cell wall may also act as signals for production of pectic enzymes. Usually, there is a low level of constitutive expression of the degradative enzymes and therefore the fungal spore would arrive at the host surface with a small amount of the enzyme. On contact with the polymeric barrier of the host, the constitutively expressed enzyme would generate small amounts of the hydrolytic products. These products would then activate the transcription of the inducible gene, generating a high level of the enzyme that can assist the pathogen to penetrate through the physical barriers of the host. The pectin lyase gene, *pelB*, in *N. haematococca* is constitutively expressed, whereas another pectin lyase gene, *pelA*, is only inducible. Antisense expression of *pelB* prevented induction of *pelA* and it suggests that the constitutively expressed *pelB* gene would have provided oligomers that induced *pelA* (Rogers et al., 2000).

5.10.7 Host Cell Wall Differs in Its Susceptibility to Pectic Enzymes

Efficacy of pectic enzymes to degrade host cell walls may also depend upon the structure of host cell wall (Cooper et al., 1981). The pectic enzymes produced by *Mycocentrospora acerina* hydrolyzed and solubilized the pectins from carrot cutivars (Lecam et al., 1994a). Pectin fractions from the cultivars most (Touchon) and least (Major) susceptible to the fungal infection were incubated with pure endo-PG. After 45 min of reaction, Touchon pectin was hydrolyzed, whereas Major pectin was not (Lecam et al., 1994a). Amounts of cell wall material and composition of the pectic fraction were not correlated with cultivar resistance, but a different distribution of methyl groups along the rhamnogalacturonan chains in the different cultivars would have contributed to the difference in behavior of endo-PG on pectin from the Touchon and Major cultivars (Lecam et al., 1994a).

Insoluble forms of pectin (protopectin) are resistant to hydrolysis by pectic enzymes. Susceptibility of strawberries (*Fragaria grandiflora*) to *Botrytis cinerea* was highly correlated with the soluble pectin content of the fruit (Hondelmann and Richter, 1973). The carrot cultivars, which are resistant to *M. acerina*, contained more insoluble form of pectin (Lecam et al., 1994a).

5.10.8 Cell Wall Proteins Modulate Pectic Enzyme Activity

Some proteins present in the host cell wall modulate pectic enzyme activity in cell walls. Degradability of chickpea cell wall preparations without ionically bound proteins was not related to host resistance or susceptibility to the wilt pathogen, *Fusarium oxysporum* f. sp. *ciceri*. However, walls containing ionically bound proteins from the chickpea susceptible cultivar PV-24 were more extensively degraded by the exo-PG and PL forms (PL I and PL II) produced by the pathogen than were similar preparations from the resistant cultivar WR 315 (Peres-Artes and Tena, 1990). The results suggest that certain proteins can modulate pectic enzyme activities on plant cell walls.

Some proteins in plant cell walls specifically inhibit PGs produced by pathogens and they are called PGIPs. PGIPs have been detected in cell walls of all plants examined (Cervone et al., 1997; Favaron et al., 1997; Yao et al., 1999; Berger et al., 2000; De Lorenzo et al., 2001; James and Dubery, 2001; Vidhyasekaran, 2002; Fish and Davis, 2004; Gazendam et al., 2004; Howell and Davis, 2005; Manfredini et al., 2005). PGIPs belong to a superfamily of leucine-rich repeat (LRR) proteins (Toubart et al., 1992; Stotz et al., 1994; Howell and Davis, 2005). PGIPs specifically inhibit PGs, and PGs have been shown to be required for full virulence of several pathogens including *Botrytis cinerea* (ten Have et al., 1998), *Colletotrichum lindemuthianum* (Benhamou et al., 1991), *Alternaria citri* (Isshiki et al., 2001), and *Claviceps purpurea* (Oeser et al., 2002). The PG activity of *B. cinerea* is inhibited *in vitro* by PGIPs from tomato, bean, apple, pear, and raspberry (Sharrock and Labavitch, 1994;

Stotz et al., 1994; Leckie et al., 1999; Yao et al., 1999). PG from *Verticillium dahliae* is inhibited by a PGIP from cotton (James and Dubery, 2001). Bean PGIP-1 inhibited PGs from *Stenocarpella maydis* (Berger et al., 2000). PvPGIP2 of *Phaseolus vulgaris* efficiently inhibited BcPG1 produced by *B. cinerea* (Manfredini et al., 2005).

PGIPs from different plants may have selective action against PGs produced by pathogens. PGIPs purified from bean, tomato, and pear show differential specificity toward PGs of different pathogens (Johnston et al., 1993, 1994; Desiderio et al., 1997). PGIP from pea selectively inhibited PG 2 (endo-polygalaturonase) and not PG 1 (exo-PG) produced by the pea pathogen *Ascochyta pisi* (Hoffman and Turner, 1982). The PGIP-1 isolated from bean inhibited PGs from *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria solani*, and *B. cinerea*, whereas it did not show inhibitory activity against *Fusarium moniliforme* (Desiderio et al., 1997). Stotz et al. (2000) reported that the PGIP isolated from bean (*P. vulgaris*) inhibited PGs from *F. moniliforme*, *B. cinerea*, and *Aspergillus niger*, whereas pear PGIP was effective only against *B. cinerea* PG. PGIPs from a single plant tissue may differentially inhibit PGs from various fungi (Cook et al., 1999; Fish and Davis, 2004).

PGIP is encoded by a small family of genes (Toubart et al., 1992; Frediani et al., 1993; Favaron et al., 1997, 2000). The different *PGIP* genes encode PGIPs with different inhibiting activities against different pathogens (Desiderio et al., 1997). At least four genes encode PGIPs in bean. *PvPGIP2* encodes a PGIP that inhibits PGs from both *Fusarium moniliforme* and *Aspergillus niger*, whereas the *PvPGIP1*-encoded protein shows inhibitory activity against *A. niger*, and not against *F. moniliforme* (Leckie et al., 1999). Two PGIPs (encoded by *AtPGIP1* and *AtPGIP2*) have been isolated from *Arabidopsis thaliana*. Both the PGIPs inhibited PG activity of *Colletotrichum gloeosporioides*, *B. cinerea*, and *Stenocarpella maydis*, but failed to inhibit that of *F. moniliforme* and *Aspergillus niger* (Ferrari et al., 2003).

Inhibitory action of PGIPs from different plants against a pathogen may vary (Cook et al., 1999). A pear fruit PGIP inhibits PGs of *B. cinerea* more effectively than a tomato PGIP (Stotz et al., 2000). The amount of PGIP detected in plant tissues may also determine effectiveness of the PGIP against pathogens. High amount of a PGIP was detected in Chinese chestnut (*Castanea mollissima*) bark, which is resistant to *Cyphonectria parasitica* (Gao and Shain, 1995). The bark of American chestnut (*Castanea dentata*), which is susceptible to the pathogen, contained less PGIP. Lafitte et al. (1984) observed that the isogenic lines of bean that are resistant to *C. lindemuthianum* contained higher levels of PGIPs than the susceptible ones.

PGIPs isolated from different cultivars of a plant species may not show any race-specificity action. The inhibitor proteins from four bean cultivars have been purified (Lorenzo et al., 1990). The molecular size of the proteins from the four cultivars was indistinguishable. The abilities of the inhibitors purified from great northern, pinto, red kidney, and small red beans to inhibit the endo-PGs purified from α, β, and γ races of *C. lindemuthianum* were assessed. The three endo-PGs were inhibited to the same extent by the same amount of the inhibitor, regardless of the bean from which the inhibitor was isolated. No significant difference in the rate and pattern of degradation of polygalacturonic acid was observed (Lorenzo et al., 1990). The results suggest that the inhibitor may not be involved in race–cultivar specificity.

PGIPs may modulate the PG activity of the pathogens and may cause the accumulation of oligogalacturonide fragments, which may act as signal molecules inducing host defense mechanisms (Ferrari et al., 2003; Zuppini et al., 2005). The dynamic regulation of production of pectic enzymes may determine susceptibility or disease resistance. For example, high production of PGs may facilitate the pathogen colonization and disease development, whereas low amount of production of the same enzyme may trigger accumulation of fungitoxic compounds such as phytoalexins and pathogenesis-related/antifungal proteins resulting in disease resistance (Wegener and Olsen, 2004). Low levels of pectic enzymes released in the

infection court can result in release of elicitors, which can activate defense mechanisms of host plants (Hammerschmidt, 2004). The extent of cell wall degradation appears to be controlled by the presence of inhibitory proteins, which counteract fungal enzyme hydrolysis (Esquerré-Tugaye et al., 2000). Some of the PGIPs have been shown to activate (instead of inhibiting) PGs produced by *Aspergillus niger* (Kemp et al., 2004). It suggests that PGIPs may include PG-modulating proteins.

5.11 PATHOGENS PRODUCE CELLULOLYTIC ENZYMES TO BREACH CELL WALL BARRIER

Cellulose is the major wall polysaccharide and is composed of glucose units in the chain configuration, connected by β-1,4-glycosidic bonds. The enzymatic hydrolysis of cellulose requires the action of endo-glucanase (endo-β-1,4-glucanase) and exo-glucanases (β-1, 4-cellobiohydrolase and β-glucosidase) (Yazdi et al., 1990; Espino et al., 2005). Degradation of cellulose in plant cell walls has been demonstrated by histogical studies. Cellulose in tobacco roots was labeled by using gold-complexed exo-glucanase (Benhamou and Cote, 1992). Labeling decreased in the tobacco root tissues inoculated with *Phytophthora parasitica* var. *nicotianae*, indicating degradation of cellulose during fungal pathogenesis (Figure 5.7; Benhamou and Cote, 1992). These histological studies demonstrated the involvement of cellulases in fungal pathogenesis.

Several cellulolytic enzymes are known to be produced by pathogens. *Venturia inaequalis*, the apple scab pathogen, produces in culture 12 cellulase isozymes with isoelectric points in the range of 3.7–5.6 (Kollar, 1994). The molecular weights were about 60 kDa for at least five enzymes and about 25 kDa for the five more prominent isozymes. These enzymes could be isolated also from apple leaves infected with the pathogen (Kollar, 1994). The maize leaf pathogen, *Cochliobolus heterostrophus*, produces a cellobiohydrolase and an

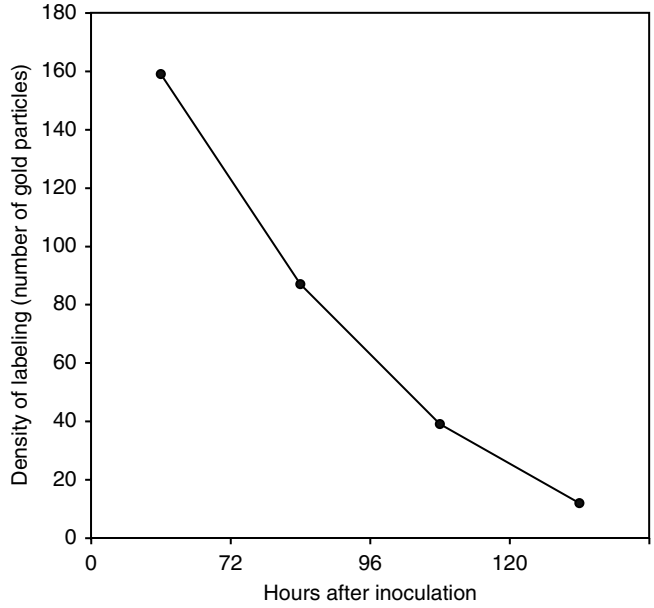

FIGURE 5.7 Intensity of degradation of cellulose in tobacco root tissues at different days after inoculation with *Phytophthora parasitica* var. *nicotianae*. (Adapted from Benhamou, N. and Cote, F., *Phytopathology*, 82, 468, 1992.)

endo-glucanase. The genes encoding these enzymes have been cloned (Lev and Horwitz, 2003). *Botrytis cinerea* produces an endo-β-1,4-glucanase, and the gene encoding this enzyme, *cel5A*, has been cloned (Espino et al., 2005). The *cel5A* mRNA could be detected during infection of tomato leaves by *B. cinerea* (Espino et al., 2005).

Gaeumannomyces graminis var. *tritici*, the causal agent of take-all disease of wheat, secretes two groups of enzymes that degrade cellulosic polymers (Dori et al., 1995). The first group contains an endo-glucanase and β-glucosidase with acidic pIs of 4.0 and 5.6, respectively. The second group contains an endo-glucanase and β-glucosidase with basic pIs of 9.3 and >10, respectively. Acidic and basic groups of endo-glucanase and β-glucosidase were also obtained from inoculated wheat roots, and they appeared to be similar to those isolated from the culture fluid (Dori et al., 1995). Several other pathogens have been reported to produce multiple cellulases (Suzuki et al., 1983; Wagner et al., 1988).

5.12 FUNGAL HEMICELLULASES IN PLANT CELL WALL DEGRADATION

Xylans, arabinans, xyloglucans, arabinoxylans, arabinogalactans, mannans, glucomannans, and galactoglucomannans are the important hemicelluloses detected in plant cell walls. Xylan is a predominant hemicellulose polysaccharide composed of a backbone of β-1,4-xylopyranosyl residues, some of which are substituted with arabinosyl, acetyl, and glucuronosyl residues (Wong et al., 1998; Saha, 2000). L-Arabinosyl residues are widely distributed in some hemicelluloses, such as arabinan, arabinoxylan, and arabinogalactan (Saha, 2000).

The complete breakdown of these hemicelluloses requires the cooperative action of several enzymes, including arabinosidases and endo-β-xylanases (Brito et al., 2006). Arabinosidases comprise the α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranohydrolase, ABF) (Crous et al., 1996). Xylanases are produced by a number of plant pathogenic fungi and they may play a role during infection. *Fusarium oxysporum* secretes a number of xylanases (Alconada and Martínez, 1994; Wu et al., 1997; Christakopoulos et al., 2000). At least three xylanase genes are expressed by *F. oxysporum* f. sp. *lycopersici* during different stages of infection on tomato plants (Gómez-Gómez et al., 2002). The xylanase genes *xyl2* and *xyl3* have been detected in *F. oxysporum* f. sp. *lycopersici* (Ruíz-Roldán et al., 1999). *Cochliobolus carbonum* secretes xylanase when grown on maize cell walls (Wegener et al., 1999). On purification, three xylanase isoenzymes and a β-xylosidase were detected (Holden and Walton, 1992). Three xylanase genes have been identified in *C. carbonum* (Apel et al., 1993). *Xyl1*, the gene for the major xylan-degrading enzyme in *C. carbonum*, has been cloned and sequenced. *Xyl1* encodes two forms of endo-xylanase activity (Xylanase I and II) (Apel et al., 1993). Two other endo-β-1,4-xylanase genes, *XYL2* and *XYL3*, have been reported in *C. carbonum* (Apel-Birkhold and Walton, 1996). A xylanase gene has been cloned from the maize pathogen *Exerohilum turcicum* (= *Helminthosporium turcicum*) (Degefu et al., 2001).

Two xylanases have been purified from *Gaeumannomyces graminis* var. *avenae*, the causal agent of take-all disease of oats (Southerton et al., 1993). Their molecular weights were 26.5 and 28 kDa and they had a pI of 10.5. These xylanases could be detected in oat and wheat roots infected with *G. graminis* var. *avenae* (Southerton et al., 1993). Two xylanases have been purified from *Magnaporthe grisea*, the rice blast pathogen. They are secreted when the fungus is grown on rice cell walls as the only carbon source. Their molecular weights were 22 and 33 kDa, and both of them were basic proteins with calculated isoelectric points of 9.95 and 9.71, respectively. These xylanase genes have been cloned and sequenced (Wu et al., 1995, 1997). Brito et al. (2006) have cloned an endo-β-1,4-xylanase gene, *xynl1A*, from *Botrytis cinerea*. Deletion of this gene had a pronounced effect on virulence and reintroducing the wild-type gene into the mutant strains reversed this phenotype back to wild type (Brito et al., 2006).

Arabinose is a major component of the arabinans. It is also found as a side-chain constituent of the xyloglucan, xylan rhamnoglacturonan I, and rhamnogalacturonan II polymers of plant cell walls (McNeil et al., 1984). The removal of the arabinose-containing side chains from these major plant cell wall polymers by arabinofuranosidase might facilitate the digestion of these polymers. *Monilinia fructigena* produces L-arabinofuranosidase *in vitro* and in infected apple fruits. The breakdown product arabinose was detected in apple fruits infected with *M. fructigena* (Laborda et al., 1974; Howell, 1975). A correlation between levels of arabinofuranosidase activity and virulence has been reported in *Monilinia fructigena* (Howell, 1975). The gene encoding α-L-arabinofuranosidase B has been cloned from *Fusarium oxysporum* f. sp. *dianthi* (Chacón-Martínez et al., 2004). This gene was found to be upregulated during the infection process in carnation (Chacón-Martínez et al., 2004). *Cochliobolus carbonum* produces a β-xylosidase (Wegener et al., 1999). It also produces α-arabinofuranosidase in culture (Ransom and Walton, 1997).

Sclerotinia trifoliorum, a pathogen of legumes, produces an arabinofuranosidase in culture (Rehnstrom et al., 1994). Three arabinofuranosidase-deficient mutants were obtained (Rehnstrom et al., 1994). The pathogenicity of the arabinofuranosidase-deficient mutants was evaluated by inoculating alfalfa and pea stems. These mutants were significantly virulent on the pea stems but not on alfalfa stems. It suggests that arabinofuranosidase may play a role in pathogenicity only under certain conditions (Rehnstrom et al., 1994).

5.13 DEGRADATION OF CELL WALL STRUCTURAL PROTEINS

Plant cell walls contain both structural and enzyme proteins, which can account for up to 15% of the cell wall (Showalter, 1993). There are five major classes of structural cell wall proteins mostly characterized from dicots. These include extensins, proline-rich proteins (PRPs), arabinogalactan proteins (AGPs), lectins, and glycine-rich proteins (GRPs). Each of them, with the exception of GRPs, contains hydroxyproline-rich glycoproteins (HRGPs). They are rich in hydroxyproline (Hyp) and serine (Ser), and they usually contain the repeating pentapeptide motif Ser–Hyp4. PRPs are characterized by the repeating occurrence of Pro–Pro repeats. They contain approximately equimolar quantities of proline and hydroxyproline. Hydroxyproline and arabinose are major constituents of lectins. AGPs are HRGPs that are highly glycosylated. The protein moiety of AGPs is typically rich in hydroxyproline, serine, alanine, threonine, and glycine. They contain Ala–Hyp repeats. GRPs lack hydroxyproline and contain Gly–X repeats, where X is most frequently Gly but can also be Ala or Ser (Showalter, 1993; Otte and Barz, 2000; Vidhyasekaran, 2002, 2004). Some of these proteins have been found in different forms in graminaceous monocots, such as HRGPs containing threonine (threonine–hydroxyproline-rich glycoproteins, THRGPs) or histidine (histidine–hydroxyproline-rich glycoproteins, HHRGPs) (Showalter, 1993; Vidhyasekaran, 2002).

Fungal pathogens produce proteases to degrade plant cell wall barrier. *Stagonospora* (*Septoria*) *nodorum*, the wheat glume blotch pathogen, produces a trypsin like protease (SNP1) when grown in liquid culture with wheat cell walls as the sole carbon and nitrogen source. The enzyme could be detected in the infected wheat leaf tissue. The protease enzyme SNP1 released hydroxyproline from wheat cell walls. These results suggest that the protease enzyme participates in the degradation of host cell walls during infection (Carlile et al., 2000). *Botrytis cinerea* produces a protease in culture and in infected carrots (Movahedi and Heale, 1990a,b). The sugarbeet pathogen, *Aphanomyces cochlioides*, and the legume pathogen, *Aphanomyces euteiches*, produce trypsin-like protease enzymes both in culture and in infected tissues (Weiland, 2004). *Pyrenopeziza brassicae*, the causal agent of light leaf spot of oilseed rape (*Brassica napus* L. ssp. *oleifera*), produces a cysteine protease with a molecular weight of 34 kDa (Ball et al., 1991). A UV-induced nonpathogenic mutant of the fungus was also

deficient in protease production *in vitro*. The protease mutant was transformed with clones from a genomic library of *P. brassicae* and a transformant obtained showed concomitant restoration of pathogenicity and proteolytic activity *in vitro* (Ball et al., 1991). It suggests the importance of protease in pathogenesis of *P. brassicae*.

Proteases are produced by several other pathogens such as *Magnaporthe poae* (Sreedhar et al., 1999), *Uromyces viciae-fabae* (Raucher et al., 1995), *Ustilago maydis* (Hellmich and Schauz, 1988), *Cochliobolus carbonum* (Murphy and Walton, 1996), and *Cladosporium cucumerinum* (Robertson, 1984). Pathogens of dicots may require arabinosidases to act synergistically with proteases to achieve extensin degradation (Carlile et al., 2000). In fact, pathogens may require a set of wall-degrading enzymes acting in concert for causing cell wall degradation and disease development (Carlile et al., 2000).

5.14 REQUIREMENT OF SEVERAL CELL WALL–DEGRADING ENZYMES TO DEGRADE THE COMPLEX-NATURED CELL WALL

Since plant cell walls contain different polysaccharides, proteins and lipids, which are interwoven, a single enzyme may not be sufficient to degrade the cell wall efficiently. Multiple cell wall–degrading enzymes may be necessary for full virulence of fungal pathogens (Valsangiacomo et al., 1992). An avirulent isolate of *F. solani* f. sp. *pisi* became highly virulent when the spore suspension was supplemented with pectinase, PME, cellulase and cutinase (Köller et al., 1982a). Supplementation by the individual enzymes was not effective suggesting that all enzymes are essential for penetration and virulence (Köller et al., 1982a). Carlile et al. (2000) suggested that the wheat pathogen *Stagonospora nodorum* might require a set of enzymes such as xylanases, arabinosidases, and proteases for its full virulence.

5.15 PRODUCTION OF SUITABLE ENZYMES IN APPROPRIATE SEQUENCE BY FUNGAL PATHOGENS

Successful pathogens produce cell wall–degrading enzymes in sequence. *Verticillium albo-atrum* grown in a medium containing tomato cell walls secreted a range of polysaccharide-degrading enzymes (Cooper and Wood, 1975). Endo-PG increased rapidly after 2 days, followed later by exo-arabinase and endo-PGTE. Endo-xylanase and cellulase were produced at low basal levels up to 6 days after which production increased rapidly; endo-xylanase productions preceded that of cellulase by 24 h (Cooper and Wood, 1975).

Fusarium oxysporum f. sp. *lycopersici* produced xylanase, proteases, PLs, and exo- and endo-PGs (Christakopoulos et al., 1995; Di Pietro and Roncero, 1996a,b; García-Maceira et al., 1997; Huertas-González et al., 1999; Ruíz-Roldán et al., 1999). The fungus produced endo-PG, endo-PGTE, cellulase, arabinase, xylanase, and β-galactosidase in sequence (Cooper and Wood, 1975). *Colletotrichum lindemuthianum* when grown on plant cell walls produced PG followed by arabinase, xylanase, and cellulase (English et al., 1971). A sequential production of enzymes appears to be needed for effective cell wall degradation. Any change in the sequence may make the cell wall resistant to the pathogen.

The pathogens may adapt themselves to the cell wall composition of their hosts and secrete suitable enzymes to degrade the host cell wall. When wheat seedlings were inoculated with *Rhizoctonia cerealis*, only xylanase activity increased. Cellulase, PG, and pectin lyase activities could not be detected in *R. cerealis*-infected wheat seedlings (Cooper et al., 1988). In contrast, when *R. cerealis* was inoculated on potato cell walls, pectin lyase activity increased severalfold, whereas xylanase activity remained low (Cooper et al., 1988). These results suggest that the pathogen may produce different enzymes depending on the type of host cell wall it comes into contact with.

Production of these cell wall–degrading enzymes aids in colonization of host tissues by pathogens. However, it is well known that saprophytes like *Aspergillus* and *Penicillium* also produce these enzymes in plenty. Probably, production of these enzymes in appropriate site, in appropriate sequence, and in appropriate amount may be important for pathogenesis.

5.16 REINFORCEMENT OF HOST CELL WALL DURING FUNGAL INVASION

The first plant defense against many potential pathogens is the interlocking network of macromolecules in the host cell wall. If this barrier is breached, the cell attempts to limit the spread of the infection by several ways. One common response is formation of cell wall appositions that have been shown to be induced quickly after the onset of fungal penetration, some times in a matter of only a few hours (Lyngkjær and Carver, 1999; Lee et al., 2000). These appositions are called papillae (Kang and Buchenauer, 2000) and they typically comprise a callose matrix (Brammall and Higgins, 1988) variously incorporating pectic materials (El Ghaouth et al., 1994), cellulose, suberin, gums, proteins (including peroxidase enzymes), calcium, and silicon (Aist, 1983). Lignification of papillae occurs and it confers extra resistance to penetration (Asiegbu et al., 1993, 1994).

Upon penetration by powdery mildew fungi, within the host cell, another reactive material, the collar, is deposited along the haustorial neck. In some cases, this collar may develop to such an extent that the entire haustorium is encased, thus reducing the haustorial function by acting as a barrier to nutrient uptake (Godwin et al., 1987; Cohen et al., 1990). The collar is made of two distinct areas, one amorphous and the other fibrillar. Neither the amorphous nor the fibrillar material contained chitin that is of fungal origin. Cellulosic β-1,4-glucans were found to be restricted to the outermost fibrillar layers. The presence of collars is usually associated with poor development of the haustoria (Hajlaoui et al., 1991). The collar may act as a barrier to apoplastic flow in rust fungi (Stumpf and Gay, 1989). The collar may develop from papillae that form before or during host cell wall penetration (Manners and Gay, 1983). In hop (*Humulus lupulus*) plants infected with *Sphaerotheca fuliginea*, the collar was found to contain callose-like deposits (Cohen et al., 1990).

The haustorium in case of powdery mildew fungi is surrounded by an extrahaustorial matrix (Manners and Gay, 1983). The extrahaustorial matrix may be composed of a mixture of plant- and fungus-derived compounds (Chong et al., 1985). When *Sphaerotheca pannosa* var. *rosae* was inoculated on rose (*Rosa hybrida*) leaves, fungal growth in the epidermis was associated with the formation of haustoria, which appeared multilobed and delimited by an extrahaustorial membrane probably originating from the host plasmalemma. In the extrahaustorial matrix, cellulose and pectin were absent (Hajlaoui et al., 1991).

The extrahaustorial matrix formed in pea leaf cells infected with *Erysiphe pisi* was a fluid that reacted less intensely than the extrahaustorial membrane with polysaccharide agents and was removed by enzyme degrading the host cell wall (Gil and Gay, 1977). The matrix around haustoria of rust fungi contained a mixture of lipids and large amounts of polysaccharides and proteins (Chong et al., 1984, 1985). In the flax (*Linum usitatissimum* L.) rust infections, the extrahaustorial matrix contained bound sugars, probably glycoproteins (Coffey and Allen, 1983).

5.17 PAPILLAE SUPPRESS FUNGAL PENETRATION

Papillae formation is seen in both compatible and incompatible interactions (Lyngkjær and Carver, 1999; Kang and Buchenauer, 2000; Gjetting et al., 2004). Papillae may function as a resistance mechanism (Brammall and Higgins, 1988; Cohen et al., 1989). Use of inhibitors of papilla formation in various host–pathogen systems has provided evidence for the role of

the papilla in restricting fungal penetration (Stewart and Mansfield, 1985; Gold et al., 1986). When coleoptiles of a barley line resistant to *Erysiphe graminis* f. sp. *hordei* were inoculated with the pathogen, more papillae were formed compared with that in a susceptible barley line. Penetration efficiency of the conidia was also less in the resistant variety and only a few haustoria were formed (Bayles et al., 1990). When the coleoptiles were treated with 2-deoxy-D-glucose (DDG), papillae formation was inhibited in the resistant variety resulting in more penetration efficiency of the conidia and more haustoria formation (Bayles et al., 1990). Thus, when papilla formation is inhibited, penetration is successful. When papilla formation is induced, the plant becomes resistant. Chitosan, when applied as a stem scar treatment, reduced lesion development in bell pepper (*Capsicum annuum* cultivar Bellboy) fruit caused by *Botrytis cinerea* (El Ghaouth et al., 1994). In the untreated tissue, deformation of primary cell wall was observed, whereas in chitosan-treated fruit tissue no cell damage was observed due to fungal inoculation. In chitosan-treated tissue inoculated with the pathogen, papilla formation in host cell was observed (El Ghaouth et al., 1994).

The time-course studies have also demonstrated the role of papillae in restricting fungal penetration (Hachler and Hohl, 1984; Stumm and Gessler, 1986). Delaying or inhibiting papilla formation induces susceptibility (Gold et al., 1986). In barley varieties resistant to *Blumeria graminis*, size of the papillae was larger than that observed in susceptible varieties at the fungal penetration sites (Yokoyama et al., 1991). Oversize papillae have been implicated in host resistance to pathogens (Smart et al., 1986a,b; Kunoh, 1990).

The efficacy of papilla formation as a resistant mechanism depends upon its early initiation. A low penetration efficiency of the fungal pathogens was correlated with papillae that were formed in advance of penetration pegs (Aist and Israel, 1977). When papilla deposition started earlier and increased faster, potato plants became resistant to *Phytophthora infestans* (Stromberg and Brishammar, 1993). Earlier papilla initiation is an important factor for papilla-mediated resistance (Gold et al., 1986; Bayles et al., 1990). The frequency of papilla formation is also important. When water extract of *Reynoutria schaliensis* was sprayed on cucumber leaves, it induced resistance against the powdery mildew fungus, *Sphaerotheca fuliginea* (Schneider and Ullrich, 1994). The treatment induced an increased frequency of papillae (Schneider and Ullrich, 1994).

The factors contributing to papilla formation in infected tissues have been studied (Inoue et al., 1992, 1993, 1994a,b). A partially purified aqueous extract from barley seedlings enhanced oversize papilla formation at fungal penetration sites and reduced penetration efficiency (Yokoyama et al., 1991). The extract was referred to as papilla-regulating extract (PRE). Coleoptiles of susceptible barley variety were floated on PRE solution (Inoue et al., 1994a). Papillae were initiated about 23 min earlier in PRE treatments than in controls. In addition, papillae were initiated 20 min before penetration peg initiation in the PRE treatments, whereas they were initiated 8 min after penetration peg initiation in the control. Mean papilla diameter at the time of initiation of penetration pegs was significantly greater in PRE treatments than in controls (Inoue et al., 1994a). The results suggest that PRE may be responsible for earlier papillae formation and development.

The PRE appears to contain potassium phosphate. Potassium phosphate, extracted from uninoculated barley leaves, induced papilla-mediated resistance against the powdery mildew fungus, *Blumeria graminis*, in barley coleoptiles (Inoue et al., 1994b). Formation of resistant, oversize papillae was observed to be induced by $Ca(H_2PO_4)_2$ solution (Smart et al., 1986b). Phosphate salts induce local and systemic resistance against northern leaf blight and common rust in maize (Reuveni et al., 1992a,b).

Both PRE-induced resistance and PRE induction of oversize papillae were Ca^{2+} mediated (Inoue et al., 1994a). Calcium and phosphorous have been reported to be highly concentrated in $Ca(H_2PO_4)$-induced oversize papillae (Kunoh et al., 1986; Kunoh, 1990). Calcium and

phosphorous are also found in both cytoplasmic aggregate vesicles and developing papillae in epidermal cells of barley leaves (Akutsu et al., 1980).

5.18 CALLOSE DEPOSITION IN CELL WALL

Papillae contain callose and papillae cannot be formed without callose in many host–pathogen interactions (Bayles et al., 1990). All papillae that were formed in barley contained callose (Bayles et al., 1990). Callose is a polymer of β-1,3-glucans, and it is a minor component of healthy plant tissue. Plants respond to infection by pathogens or treatment with elicitors by the rapid deposition of callose (Schmele and Kauss, 1990; Robertson et al., 1999; Trillas et al., 2000; Ton and Mauch-Mani, 2004). Callose deposition around the infection sites is an important cell wall fortification process in plants (Hamiduzzaman et al., 2005; He and Wolyn, 2005).

The paratracheal parenchyma cells adjacent to infected xylem vessels in egg plant (*Solanum melongena*) stem tissues inoculated with *Verticillium albo-atrum* responded by rapidly sealing off attempted sites of penetration (Benhamou, 1995). Electron-opaque, globular structure accumulated in paramural spaces. Large amounts of callose were detected in the electron-opaque globules (Benhamou, 1995). Infected epidermal cells of mushmelon showed deposition of callose-like materials around penetration pegs of the fungal pathogen *Sphaerotheca fuliginea* (Cohen et al., 1990). Cucumber leaves inoculated with *Colletotrichum lagenarium* showed induction of callose-containing papillae (Binder et al., 1989).

Biosynthesis of callose involves the enzyme β-1,3-D-glucan synthase (callose synthase). The UDP-glucose is converted into a β-1,3-glucan (callose) by the enzyme (Lawson et al., 1989). β-1,3-Glucan synthase has been purified from plasma membranes of several host cells (Fink et al., 1990; Frost et al., 1990; Dhugga and Ray, 1991; Dugger et al., 1991; Fredrikson et al., 1991). The purified β-1,3-glucan synthase from *Beta vulgaris* contains polypeptides with molecular masses of 92, 83, 70, 57, 43, 35, 31/29, and 27 kDa suggesting the existence of a multisubunit enzyme complex (Wu et al., 1991). A 31 kDa polypeptide has been reported in purified preparations of soybean β-1,3-glucan synthase (Fink et al., 1990). In cotton, eight polypeptides have been found in the purified preparation of β-1,3-glucan synthase (Delmer et al., 1991).

Callose synthesis has been shown to be induced by Ca^{2+} (Kohle et al., 1985), and this induction is associated with a rise in cytoplasmic Ca^{2+} (Kauss, 1990). The fungal elicitors such as chitosan and β-1,3-glucans have been shown to increase Ca^{2+} level in the cytoplasm and induce callose deposition (Kohle et al., 1985; Masuta et al., 1991). Ca^{2+} stimulates the activity of β-1,3-glucan synthase (Kohle et al., 1985). Chitosan induced callose synthesis, and the synthesis was immediately stopped when external Ca^{2+} was bound by ethylene glycol-bis-2-aminoethyl ether-*N*,*N*′-tetracetate or cation exchange beads and partly recovered upon restoration of 15 μmol Ca^{2+}. Chitosan treatment induced callose synthesis in soybean cells; however, it did not induce β-1,3-glucan synthase activity in the soybean cells. In the presence of Ca^{2+}, however, the activity of the enzyme was about 15-fold to 25-fold stimulated. The first callose formation was detected in soybean cells in about 20 min after addition of chitosan. The speed of this response suggests that β-1,3-glucan synthase may be at the site before callose formation begins, but in an inactive state as this process may be too rapid to involve transcriptional de novo synthesis of enzyme proteins (Kohle et al., 1985).

β-1,3-Glucan synthase is localized on the cytoplasmic side of the plasma membrane (Fredrikson and Larsson, 1989). When the fungal pathogen attempts to penetrate the cell wall, the Ca^{2+} permeability of the plasma membrane is perturbed (Schmele and Kauss, 1990), and this perturbation would have increased activity of β-1,3-glucan synthase resulting in accumulation of callose (Ohana et al., 1992). Microsomes and plasma membranes, prepared from first leaves of cucumber, exhibited increased specific activity of the Ca^{2+}-regulated

β-1,3-glucan synthase. This enzyme activity was latent in epidermal cells of healthy leaves, but was activated by attempted penetration by *Colletotrichum lagenarium*. It resulted in rapid production of callose-containing papillae in cucumber leaves (Schmele and Kauss, 1990; Ohana et al., 1992).

Elevation of Ca^{2+} does not always lead to callose synthesis, and other unknown effectors may be required in addition to Ca^{2+} for elicitation of callose synthesis (Kauss et al., 1991). UDP-glucose:(1–3)-β-glucan synthesis is synergistically activated by both Ca^{2+} and a β-glucoside (Callaghan et al., 1988). β-Furfuryl-β-glucoside (FG) is a specific endogenous activator of plant β-1,3-glucan synthase (Ohana et al., 1992) and is present in a variety of plants (Ohana et al., 1991, 1992). However, glycosides are localized within the vacuole (Wagner, 1982) and hence may be inaccessible to the active site of β-1,3-glucan synthase, which is localized on the cytoplasmic side of the plasma membrane (Fredrikson and Larsson, 1989). In barley suspension-cultured cells, FG was found to be sequestered in the vacuole (Ohana et al., 1993). Lowering of cytoplasmic pH led to induction of callose synthesis. Addition of propionic acid to soybean cells elevated the percentage of FG found in the cytoplasmic compartment of soybean cells. Addition of an oligogalacturonide elicitor of DP 10–13 lowered cytoplasmic pH and caused a similar redistribution of FG. When the medium was supplemented with 100 mM $CaCl_2$, intracellular Ca^{2+} level was elevated. This treatment led to a relative elevation of FG in the cytoplasm. All treatments that led to a relative increase in cytoplasmic FG also led to stimulation of callose synthesis *in vivo* (Ohana et al., 1993). These results suggest that a relative redistribution of FG between cytoplasm and vacuole is a possible component of the signal transduction pathway for elicitation of callose synthesis *in vivo*. FG may be the required second signal, in addition to Ca^{2+}, and it should undergo redistribution to elicit callose synthesis. These studies suggest that callose accumulation in the infected tissues occurs due to changes in Ca^{2+} and pH levels, the processes triggered by fungal elicitors released during fungal pathogenesis.

5.19 HOW DO PATHOGENS OVERCOME THE PAPILLAE AND CALLOSE BARRIERS?

5.19.1 Pathogen Delays Papillae Formation

Papillae formation and callose deposition are common in both susceptible and resistant interactions. However, in susceptible hosts, pathogens appear to delay papillae formation in host cell. In susceptible barley leaves inoculated with *Blumeria graminis* f. sp. *hordei*, the processes leading to papilla formation were too slow for papillae to be effective as a resistance mechanism (Bayles et al., 1990). When tobacco plant is infected by *Phytophthora parasitica* var. *nicotianae*, cell appositions formed in response to infection, but expressed too late to account for an effective resistance to fungal colonization (Benhamou and Cote, 1992). Some of the chemicals are known to delay papilla formation and they are used to demonstrate that a delay in papillae formation may enhance penetration efficiency of pathogens. Chlortetracycline treatment delayed papillae formation in barley, resulting in increased penetration efficiency of *B. graminis* f. sp. *hordei* (Gold et al., 1986). DDG treatment delayed the papillae formation induced by *B. graminis* f. sp. *hordei* in barley, and this treatment resulted in an acceleration of the initiation of the penetration peg and haustorium formation (Bayles et al., 1990).

Papilla formation is induced by Ca^{2+} (Waldmann et al., 1988), and Ca^{2+} chelators induce susceptibility (Gold et al., 1986; Bayles and Aist, 1987). Less rapid triggering of increase in Ca^{2+} delays secretion of papilla precursors (Bayles et al., 1990). The fungal elicitor activates Ca^{2+} signal transduction system, and slower release of elicitor in susceptible interactions would have delayed the action of Ca^{2+}-induced papilla formation.

5.19.2 Pathogens May Suppress Callose Synthesis in Susceptible Interactions

Pathogens appear to suppress callose synthesis during their penetration process. Wheat line CS 2D/2M and cultivar Thew responded differently to *Puccinia triticina* strains 104-2,3,6,7,8 and 104-1,2,3,6. Callose deposition was suppressed in compatible interactions, whereas it was abundant in incompatible interactions (Table 5.3; Southerton and Deverall, 1990).

When *Plasmopara lactucae-radicis* was inoculated on susceptible and resistant cultivars of lettuce (*Lactuca sativa*), intercellular runner hyphae and intracellular haustoria could be seen within roots of both the cultivars within 96 h after inoculation. In the resistant cultivar, approximately 94% of the haustoria exhibited intense fluorescence when stained with aniline blue, indicating the deposition of callose and they appeared to be totally encased in callose (Stanghellini et al., 1993). In the susceptible cultivar, however, haustoria exhibited only a faint fluorescence that was localized around haustorial necks indicating less amount of deposition of callose (Stanghellini et al., 1993). In *Sphaerotheca fuliginea*–muskmelon interactions, callose deposition was observed only around the penetration pegs in the compatible interactions, whereas heavy callose deposition was observed in epidermal and mesophyll cells in resistant interactions (Cohen et al., 1990). Less callose deposition appears to favor pathogenesis in susceptible interactions (Cohen and Eyal, 1988). Papillae in susceptible interactions are smaller than those formed in resistant interactions (Stolzenburg et al., 1984a,b; Gold et al., 1986), and slower deposition of callose deposition has been reported in various susceptible reactions (Skou et al., 1984; Skou, 1985; Gold et al., 1986; Stumm and Gessler, 1986; Binder et al., 1989; Trillas et al., 2000).

Less amount of callose deposition appears to be due to inhibition of callose synthesis. Treatment of a genetically resistant lettuce cultivar with DDG, an inhibitor of callose synthesis by the plant, results in suppression of callose synthesis and susceptibility to *Plasmopara lactucae-radicis* (Stanghellini et al., 1993). β-1,3-Glucan synthase, the enzyme involved in synthesis of callose, is activated only in resistant interactions. *Colletotrichum lagenarium* inoculation systemically induced resistance in cucurbits against *C. lagenarium*. The induced resistance was associated with an increased ability to prevent penetration of the epidermal cell wall (Stumm and Gessler, 1986). Increased β-1,3-glucan synthase activity was detected in the induced resistant cucumber plants challenge-inoculated with *C. lagenarium* (Schmele and Kauss, 1990). Fungal cell wall components (elicitors) induce callose deposition (Kohle et al., 1985; Masuta et al., 1991). It is possible that in the susceptible interactions these

TABLE 5.3
Callose Deposition in Wheat Cells at Different Hours after Inoculation with *Puccinia triticina*

			Callose Deposition at Times after Inoculation (h)		
Wheat Variety	***P. triticina* Strain**	**Disease Reaction**	**20**	**36**	**48**
Line CS2D/2n	104-2,3,6,7,8	Compatible	−[a]	−[a]	−[a]
	104-1,2,3,6	Incompatible	+[b]	+[b]	+[b]
Cultivar thew	104-1,2,3,6	Compatible	−[a]	−[a]	−[a]
	104-2,3,6,7,8	Incompatible	−[a]	+[b]	+[b]

Source: Adapted from Southerton, S.G. and Deverall, B.J., *Physiol. Plant Pathol.*, 36, 483, 1990.

[a] − indicates no deposition.
[b] + indicates deposition as indicated by aniline blue staining.

elicitors would not have been released or released slowly (see Chapter 2), resulting in reduced callose deposition in the susceptible interactions.

5.19.3 Pathogens May Be Able to Penetrate the Papillae Barrier

In compatible interactions, pathogens have been shown to penetrate papillae. When *Heterobasidion annosum* penetrated cells in cortical and endodermal regions of Norway spruce (*Picia abies*), papillae were observed. However, not all hyphae in this region were restricted by papillae, and at a later stage of infection, fungal hyphae were able to penetrate through papillae (Asiegbu et al., 1994). *Sphaerotheca fuliginea*, the powdery mildew fungus, was inoculated on muskmelon (*Cucumis melo*) plants, and basic aniline blue calcofluor double staining technique was used for locating the penetrations induced by the fungus on the surface of the inoculated leaves (Cohen et al., 1990). Fungal structures (conidia, germ tubes, and hyphae) fluoresced blue under UV epifluorescence microscopy, whereas perforations of the host surface resulting from penetration by the fungus fluoresced intensive yellow due to the accumulation of callose material in the collar region surrounding the penetration peg of the fungus. A total of about 14 perforations per colony resulting from a single conidium were observed at 72 h after inoculation (Cohen et al., 1990). Similar results have been obtained in muskmelon infected with *Pseudoperonospora cubensis* (Cohen et al., 1989) and in hop cultivars infected with *Sphaerotheca humuli* (Godwin et al., 1987).

5.19.4 Pathogens May Degrade Callose by Producing β-1,3-Glucanase

Pathogens are known to produce β-1,3-glucanase to degrade callose (β-1,3-glucan). *Fusarium culmorum*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia cerealis* (Cooper et al., 1988), *Cochliobolus carbonum* (Van Hoof et al., 1991), *Cochliobolus heterostrophus* (Anderson, 1978), *Stagonospora nodorum* (Lehtinen, 1993), and *Claviceps purpurea* (Brockmann et al., 1992) have been reported to produce β-1,3-glucanases *in vitro*. All plant β-1,3-glucanases with a few exceptions are endoacting. β-1,3-Glucanase produced by the maize pathogen *Cochliobolus carbonum* is exo-acting and the molecular mass of the enzyme is 63,000 (Van Hoof et al., 1991). The enzyme could digest callose almost completely (Figure 5.8; Van Hoof et al., 1991).

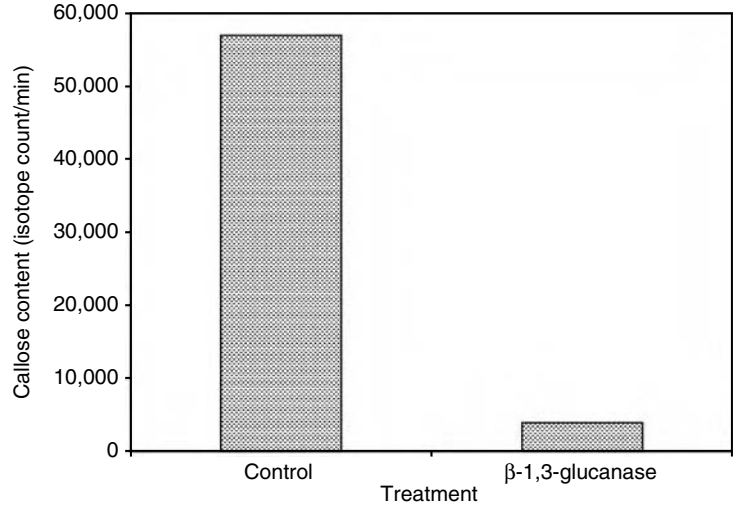

FIGURE 5.8 Digestion of radiolabeled callose by β-1,3-glucanase from *Cochliobolus carbonum*. (Adapted from Van Hoof, A., Leykam, J., Schaeffer, H.J., and Walton, J.D., *Physiol. Mol. Plant Pathol.*, 39, 259, 1991.)

Maize papillae contain callose (Hinch and Clarke, 1982) and *C. carbonum* efficiently penetrated the host cell wall probably by producing β-1,3-glucanase (Van Hoof et al., 1991).

Honey dew of *Claviceps purpurea*-infected rye (*Secale cereale*) plants showed high levels of β-1,3-glucanase activity (Dickerson and Pollard, 1983). The pathogen produces an extracellular endo-β-1,3-glucanase. The enzyme was purified and the molecular weight was about 90 kDa. It had an optimum pH of 4.5 and there was a rapid decline of activity at pH values beyond 5.0. The enzyme was identified as a glycoprotein, containing glucose and mannose units. The enzyme was strictly specific for β-1,3-linkages characteristic for callose (Brockmann et al., 1992). During the infection process callose was deposited in several areas of the ryeflower, namely at the top of the style and in the outer cell walls of stigma trichome cells. Secretion of β-1,3-glucanases at the interface between mycelium and plant tissue may result in degradation of callose (Brockmann et al., 1992).

5.20 ACCUMULATION OF HYDROXYPROLINE-RICH GLYCOPROTEINS IN PLANT CELL WALLS

5.20.1 Host Cell Wall Responds to Fungal Invasion by Accumulating HRGP

Although the fungal pathogen attempts to penetrate, HRGP accumulates in the host cell wall (Mazau et al., 1987). HRGP increased markedly in the cell walls of melon seedlings during infection by *Colletotrichum lagenarium* (Esquerré-Tugaye and Mazau, 1984). When *Colletotrichum lindemuthianum* was inoculated, a few intracellular hyphae became encased in a large deposit of host wall-like material (papillae) and did not develop further. HRGPs accumulated in these papillae (O'Connell et al., 1990). HRGPs accumulated in many other dicotyledonous plants due to infection with various pathogens (Table 5.4; Mazau and Esquerre-Tugaye, 1986). Similar accumulation of HRGPs has not been observed in monocotyledonous plants such as rice, barley, and wheat (Mazau and Esquerre-Tugaye, 1986).

5.20.2 Signals Triggering Accumulation of HRGPs

The accumulation of HRGPs in host cell walls due to fungal invasion appears to depend upon the signal generated by the fungal elicitor and ethylene as second messenger (Toppan et al., 1982). Elicitor from *Colletotrichum lindemuthianum* induced accumulation of HRGPs in bean (Bradley et al., 1992). HRGPs accumulated in soybeans inoculated with an elicitor from

TABLE 5.4
Changes in Levels of HRGPs in Cell Walls of Dicotyledonous Plants Infected by Pathogens

		HRGPs Expressed as Hydroxyproline Levels (μg/100 μg Cell Wall)	
Host	**Pathogen**	**Healthy**	**Infected**
Cucumber	*Colletotrichum lagenarium*	0.08	0.78
Bean	*Colletotrichum lindemuthianum*	0.17	0.46
Lucerne	*Colletotrichum trifolii*	0.25	0.49
Melon	*Fusarium oxysporum* f. sp. *melonis*	0.20	0.54
Grapevine	*Plasmopara viticola*	0.16	0.25
Tobacco	*Peronospora tabacina*	0.07	0.18

Source: Adapted from Mazau, D. and Esquerre-Tugaye, M.T., *Physiol. Mol. Plant Pathol.*, 29, 147, 1986.

Phytophthora megasperma f. sp. *glycinea* (Roby et al., 1985). Elicitor treatment stimulates transcription of genes encoding HRGPs (Corbin et al., 1987; Sauer et al., 1990). Ethylene treatment induces HRGPs accumulation in cell walls. When the petioles of melon plants were treated with *C. lagenarium* elicitor, the elicitation of ethylene occurred within the first few hours after the addition of elicitors, whereas elicitation of HRGPs was observed only after 18 h (Roby et al., 1985). An inhibitor of ethylene synthesis, aminoethoxy-vinylglycine (AVG), inhibited synthesis of HRGPs (Roby et al., 1985). Other specific inhibitors of the ethylene pathway such as 1-canalin inhibited both ethylene and hydroxyproline deposition in the cell wall of melon plants infected with *C. lagenarium* (Toppan et al., 1982). 1-Aminocyclopropane-1-carboxylic acid, the precursor of ethylene, triggered the synthesis of HRGP to the same extent as the elicitor of *C. lagenarium* (Roby et al., 1985). Wounding also induced HRGP accumulation (Ludevid et al., 1990; Tagu et al., 1992), and ethylene is known to increase due to wounding (Ecker and Davies, 1987). These results suggest that ethylene may be involved in the accumulation of HRGPs in plant cell walls due to fungal infection.

Ethylene induces HRGP mRNA accumulation. When mesocotyls of young maize plantlets were exposed to ethylene, HRGP mRNA accumulation was observed (Tagu et al., 1992). A 3-h ethylene treatment produced a 17-fold increase in HRGP mRNA (Tagu et al., 1992). The HRGP gene was detectable at 15 min with a maximum expression between 1 and 2 h (Tagu et al., 1992). These results suggest that the fungal elicitor may trigger ethylene biosynthesis and ethylene may trigger transcription of HRGP genes, which result in accumulation of HRGPs.

5.20.3 Host Cell Wall Responds to Fungal Invasion by Strengthening Its HRGPs by Glycosylation

Pathogens induce glycosylation of HRGP. The extent of glycosylation of hydroxyproline appears to offer resistance to pathogens. Infection of muskmelon seedlings by *Colletotrichum lagenarium* causes a 10-fold increase in the amount of cell wall HRGP, and the extent of glycosylation of hydroxylation was higher in the fungus-infected muskmelon cell walls (Esquerré-Tugaye and Lamport, 1979). The glycosylation is initiated by a protein:arabinosyl-transferase in the Golgi apparatus of the host cell (Bolwell et al., 1985). Elicitor from *Colletotrichum lindemuthianum* induced rapid induction of the protein:arabinosyl-transferase and increased levels of an arabinosylated hydroxyproline-rich protein in bean cells (Bolwell et al., 1985). It suggests that the pathogen induces glycosylation of HRGP by activating protein:arabinosyl-transferase, and the fungal elicitor may be involved in this process.

5.20.4 Insolubilization of HRGPs in Host Cell Wall

HRGPs become insolubilized in plant cell walls when attacked by pathogens (Chen and Varner, 1985). The insolubilization may be caused by the induced cross-linking of HRGPs by intermolecular isodityrosine residues and by a diphenyl ether linkage (Fry, 1982; Chen and Varner, 1985). The increased HRGP cross-linking may lead to a more impenetrable cell wall barrier, thus impeding pathogen infection. Cell walls, which undergo ultra rapid HRGP cross-linking, are tougher than cell walls of untreated cells for penetration (Showalter, 1993).

5.20.5 Enrichment of HRGPs by Lignin Deposition

HRGPs may act as matrices for lignification. When fungal pathogen invades host tissues, HRGPs accumulate and they provide a template for the subsequent deposition of lignin (Ride, 1983). Lignified papillae containing HRGP offer resistance to fungal penetration (Benhamou et al., 1990). The association between lignin deposition and HRGP accumulation

has been reported in several fungus-infected plants (Mazau and Esquerre-Tugaye, 1986; Corbin et al., 1987). Several cultivars of cucumber were inoculated with *Cladosporium cucumerinum*, and simultaneous accumulation of HRGP and lignin was observed in all cultivars that showed resistance to *C. cucumerinum* (Hammerschmidt et al., 1984). Enrichment of HRGPs with lignin in plant cell walls may offer resistance to pathogens.

5.20.6 Some HRGPs May Immobilize Plant Pathogens

Extensin, a type of HRGP, immobilizes certain plant pathogens. This agglutination response may result from positively charged extensin molecules interacting ionically with negatively charged surfaces of certain plant pathogens (Leach et al., 1982; Mellon and Helgeson, 1982).

5.20.7 How Does Pathogen Overcome HRGP Barrier?

5.20.7.1 Less Accumulation of HRGPs in Compatible Interactions

Fungal elicitor induces HRGP accumulation. In some of the susceptible interactions, action of the elicitors may be suppressed, as discussed in Chapter 2. When cucumber plants were inoculated with *Cladosporium cucumerinum*, HRGP accumulated only in resistant varieties. In all susceptible interactions the accumulation of HRGP in the cell wall was negligible (Table 5.5; Hammerschmidt et al., 1984). The amount of HRGP may not be sufficient for prevention of fungal penetration in the susceptible interactions.

5.20.7.2 Pathogen Overcomes HRGP Barrier by Delaying Accumulation of HRGPs in Host Cell Wall

In many host–pathogen interactions, HRGP accumulates almost equally in both compatible and incompatible interactions. In bean, cucumber, and lucerne (*Medicago sativa*) plants infected by *Colletotrichum lindemuthianum*, *Fusarium oxysporum* f. sp. *melonis*, and *Colletotrichum trifolii*, respectively, HRGP accumulates almost equally in both susceptible and resistant varieties (Mazau and Esquerre-Tugaye, 1986). Elicitor from *C. lindemuthianum* cell wall caused a marked and prolonged accumulation of three HRGP mRNAs from relatively low basal levels in unelicited cells (Showalter et al., 1985). In the compatible bean–*C. lindemuthianum* interactions, marked accumulation of HRGP mRNA occurred at about

TABLE 5.5
Accumulation of Hydroxyproline in Cucumber Cell Walls Inoculated with *Cladosporium cucumerinum*

		Hydroxyproline ($\mu g\ mg^{-1}$ Cell Wall)		
Cultivar	**Host Response**	**Control**	**Inoculated**	**% Increase**
SMR 58	Resistant	2.0	3.6	83
Salty	Resistant	2.2	3.9	79
Marketmore 70	Resistant	1.7	3.0	74
Marketer	Susceptible	1.8	1.7	−3
Shamrock	Susceptible	2.9	3.2	9
Gemini	Susceptible	2.0	2.3	12

Source: Adapted from Hammerschmidt, R., Lamport, D.T.A., and Muldoon, E.P., *Physiol. Plant Pathol.*, 24, 43, 1984.

150 h after inoculation, whereas 10-fold to 20-fold increase in HRGP mRNA was seen in about 50 h after inoculation in the incompatible interactions (Showalter et al., 1985).

When susceptible cucumber plants were inoculated with *Cladosporium cucumerinum*, HRGP accumulated at 48–72 h after inoculation, whereas similar accumulation was observed even at 12–18 h after inoculation in resistant cucumber plants (Hammerschmidt et al., 1984). In the resistant variety, the HRGP accumulation was observed at the time of initial penetration by the hyphae, whereas in the susceptible variety the HRGP enhancement was observed when water soaking and necrosis symptoms were observed (Hammerschmidt et al., 1984).

When tomato roots were inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*, cell walls of tomato roots were markedly enriched with HRGPs (Benhamou et al., 1991). Accumulation of HRGPs was seen in tomato cell walls invaded by the pathogen mostly during its necrotrophic phase. These observations suggest that the accumulation of HRGPs in susceptible tomato plants may be a late biochemical event (Benhamou et al., 1991).

Templeton et al. (1990) showed that *C. lindemuthianum* infection in an incompatible bean cultivar was characterized by an early (within 4 days) and massive accumulation of HRGP transcript in the epidermal, cortical, and perivascular tissues immediately adjacent to the inoculation site. In the compatible interaction, such accumulation was seen only 7 days after inoculation. These studies suggest that the fungal pathogens may be able to penetrate host cell wall, which does not have high amount of HRGP, and delayed accumulation of HRGP only during necrotrophic phase is not able to suppress the fungal development.

5.21 CELL WALL–BOUND PHENOLICS AND LIGNINS

5.21.1 Fortification of Plant Cell Wall by Phenolics and Lignin

Plant cell walls respond to invasion by fungal pathogens by accumulating phenolics and phenolic polymers such as lignins (Coffey and Cassidy, 1984; Carver et al., 1998a,b). Induced resistance in barley against *Blumeria graminis* f. sp. *hordei* has been shown to be due to deposition of phenolics in the cell walls (Lyngkjær and Carver, 1999). Phenolics accumulated in epidermal cells attacked by appressoria in oats infected by *Blumeria graminis* (Carver et al., 1998a). Ferulic acid, *p*-coumaric acid, and sinapic acid are the predominant cell wall–bound phenolics, and lignins are the wall-bound polymerized phenolics (Bily et al., 2003). Cell wall–bound phenolics and lignins may contribute to disease resistance. Structural phenolics in grain have been implicated in resistance to ear rot of maize (Assabgui et al., 1993; Miller et al., 1997). Ferulic acid is the predominant phenolic compound of maize tissue and is mainly found bound to cell wall polysaccharides as a complex such as *O*-(5-*O*-[(E)-feruloyl]-α-L-arabinofuranosyl)-(1,3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylose (Bily et al., 2003). *p*-Coumaric acid is the second most frequent phenolic acid in maize walls and is generally bound to lignin. Bound forms of ferulic acid can be dimerized by peroxidases to form cross-links between arabinoxylans chains that fortify the cell wall (Ralph et al., 1994; Fry et al., 2000). Several forms of dehydrodimers of ferulic acid have been discovered in plant cell walls (Grabber et al., 1995). Dehydroferulic acid content was found to be correlated with resistance to *Fusarium graminearum* in maize (Bily et al., 2003). Dehydroferulic acids may strengthen the physical integrity of the cell wall by linking hemicellulose chains together (Bily et al., 2003). The dehydroferulic acids may impede cell wall degradation caused by cell wall–degrading enzymes produced by pathogens (Grabber et al., 1998). Resistance to the brown rot fungus, *Monilinia fructicola*, was found to be related to high concentrations of chlorogenic and caffeic acids found in the cell layers of peach genotypes. These phenolics inhibited cutinase production by the pathogen (Bostock et al., 1999). These observations suggest that the wall-bound phenolics may induce resistance by suppressing the activities of wall-degrading enzymes of the pathogen.

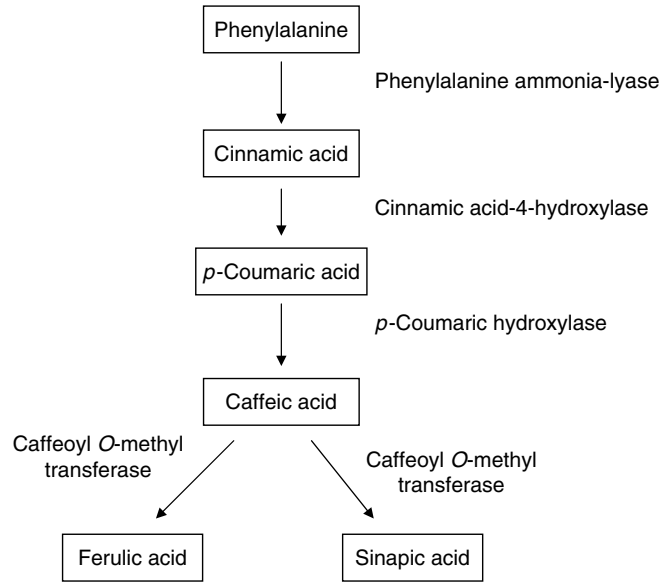

FIGURE 5.9 Biosynthesis of wall-bound phenolics. (Adapted from Vidhyasekaran, P., *Physiology of Disease Resistance in Plants*, Vol. I, CRC Press, Boca Raton, 1988; de Ascensao, A.R.F.D.C. and Dubery, I.A., *Phytochemistry*, 63, 679, 2003.)

5.21.2 Biosynthesis of Phenolics and Lignins

The pathway of synthesis of major cell wall–bound phenolics is presented in Figure 5.9. Phenylalanine ammonia-lyase (PAL), cinnamic acid-4-hydroxylase (CAH), *p*-coumaric hydroxylase (CH), and caffeoyl-CoA 3-*O*-methyltransferase (CCOAMT) are the key enzymes involved in biosynthesis of wall-bound phenolics.

The lignin biosynthetic pathway has been studied for more than a century but has undergone major revisions over the past decade (Boerjan et al., 2003). The pathway of lignin synthesis is not yet completely understood and the lignin roadmap is rewritten frequently (Humphreys and Chapple, 2002). The possible pathway of synthesis of lignins is given in Figure 5.10. Monolignols are the precursors of lignin biosynthesis. Coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol are the important monolignols, and their mode of biosynthesis is presented in Figure 5.10.

p-Hydroxyphenyl alcohol and cinnamyl alcohol are also considered as monolignols (Humphreys and Chapple, 2002). PAL, 4-coumarate:CoA ligase (4CL), coumarate alcohol dehydrogenase, CCOAMT, coniferyl-CoA reductase, coniferyl alcohol dehydrogenase, sinapoyl-CoA reductase, sinapoyl alcohol dehydrogenase, and peroxidases are the important enzymes involved in biosynthesis of lignins. CCOAMT is a bifunctional enzyme and acts on caffeic/5-hydroxyferulic acid (Cui et al., 2000).

5.21.3 Phenolic Deposition in Host Cell Wall in Response to Fungal Invasion

Phenolic compounds are fluorescent, and appearance of fluorescent materials in diseased plant tissue is considered to be due to the presence of phenolic materials that accumulate in the tissue as the host attempts to limit the development of the pathogen (Cohen et al., 1990). Autofluorescence of host cells is an early response of host to fungal infection (Kunoh et al., 1982, 1983). The autofluorescence occurs early in the interaction of the host and the pathogen and is observed in association with attempted penetration of the host cell by the primary germ

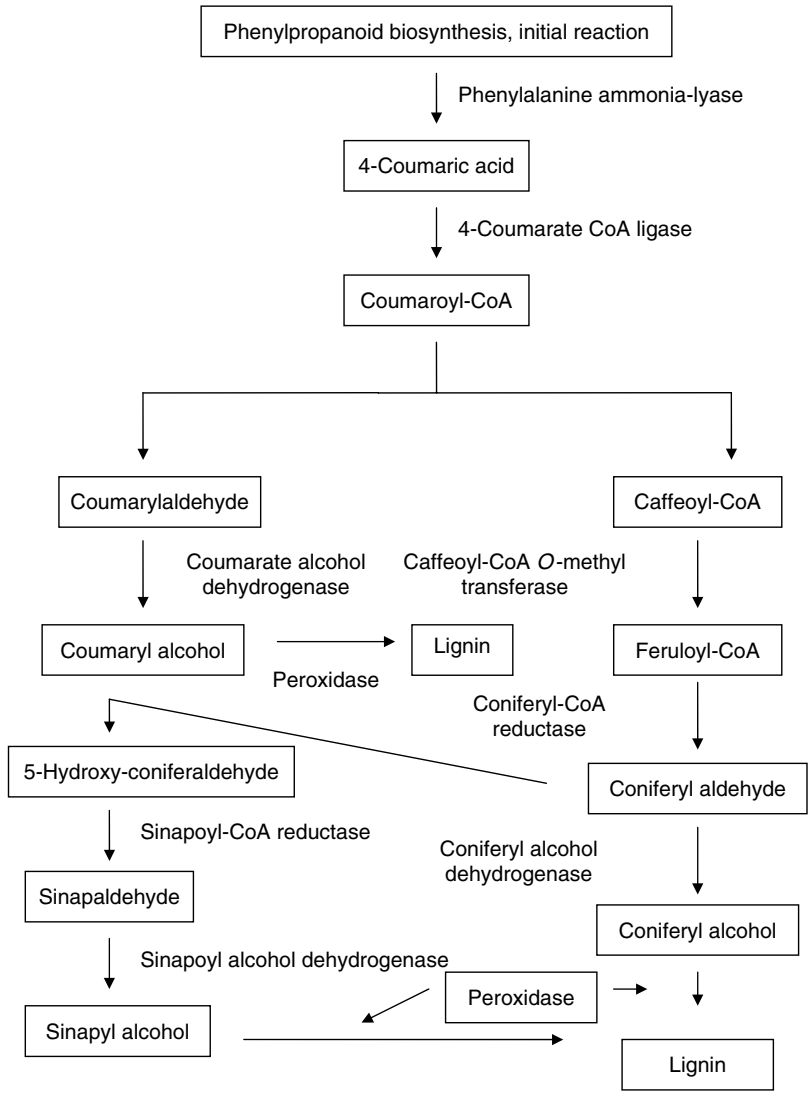

FIGURE 5.10 Biosynthesis of lignin. (Adapted from Humphreys, J.M. and Chapple, C., *Curr. Opin. Plant Biol*., 5, 224, 2002; Boerjan, W., Ralph, J., and Baucher, M., *Annu. Rev. Plant Biol*., 54, 519, 2003.)

tube and the appressorium. Localized autofluorescence is a common epidermal cell response to *Blumeria graminis* appressoria on barley (Kunoh et al., 1982; Koga et al., 1983), oat (Carver et al., 1998a), and wheat (Tosa et al., 1990; Carver et al., 1991, 1992a,b). This autofluorescence is localized in limited regions of the host cell wall surrounding fungal germ tube contact sites and in papillae deposited by host epidermal cells as a response to primary and appressorial germ tube contact (Aist and Israel, 1986). PAL inhibitors suppressed accumulation of localized autofluorogens in oats (Carver et al., 1992a) suggesting the accumulation of phenolics.

Tomato cell cultures inoculated with *Verticillium albo-atrum* accumulated up to fivefold higher level of wall-bound phenolics than were found in uninoculated control cultures (Figure 5.11; Bernards and Ellis, 1991). The analysis of this cell wall–bound material revealed that two populations of phenolic material existed. The first comprised esterified compounds

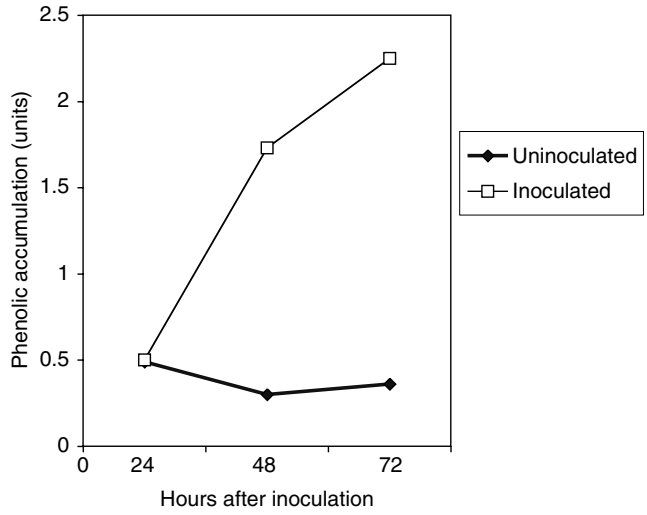

FIGURE 5.11 Accumulation of wall-bound phenolics in *Verticillium albo-atrum*-inoculated tomato cell suspension cultures. (Adapted from Bernards, M.A. and Ellis, B.E., *Plant Physiol*., 97, 1494, 1991.)

and the second comprised nonbase-labile polymeric material (Bernards and Ellis, 1991). An array of phenolic compounds was detected in wall preparations of inoculated cell cultures, whereas walls from uninoculated cells yielded only traces of esterified phenolics. A major component in this hydrolyzable phenolic population was sinapic acid (Bernards and Ellis, 1991).

When potato leaves were inoculated with *Phytophthora infestans*, two classes of phenolics accumulated in host cell wall. One class consisted of hydroxybenzoic and hydroxycinnamic acids, such as 4-hydroxybenzoic, 4-coumaric, and ferulic acids. The other class consisted of hydroxycinnamic amides, primarily 4-coumaroyltyramine and feruloyltyramine (Friend, 1981; Clarke, 1982). Accumulation of cell wall–bound phenolics in *Fusarium oxysporum* f. sp. *dianthi*-infected carnation stems has been reported (Niemann and Baayer, 1988; Niemann et al., 1991a,b).

5.21.4 Host Cell Wall Responds to Fungal Invasion by Activating Enzymes Involved in Synthesis of Wall-Bound Phenolics

PAL, the first enzyme in phenylpropanoid pathway, was induced in tomato cell cultures, both at the enzyme and mRNA level by *Verticillium albo-atrum* inoculation (Bernards and Ellis, 1991). Maximum enzyme activity occurred by 36–48 h postinoculation, concomitant with a marked accumulation of PAL mRNA. Only a low level of PAL and its mRNA were detectable from uninoculated cell cultures (Bernards and Ellis, 1991). The accumulation of wall-bound phenolics in inoculated tomato cell cultures was inhibited by the PAL inhibitor 2-amino-2-indanephosphate (AIP) (Bernards and Ellis, 1991). Benzothiadiazole (BTH) treatment induced systemic resistance in the susceptible wheat to *Blumeria graminis* f. sp. *tritici* (Stadnik and Buchenauer, 2000). It induced rapid synthesis of phenolic acids esterified to cell wall in wheat leaves inoculated with *B. graminis* f. sp. *tritici* (Stadnik and Buchenauer, 2000). When oat plants were inoculated with *B. graminis*, PAL activity increased, resulting in accumulation of phenolics (Carver et al., 1998a). When potato leaves were inoculated with *Phytophthora infestans*, rapid increase in the rate of PAL transcription was observed within 1–2 h (Cuypers et al., 1988). Increases in activities of PAL and tyrosine carboxylase in

the *P. infestans*-infected tissues led to the synthesis of wall-bound phenolics (Hahlbrock and Scheel, 1989). Within 3–4 h after inoculation with *B. graminis*, there was an increase in synthesis of cinnamic acid, and increase in the level of PAL activity occurred by as early as 2 h after inoculation in barley leaves (Shiraishi et al., 1989). These events corresponded with the time of attempted penetration by the *B. graminis* primary germ tube (Shiraishi et al., 1989).

Shiraishi et al. (1995) observed increases in PAL activity at two different times in barley cultivars inoculated with *B. graminis*. The first increase in enzyme activity began at 3 h after inoculation and this was followed by a second increase in activity between 12 and 15 h after inoculation. The conidium produces a primary germ tube that attempts penetration beginning about 2 h after inoculation and an appressorium that attempts to penetrate beginning 9–10 h after inoculation. Thus, it appears that the elevation of PAL enzyme levels by hosts is a direct response to attempted penetration by the fungus.

The first detectable increase in PAL mRNA occurred at 30 min after inoculation with *B. graminis* in barley leaves (Shiraishi et al., 1995). This increase in the level of mRNA was at a time considerably earlier than the time of attempted penetration by the fungus. The increase in mRNA may be due to release of elicitor from the fungal cell wall (Shiraishi et al., 1995). Clark et al. (1994) showed that PAL transcript accumulated in barley cultivars inoculated with *B. graminis*. The initial PAL transcript accumulation occurred between 4 and 6 h and declined to near constitutive levels by 8–10 h. The second peak was from 10 h until 12 h and then declined until 15–18 h. The first increase occurred due to response to the fungal germ tube contact, whereas the second increase was due to appressorial contact (Clark et al., 1994). The increase in PAL transcripts followed increase in PAL activity (Clark et al., 1994).

The monomeric precursors for the deposition of wall-bound phenolics are hydroxylated and methoxylated cinnamyl alcohols synthesized in two steps from the corresponding coenzyme A esters by cinnomyl-CoA reductase and cinnamyl alcohol dehydrogenase (CAD) (Grisebach, 1981). CAD mRNA appeared within 90 min after elicitor treatment in bean cell cultures (Grand et al., 1987).

Challenge of parsley cell suspension cultures with fungal elicitors induced the formation of ferulic cell wall esters (Hahlbrock and Scheel, 1989; Pakusch et al., 1989). The incorporation of ferulic acid and related acids into cell walls is a widespread phenomenon and invariably requires activation of *S*-adenosyl-L-methionine:*trans*-CCOAMT, which is responsible for the formation of feruloyl-CoA (Pakusch et al., 1989). CCOAMT has been characterized from elicitor-treated parsley cells (Pakusch et al., 1990). This enzyme possesses a narrow specificity for caffeoyl-CoA (Pakusch and Matern, 1991). CCOAMT is known to be induced de novo in cultured parsley cells upon the addition of elicitors, although noninoculated control cultures already contain fairly high background activity (approximately one-fifth of maximum) (Pakusch et al., 1989). In cultured parsley cells treated with an elicitor from *Phytophthora sojae*, the CCOAMT enzyme activity is rapidly induced by a transient increase in the rate of de novo transcription (Pakusch and Matern, 1991).

The rapid synthesis of phenolics and their polymerization in the cell wall is generally regulated by *p*-coumaric hydroxylase that is extremely pH dependent, and not by de novo enzyme synthesis. Membrane damage leads to decrease in cytoplasmic pH, which activates the hydroxylase (Matern and Kneusel, 1988).

5.21.5 How Does the Pathogen Overcome the Cell Wall–Bound Phenolics to Cause Disease?

5.21.5.1 Pathogen Suppresses Accumulation of Phenolics in Host Cell Wall

Several studies have indicated that successful pathogens may be able to suppress accumulation of phenolics in the plant cell wall. When *S.phaerotheca fuliginea*, the powdery

mildew fungus, was inoculated on resistant muskmelon plants, the infected epidermal cells emitted autofluorescence from their lumen. Autofluorescence indicates the presence of phenolics. The phenolics were present in cell walls of the resistant variety at 24–96 h after inoculation, but penetrated cells of the susceptible variety did not show any autofluorescence (Cohen et al., 1990).

Autofluorescence in cell walls has been shown to be associated only with the manifestations of resistance to *Pseudoperonospora cubensis* in muskmelon (Cohen et al., 1989), *Sphaerotheca humuli* in hop cultivars (Godwin et al., 1987), and to *Sphaerotheca pannosa* in rose leaves (Conti et al., 1986). In the susceptible varieties all these pathogens suppressed the accumulation of phenolics in epidermal cell walls (Conti et al., 1986; Godwin et al., 1987; Cohen et al., 1989).

Suppression of PAL has been shown to induce susceptibility in resistant varieties. Oat leaves were infused with α-aminooxy acetic acid (AOA) or α-aminooxy-β-phenylpropionic acid (AOPP), both of which are inhibitory to PAL. The PAL inhibitors suppressed localized autofluorescent host cell responses to contact of germ tube of *B. graminis* f. sp. *avenae* and increased susceptibility to penetration from appressoria (Carver et al., 1992b). AOPP suppressed the phenolic accumulation in epidermal cell walls in barley and wheat also and this treatment made the hosts susceptible to their pathogens *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici*, respectively (Carver et al., 1992b). These observations suggest that in susceptible interactions, phenolic accumulation in plant cell wall is suppressed.

The amount of phenolic accumulation in plant cell wall in susceptible interactions is less than that in resistant interactions. All barley cultivars showed autofluorogenic compound accumulation at 24 h after inoculation with *B. graminis*. In the resistant interactions, higher frequency of intense autofluorescence at appressorium contact sites was observed when compared with the susceptible interactions (Clark et al., 1994). When barley leaves were inoculated with a nonpathogen, *Erysiphe pisi*, increase in the level of PAL mRNA was observed and the magnitude of PAL mRNA was higher when inoculated with *E. pisi* than that observed in barley leaves inoculated with the pathogen *B. graminis* (Shiraishi et al., 1995).

5.21.5.2 Pathogen Delays Synthesis of Cell Wall–Bound Phenolics

Synthesis of cell wall–bound phenolics may be delayed at the fungal penetration site in the susceptible interaction. When potato leaves were inoculated with *Phytophthora infestans*, rapid accumulation of PAL mRNA in a sharply confined area around the fungal penetration site was observed in incompatible interaction, whereas in the compatible interaction PAL mRNA accumulated more slowly and in a more diffuse and further spreading area (Cuypers et al., 1988). In the barley line susceptible to powdery mildew fungus, papillae were formed much later than those in the resistant line (Aist et al., 1988). Yellow autofluorescence (indicating phenolic synthesis) after excitation at 365 nm was emitted by stomatal cells and the cell walls around the necrotic stomata as early as 2 days after inoculation with *Plasmopara viticola* in the resistant *Vitis rotundifolia* (Dai et al., 1995). In contrast, a few stomatal cells with yellow autofluorescence were detected only 8 days after inoculation in lesions on the susceptible *Vitis vinifera* (Dai et al., 1995). The results suggest that delayed accumulation of phenolics in host cell walls favors pathogenesis.

5.21.6 Lignification during Fungal Pathogenesis

5.21.6.1 Host Cell Wall Responds to Fungal Invasion by Increasing Lignification Process

Lignification is a common response to plant infection (Carver et al., 1998a). Lignin is a difficult polymer for pathogens to degrade (Nicholson and Hammerschmidt, 1992). Lignification increased in potato inoculated with *Phytophthora infestans* (Henderson and

Friend, 1979) and in *Raphanus japonica* inoculated with *Peronospora parasitica* (Asada and Matsumoto, 1972) and *Alternaria japonica* (Asada and Matsumoto, 1967). The increased lignification is reflected in cucumber, potato, castor bean, pepper, and *Raphanus japonica* by enhanced peroxidase (Asada and Matsumoto, 1972; Hammerschmidt et al., 1982; Bruce and Galston, 1989; Ray et al., 1998; Zheng et al., 2005), in muskmelon by increased hydroxycinnamate:CoA ligase (Grand and Rossignol, 1982), in melon by increase in *p*-coumarate:CoA ligase (4CL) (Grand and Rossignol, 1982), and in potato by PAL (Henderson and Friend, 1979). PAL activity increased by more than 10-fold in pine (*Pinus* sp.) cell cultures after treatment with a fungal elicitor at 24 h after treatment, coinciding with the initiation of cell wall lignification (Campbell and Ellis, 1992). Treatment of pine cell cultures with the PAL inhibitor AIP suppressed lignification (Campbell and Ellis, 1992), indicating that PAL is involved in lignin biosynthesis. Inhibition of PAL by another PAL inhibitor, AOPP (α-aminooxy-β-phenylpropionic acid), reduced the localized accumulation of autofluorogenic compounds (indicating accumulation of phenolics) and suppressed the resistance induced by BTH in wheat against *B. graminis* f. sp. *tritici* (Stadnik and Buchenauer, 2000).

Treatment of soybean cotyledons with *Phytophthora sojae* wall glucan elicitor resulted in significant phenolic polymer deposition in the uppermost cell layers as early as 4 h. Phenolic polymer deposition continued rapidly and leveled off by 24 h (Graham and Graham, 1991). The phenolic polymers have been identified as lignin- and suberin-like polymers (Graham and Graham, 1991).

5.21.6.2 Pathogen Suppresses Lignin Deposition

Lignification is suppressed in compatible interactions and only in resistant interactions lignification is predominant (Cadena-Gomez and Nicholson, 1987; Dean and Kuc, 1987; Moerschbacher et al., 1989). Wheat line CS2D/M and cultivar Thew responded differently to *Puccinia triticina* strains 104-2,3,6,7,8 and 104-1,2,3,6. Lignin deposition was observed only in resistant interactions (Southerton and Deverall, 1990). The lignin content in the cell walls of the *Fusarium culmorum*-infected tissues of the susceptible wheat cultivar increased slightly, whereas the lignin accumulated intensely in the host cell walls of the infected wheat spikes of the resistant cultivars (Kang and Buchenauer, 2000). In the susceptible interaction between *Sphaerotheca fuliginea* and muskmelon, the fungal spore developed one or two germ tubes that penetrated into one or two epidermal cells. The penetration zones were surrounded in callose-like material, but no autofluorescence or lignin-like materials were observed in the penetrated epidermal cells. In the resistant varieties, however, the fungus developed a single germ tube that induced autofluorescence, callose accumulation, and lignification in the penetrated epidermal cells. In susceptible tissues, suppression of phenolics and lignification was observed (Cohen et al., 1990).

The metabolism of radiolabeled aromatic compounds in healthy and *Puccinia graminis* f. sp. *tritici*-infected wheat plants was studied. More radiolabel was found in the alcohol-insoluble nonhydrolyzable material of infected resistant leaves than in infected susceptible leaves suggesting that lignification is suppressed in the susceptible reaction of wheat to the stem rust fungus (Fuchs et al., 1967; Rohringer et al., 1967). Histochemical tests indicated the presence of lignin or lignin-like material in wheat cells responding hypersensitively to penetration by haustoria of *P. graminis* f. sp. *tritici* (Tiburzy, 1982).

In wheat plants resistant to *P. graminis* f. sp. *tritici*, inoculation with the pathogen resulted in accumulation of lignin in epidermal cells. Histochemical tests for lignin using phloroglucinol and chlorine-sulfite were positive in the infected wheat cells indicating the presence of coniferyldehyde group and syringylpropane units (Tiburzy and Reisener, 1990). Observation of autoradiograms of rust-infected leaves by transmission light and fluorescence

microscopy showed that the radiolabel of the infiltrated (^{14}C)-cinnamic acid was incorporated into autofluorescing necrotic epidermal cells. The incorporated radiolabeled material exhibited the solubility characteristics of lignin. No accumulation of label was found in rust-infected cells of the susceptible wheat cultivar (Tiburzy and Reisener, 1990). Lignification was observed in resistant interactions in cotton infected by *Verticillium dahliae*, and this was due to elevation of caffeic-*O*-methyltransferase, an enzyme involved in synthesis of lignin. Greater and earlier initial response was detected in the wilt-resistant cultivar than in the susceptible cultivar (Cui et al., 2000).

Lignification is common is healthy plants also. The increased lignification observed in resistant varieties appears to be a new type of lignin. Number of histochemical tests such as phloroglucinol–HCl test, toluidine blue-O-test, chlorine-sulfite test, and modified chlorine-sulfite test are being employed to detect the presence of lignin. When these tests were used to detect the deposition of lignin in infected leaves, additional lignin deposition was observed (Southerton and Deverall, 1989, 1990). The additional lignin observed in walls of wheat leaves due to leaf rust incompatible interaction may be different from that in uninfected leaves because of its green rather than blue-green response to toluidine blue. The failure to react to phloroglucinol may indicate an absence of substituted cinnamaldehyde groups. The additional lignin formed in the incompatible interaction may not be rich with syringic groups because of its failure to stain with chlorine-sulfite (Southerton and Deverall, 1989, 1990). This additional lignin was not detected in susceptible interaction.

5.21.6.3 Pathogen Suppresses Enzymes Involved in Lignin Biosynthesis

PAL, 4-coumarate:CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), and peroxidase are the important lignin biosynthetic enzymes. All these enzymes exhibited increased activities at very early stages of infection (8–16 h after inoculation) by *P. graminis* f. sp. *tritici* in both susceptible and resistant wheat lines. Subsequently, both PAL and 4CL activities decreased to the level of uninoculated controls in the susceptible interaction. In the resistant interaction, however, their activities continued to increase from 32 h onward until 6–7 days after inoculation (Moerschbacher et al., 1988). During this period, lignification of penetrated resistant host cells were observed (Reisener et al., 1986). In contrast, in the compatible interaction, PAL and 4CL activities stayed at the level of uninfected controls or frequently declined below this level (Moerschbacher et al., 1988). CAD and peroxidase activities followed the changes in activities of PAL and 4CL (Moerschbacher et al., 1988). Thus the pathogen appears to suppress the increases in all lignin biosynthetic enzymes.

Eucalyptus calophylla is resistant to *Phytophthora cinnamomi*, whereas *Eucalyptus marginata* is susceptible. In *E. calophylla*, PAL activity increased within 24 h of inoculation and the amount of lignin increased by as much as 53% above control levels due to infection. However, in the susceptible *E. marginata*, there was no increase in PAL activity when inoculated with the pathogen. The suppression of increase in PAL activity reflected in unchanged concentrations of lignin in the infected susceptible variety (Cahill and McComb, 1992). When the roots of the resistant variety were treated with the PAL inhibitor AOA, the synthesis of lignin and phenolics was suppressed and the resistant variety became susceptible. Typical susceptible symptoms were observed in the resistant variety (Cahill and McComb, 1992). Similarly application of AOA efficiently inhibited the accumulation of lignin in epidermal cells of *P. graminis* f. sp. *tritici*-infected wheat variety resistant to the pathogen. This treatment changed the resistant reaction to a susceptible one (Moerschbacher et al., 1990; Tiburzy and Reisener, 1990).

Application of inhibitors of CAD also inhibited lignification in wheat (Tiburzy and Reisener, 1990) and barley (Carver et al., 1994a,b) leaves and conferred susceptibility to rust and powdery mildew pathogens, respectively. When oat leaves were treated with

the CAD inhibitor OH-PAS (2-hydroxyphenyl amino sulfinyl acetic acid,1,1-dimethyl ester), the leaves became more susceptible to *B. graminis* f. sp. *avenae* and the treatment reduced localized autofluorescent cell responses (Carver et al., 1994a). These results suggest that suppression of CAD may contribute to suppression of lignin synthesis by the pathogen.

Peroxidase is associated with increased lignin synthesis in many host–pathogen interactions (Moerschbacher et al., 1988; Smith and Hammerschmidt, 1988; Zheng et al., 2005). Several peroxidase isozymes have been detected, and not all peroxidase isozymes are involved in enhancing lignin synthesis. Specific lignin-forming peroxidases have been identified. Several anionic and cationic peroxidases were detected in soybean cotyledons. Induction of cationic peroxidases was not correlated with lignin deposition, whereas induction of the second group of anionic peroxidases correlated with the lignin deposition (Graham and Graham, 1991). Stem infection of tobacco cultivar with *Peronospora tabacina* induced systemic resistance to *P. tabacina* (Ye et al., 1990). In tobacco cell walls, 12 peroxidase isozymes were detected, but two anionic isozymes, P37 and P35, alone increased markedly in induced resistant plants (Ye et al., 1990). These two anionic peroxidases appear to be involved in increased lignification, and disease resistance and their levels were not elevated in susceptible interactions (Ye et al., 1990). A new peroxidase isozyme (IN3) appeared in wheat inoculated with incompatible strains of *P. graminis* f. sp. *tritici* and this peroxidase was not detected in compatible interactions (Flott et al., 1989). When barley plants were inoculated with an avirulent strain of *B. graminis* f. sp. *hordei*, increased transcription of two peroxidase genes was observed, and this increase was not observed when inoculated with virulent strain of the pathogen (Thordal-Christensen et al., 1992). Thus specific peroxidases, which are involved in lignification, appear to be suppressed by pathogens in the compatible interactions.

5.21.6.4 How Does Pathogen Suppress Lignification in Host Cell Wall?

Elicitors of lignification have been detected in both susceptible and resistant plants. This host cell wall component is released by the incompatible pathogen; but the compatible pathogen prevents release of the component from the host cell wall (Robertsen, 1986). The elicitors have been identified as oligogalacturonides (Ridley et al., 2001; Boudart et al., 2003). The dodecamer and tridecamer of galacturonic acid are the most potent elicitors, and oligomers with less than eight galacturonosyl residues do not have elicitor activity (Jin and West, 1984; Robertsen, 1986). In the susceptible variety, quicker degradation of pectic substances by the pectolytic enzymes produced by the pathogen would have resulted in accumulation of pectic polymer of less than eight galacturonosyl residues and hence no lignification would have occurred (Robertsen, 1986).

Fungal cell wall also contains a molecule that elicits lignification. This molecule is released by host enzyme, and activation of the host enzyme is under the control of fungal cell wall component. The incubation of insoluble mycelial walls of *Chaetomium globosum* with carrot homogenates released heat-stable soluble factors that stimulated lignification in carrot cells (Kurosaki et al., 1986a). The activity of these substances was reduced after digestion by chitinase, and they were released by mild hydrolysis with the enzyme. The chitinase activity was induced in cultured carrot cells incubated with the fungal walls, and the soluble fragments liberated from the walls stimulated the biosynthesis of phenolic acids, which are precursors of the lignin synthesized in the cells (Kurosaki et al., 1986a).

A marked increase of chitinase activity was observed in cultured carrot cells incubated with insoluble mycelial walls of *C. globosum* (Kurosaki et al., 1986b). The enzyme activity increased almost immediately after the mycelial wall treatment. After a lag of 2–4 h, PAL activity increased and it was followed by an increase in phenolic acids (*p*-hydroxybenzoic acid, caffeic acid, and ferulic acid), which are precursors of lignins (Kurosaki et al., 1986b). The results showed that in cultured carrot cells incubated with insoluble mycelial walls,

chitinase activity increased first, followed by an increase in PAL. Phenolic acids accumulated in parallel with the induction of PAL activity (Kurosaki et al., 1986b). Peach canker disease is caused by *Leucostoma persooni* and *L. leucostoma*. The cell wall extracts of both the pathogens induced lignification in peach and induced resistance against the pathogen (Biggs and Peterson, 1990). Thus, fungal cell wall component can induce lignification. However, the release of this fungal molecule would have been suppressed in susceptible interactions.

5.22 SUBERIZATION DURING FUNGAL PATHOGENESIS

5.22.1 Host Cell Wall Responds to Fungal Invasion by Suberization

Suberin is deposited on cell walls of various plant tissues when the pathogen invades host tissues (Kolattukudy, 1981). Suberization was observed in both susceptible and resistant interactions in tomato (Chen et al., 2004a). Suberin is highly resistant to enzymatic degradation by pathogens and hence it is an effective barrier to penetration by fungal pathogens (Kolattukudy, 1981). When potato tubers were inoculated with *Verticillium dahliae*, a rapid increase in the number of layers of suberized cells over that found in controls was observed (Vaughn and Lulai, 1991). Several workers have shown that suberization is responsible for reinforcement of cell walls limiting ingress of pathogens into host (Espelie and Kolattukudy, 1985; Espelie et al., 1986; Biggs and Miles, 1988; Kolattukudy et al., 1989).

5.22.2 Biosynthesis of Suberin in Pathogen-Inoculated Host Cell Wall

Suberin is a complex biopolyester that comprises a phenolic (aromatic or lignin-like) domain attached to the cell wall and an aliphatic (lipid, hydrophobic) domain, which is probably attached to the phenolic domain (Lulai and Corsini, 1998). Synthesis of very long chain acids and alcohols, ω-hydroxylation, and conversion of the ω-hydroxyacids to the corresponding dicarboxylic acids are the major steps involved in the formation of the aliphatic components of suberin. ω-Hydroxyacid dehydrogenase and another dehydrogenase are involved in the suberization. Oxidation of the ω-hydroxy acid to dicarboxylic acid, which is unique to suberin, is catalyzed by a specific dehydrogenase induced for suberization (Kolattukudy, 1985). Anionic peroxidase present in cell walls catalyzes polymerization of phenolic monomers and covalent bond formation between cell walls and the phenolic polymer (Kolattukudy and Soliday, 1985). The production of suberin coatings is dependent on PAL activity (Street et al., 1986). Fungal infection activates all these enzymes (Mohan and Kolattukudy, 1990; Robb et al., 1991; Lee et al., 1992).

PAL induced synthesis of many phenolic acids, which are required for synthesis of suberin. Infection with *Fusarium oxysporum* f. sp. *dianthi* increased concentration of vanillic and ferulic acids and induced accumulation of amides of *p*-coumaric, benzoic, salicylic, methoxysalicyclic, *p*-anisic, and β-resorcyclic acids in carnation (Niemann et al., 1991b). Cross-linking of such phenolics forms a polymeric matrix. Such a matrix is made hydrophobic by attachment of aliphatic polyester domains and by deposition of highly nonpolar waxes into this layer. The formation of this layer is called suberization (Pearce and Rutherford, 1981; Kolattukudy et al., 1987). The formation of the aromatic matrix is the first step in suberization. This matrix is catalyzed by a highly anionic peroxidase in potato (Espelie and Kolattukudy, 1985). This peroxidase has been purified (Espelie and Kolattukudy, 1985) and it was found to be localized in the cell walls of the suberizing cells only (Espelie et al., 1986). This peroxidase cDNA has been cloned and sequenced (Roberts et al., 1988). The peroxidase gene was absent in normal tissue (Kolattukudy et al., 1989).

The aromatic domain of suberin is similar to lignin, and the polymerization of the aromatic components of suberin involves an isoperoxidase in a manner similar to that

involved in lignin biosynthesis. In potato slices, both the time course of appearance and the spatial distribution of an anodic isoperoxidase were highly correlated with suberization (Espelie and Kolattukudy, 1985).

5.22.3 Pathogen Delays Suberin Accumulation

During pathogenesis, suberization appears to be delayed in compatible interactions. In tomato, one of the earliest defense responses against *Verticillium albo-atrum* is the coating of xylem vessels and pit membranes with suberin, produced by the xylem parenchyma cells (Street et al., 1986; Robb et al., 1989, 1991). In the resistant variety, suberization was very rapid, beginning at 8–10 h after inoculation with the pathogen. In sharp contrast, almost none were visible in the susceptible interactions at that time. After 24 h, suberization was visible in the susceptible plants; but it was much less than that in resistant plants (Lee et al., 1992).

When *Verticillium dahliae* infects a resistant potato variety, a complete layer of suberized cells was formed within 2 days after inoculation, and on fourth day three or more suberized layers were formed. When tubers of susceptible potato variety were inoculated with the pathogen, however, no suberization was detected in the first few days following inoculation (Vaughn and Lulai, 1991). This delay would have helped the pathogen to penetrate host tissues. The ability of the plants to accumulate suberin also appears to determine susceptibility or resistance. Peach cultivars, which accumulate suberin faster after wounding, become resistant to *Leucostoma cincta*, the pathogen causing canker (Biggs, 1989). Deposition of the suberin provided protection against *Fusarium sambucinum* in potato. However, resistance to fungal infection did not begin to develop until after deposition of the suberin aliphatic domain was initiated. Total resistance to fungal infection was attained after completion of deposition of the suberin aliphatic domain within the first layer of suberizing cells (Lulai and Corsini, 1998). These results suggest that the deposition of the suberin aliphatic domain is responsible for resistance to *F. sambucinum*.

5.22.4 Pathogen May Suppress Suberin-Synthesizing Enzymes

The delay in suberin accumulation in compatible interactions appears to be due to suppression of suberin-synthesizing enzymes by the pathogen. PAL, the first enzyme involved in suberin synthesis, increased in both susceptible and resistant tomato plants because of infection with *V. albo-atrum* (Lee et al., 1992). There was an initial increase of about 30% in the PAL mRNA with a subsequent gradual drop to approximately normal levels in the resistant tomato plant. In contrast, the level in the susceptible plant did not increase and proceeded to drop until after 15 h when it was only 30% of normal (Lee et al., 1992). The results suggest that the fungal component may suppress or depress PAL mRNA levels in susceptible plants. It also indicates that the fungal pathogen may gain access to its host not simply by passively avoiding defense mechanisms but actually by actively inhibiting them (Lee et al., 1992).

Anionic peroxidase is involved in suberization, and in the susceptible tomato line, anionic peroxidase was induced 1 day later than that in a near isogenic line resistant to *V. albo-atrum* (Street et al., 1986). Cell suspension cultures from the resistant and susceptible tomato lines were incubated with an elicitor preparation from *V. albo-atrum*. Within 15 min, the anionic peroxidase mRNA could be detected in the resistant line. The resistant line responded to the elicitor by producing high levels of anionic peroxidase mRNA, which reached a maximum by 16 h and then the level decreased. The susceptible line showed only a very little induction (Mohan and Kolattukudy, 1990). The results suggest that the induction of suberization may be due to the fungal elicitor and the elicitor may not be active in the susceptible cultivar or a suppressor may suppress the elicitor activity in the susceptible interaction as discussed in Chapter 2.

5.22.5 Pathogens May Penetrate the Suberized Walls of Host Cells

Some fungal pathogens have been reported to penetrate suberized cell walls (Peterson et al., 1980; Chen et al., 2004a). Culture filtrates of *Fusarium solani* f. sp. *pisi* released soluble radioactivity from insoluble labeled suberin enriched preparations obtained from potato tuber periderm, indicating that the extracellular fluid contained enzymes that degraded the aliphatic and aromatic domains of suberin (Fernando et al., 1984; Kolattukudy, 1985). The extracellular enzyme responsible for releasing the aliphatic components of suberin was isolated in a highly purified state. This esterase, induced as a result of growth of the organism on suberin, was found to be identical to cutinase generated by the same fungus on cutin. They both had identical amino acid composition, molecular weight, kinetic properties, and sensitivity to inhibitors (Fernando et al., 1984). Probably, the esterase may be involved in cutin and suberin degradation.

5.23 DEPOSITION OF MINERAL ELEMENTS IN HOST CELL WALL IN RESPONSE TO FUNGAL INVASION

5.23.1 Silicon Deposition

Many mineral elements accumulate in host cell wall when inoculated with pathogens. Silicon deposition at fungal penetration sites has been reported on epidermal cells of barley attacked by *Blumeria graminis* f. sp. *hordei* (Carver et al., 1987) and on bean mesophyll cells infected by the bean rust fungus (Heath, 1979). Silicon was found deposited around the points of pathogen (*Sphaerotheca fuliginea*) penetration of cucumber leaf tissue (Samuels et al., 1991). Silicon has been implicated in the resistance of wheat, barley, beans, rose, and *Arabidopsis* against various fungal pathogens (Perera and Gay, 1976; Stumpf and Heath, 1985; Koga et al., 1988; Leush and Buchenauer, 1989; Ghanmi et al., 2004). Accumulation of silicon in leaves of cucumber decreases the severity of powdery mildew caused by *S. fuliginea* (Menzies et al., 1991). Rice variety resistant to *Magnaporthe grisea* showed higher accumulation of silicon in epidermal cell walls than the susceptible variety (Kim et al., 2002). Silicon has been reported to accumulate in the papillae of various plants because of infection (Koga et al., 1988; Blaich and Grundhofer, 1990; Menzies et al., 1991). Silicon has been shown to be a reinforcer of mechanical resistance against pathogens (Ghanmi et al., 2004).

Pathogens may suppress silicon deposition in host cell walls and induce susceptibility. When a nonpathogen of bean (*Uromyces vignae*) was inoculated, penetration of plant cell walls was prevented by rapid deposition of silica in walls of the bean cells (Heath, 1981a,b). When the pathogen (*Uromyces appendiculatus*) was inoculated, deposition of silicon was not seen or occurred too late or at levels too low to stop fungal infection (Fernandez and Heath, 1989; Ryerson and Heath, 1992). Several studies have suggested that silicon may act as a mechanical barrier to fungal ingress by accumulation at sites of fungal penetration (Ryerson and Heath, 1992). Silicon accumulation may trigger accumulation of phenolics, lignin, callose, and suberin in the cell wall (Menzies et al., 1991; Cherif et al., 1992a). Successful pathogens are able to suppress the silicon deposition in host cell walls (Cherif et al., 1992b; Ryerson and Heath, 1992).

5.23.2 Calcium Deposition in Papillae

Calcium accumulates in papillae during fungal infection. When roots of pine seedlings of a resistant variety were inoculated with *Cylindrocarpon destructans*, papillae were formed. The papillae contained high amount of calcium (Figure 5.12; Bonello et al., 1991). The insoluble

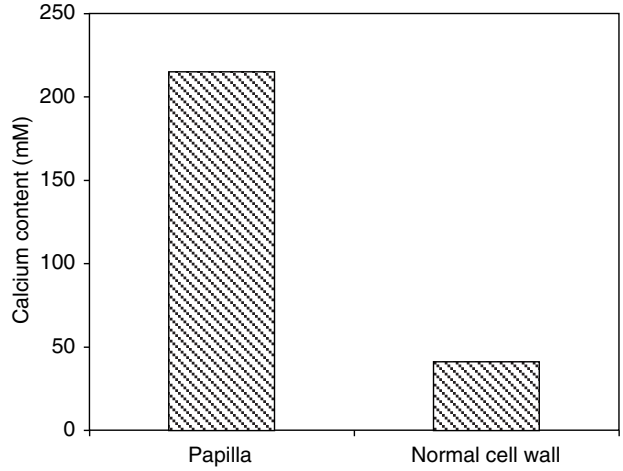

FIGURE 5.12 Concentration of calcium detected in papillae and normal cell walls of pine seedlings inoculated with *Cylindrocarpon destructans*. (Adapted from Bonello, P., Pearce, R.B., Watt, F., and Grime, G.W., *Physiol. Mol. Plant Pathol.*, 39, 213, 1991.)

calcium observed in the papillae may be a component of pectic materials. Calcium may harden plant primary walls by cross-linking of pectic polymers and confer resistance to pathogen attack (Akai and Fukutomi, 1980). Calcium is a mediator in polysaccharide synthesis (Aist, 1983; Kohle et al., 1985). Calcium is also known to induce callose synthesis in plant cell walls (Kohle et al., 1985; Kauss et al., 1989; Ohana et al., 1992, 1993).

5.23.3 Manganese Accumulation in Papillae

Manganese also accumulates in the papillae along with silicon and calcium (Carver et al., 1998b). Mn may be involved in phenolic synthesis in the cell wall (Zeyen et al., 1983). Mn is an inducer of PAL, and it also stimulates the production of hydrogen peroxide required for the polymerization of monophenols during lignin formation (Burnell, 1988). Thus, Mn may be involved in conferring resistance.

5.24 CONCLUSION

Plant cell wall appears to be an important barrier preventing penetration of fungal pathogens into host cells to obtain nutrition. The cell walls are made up of several complex carbohydrates, including cellulose, hemicellulose, and pectic polysaccharides, including homogalacturonans and rhamnogalacturonans. Cellulose is composed of several glucose units arranged in a chain configuration, connected by β-1,4-glycosidic bonds. Xylans, arabinans, xyloglucans, arabinoxylans, arabinogalactans, mannans, glucomannans, and galactoglucomannans are the complex hemicelluloses detected in plant cell walls. The carboxyl groups of the galacturonosyl residues of the cell wall pectic polysaccharides are highly esterified. Insoluble forms of pectin (protopectin) offer high resistance to the entry of fungal pathogens into host cells. Some cell wall proteins inhibit the attempt of fungal pathogens to degrade the pectic barrier producing pectolytic enzymes. Several enzymes including cutinases, PMEs, exo- and endo-PGs, PLs, pectin *trans*-eliminases, β-1,4-glucanases, β-1,4-cellobiohydrolases, β-glucosidases, xylanases, arabinosidases, arabinofuranosidases, xylosidases, and proteases are needed to breach this complex structure of plant cell walls. Further, these enzymes have to be produced in a defined

sequence and in sufficient amounts to degrade the complex components of cell wall. A delicate balance between the amounts of enzymes produced appears to exist. A low amount of the enzyme production results in oligomers triggering defense genes resulting in production of several defense-related compounds, such as phytoalexins and pathogenesis-related proteins, whereas high amount of production may result in tissue disintegration and rapid disease symptom development. Several cell wall components are known to inhibit the action of the fungal cell wall–degrading enzymes. Wall-bound phenolics and proteins inhibit the fungal enzymes.

Pathogens produce several cell wall–degrading enzymes on sensing host surface. A small quantity of constitutive enzymes is released on the host cell wall, and this may release a host signal/inducer from the host cell wall. It may result in production of bulk amount of these enzymes resulting in degradation of plant cell wall. Although not much work has been done to identify these signals, some signal molecules have been identified. When the pathogen tries to penetrate the plant cell wall, the plant tries to fortify its cell wall with callose, lignin, suberin, wall-bound phenolics, HRGPs, and some minerals. This fortified cell wall barrier is difficult to be penetrated by pathogens. However, potential pathogens try to postpone the fortification process and delayed fortification facilitates entry of the pathogens into host cell. The actual process of delaying the cell wall fortification events in susceptible interactions is still not known. Cell wall degradation and delay in cell wall fortification appear to be the key events during the fungal pathogenesis.

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6 Induction and Evasion of Pathogenesis-Related Proteins

6.1 INTRODUCTION

When fungal pathogens invade plant tissues, some new proteins appear and accumulate in the infected tissues. These proteins are called pathogenesis-related (PR) proteins, because they appear during pathogenesis (Vidhyasekaran, 2004, 2007). PR proteins are defined as proteins encoded by a host plant's genome that are induced specifically in pathological situations (Vidhyasekaran, 2002). Van Loon et al. (1994) suggested that to call a protein as a PR protein, the protein has to be newly expressed upon infection. This seems to be the most important criterion to define a PR protein (Van Loon, 1999). These PR proteins have been detected during pathogenesis in almost all plant species so far studied (Funnell et al., 2004; Piggott et al., 2004; Coram and Pang, 2005; Espino et al., 2005; Ito et al., 2005; Rodrigues et al., 2005; Santén et al., 2005; Wang et al., 2005; Wilkinson et. al., 2005; Makandar et al., 2006). PR proteins are induced not only by fungal pathogens (Faize et al., 2004; Funnell et al., 2004; Wang et al., 2005), but also induced by viral (Gordon-Weeks et al., 1997), bacterial (Strobel et al., 1996; Vidal et al., 1997), and viroid (Garcia Breijo et al., 1990; Domingo et al., 1994) pathogens. Besides these pathogens, insect pests (Fidantsef et al., 1999) and nematodes (Rahimi et al., 1996) also induce PR proteins.

Although PR proteins are considered as newly expressed proteins, many reports have appeared indicating that PR proteins can be detected even in uninfected control tissues, when Western blot analyses were made using antisera or when cDNA probes were used (Eyal et al., 1992; Beerhues and Kombrink, 1994; Dixelius, 1994; Lawrence et al., 1996). It suggests that these PR proteins may be present in uninfected tissues in amounts too small for detection on gels by general protein stains. Hence, PR proteins may better be defined as proteins that are readily detected in infected tissues but not in uninfected ones (Van Loon, 1999).

Although PR proteins are expressed in diseased plants, these proteins are also induced by several chemical treatments (Piggott et al., 2004; Qiu et al., 2004). Growth regulators, such as ethylene, indoleacetic acid, and abscisic acid, induce PR proteins in various plants (Grillo et al., 1995; Clarke et al., 1998; Wilkinson et al., 2005). Several biological products, such as elicitors (Halim et al., 2004), toxins (Reiss and Bryngelsson, 1996; Nishiuchi et al., 2006), and enzymes (Chang et al., 1995), are known to induce PR proteins. Even some saprophytes induce PR proteins (M'Piga et al., 1997; Meena et al., 2000). Several environmental factors, such as temperature (Gaudet et al., 2000), light (Asselin et al., 1985), and ozone (Ernst et al., 1992; Karlempi et al., 1994), also induce PR proteins. Any injury or mechanical wounding induces PR proteins (Piggott et al., 2004). Thus, the so-called PR proteins can be induced by several stresses besides pathogens.

In some cases, the PR proteins have been detected in healthy tissues without any stress. Several PR proteins appeared in the leaves of potato plants maturing in the glasshouse without any stress application (Pierpoint et al., 1990; Garcia-Garcia et al., 1994). PR proteins

appear in leaves of healthy tobacco plants during flowering (Neale et al., 1990). Some PR proteins appear constitutively in bean leaves (Clarke et al., 1998). In some plants, PR proteins, which appear in leaves only after a stress, naturally occur in other parts of plants like roots (Koiwa et al., 1994), flowers (Constabel and Brisson, 1995), pollens (Breitender et al., 1989), and seeds (Hejgaard et al., 1992; Zu et al., 1992). Thus some proteins, induced by pathogens in one type of plant organ like leaf, have been found to be constitutive components in other organs.

Some PR proteins, which are induced-proteins in some varieties, occur constitutively in other varieties. Constitutive expression of PR-1, PR-2, and PR-5 genes has been reported in *Solanum microdontum*, *S. sucrense*, and *S. tuberosum* (Vleeshouwers et al., 2000). The hybrid produced from *Nicotiana glutinosa* and *Nicotiana debneyi* produces PR proteins constitutively, whereas in *N. glutinosa* and *N. debneyi* the PR proteins were induced only after infection by pathogens (Pierpoint et al., 1992). Even in the same plant, PR proteins appear in lower old leaves without any stress (Keefe et al., 1990), whereas these proteins could not be detected in young leaves near top of the tobacco plant (Memelink et al., 1990; Neale et al., 1990). In these young leaves, the PR proteins are induced by pathogens (Brederode et al., 1991; Stintzi et al., 1991; Ward et al., 1991).

The PR proteins are commonly expressed during fungal pathogenesis in rice (Schweizer et al., 1997), wheat (Ergon et al., 1998), barley (Reiss and Bryngelsson, 1996), corn (Batalia et al., 1996), tomato (Christ and Mosinger, 1989), potato (Pan et al., 1989), beans (Awade et al., 1989), pea (Chang et al., 1992), strawberry (Asao et al., 1997), and in many other crops (Vidhyasekaran, 2007). Several signaling systems are involved in induction of these PR proteins (Brown et al., 2003). Although the role of PR proteins in fungal pathogenesis is not yet clearly understood, these proteins have been often implicated in disease resistance (Faize et al., 2004). Transgenic plants overexpressing some of these PR proteins show enhanced disease resistance (Chen et al., 1999; Datta et al., 1999, 2001; Gao et al., 2000; Donaldson et al., 2001). The role of PR proteins in fungal pathogenesis is discussed in this chapter.

6.2 MULTIPLICITY OF PR PROTEINS

Several PR proteins appear in infected plant tissues. More than 16 PR proteins have been detected in potato (Kombrink et al., 1988; Pierpoint et al., 1990; Sharma et al., 1992). At least 33 PR proteins have been described in tobacco (Stintzi et al., 1993), whereas 20 PR proteins have been detected in sugar beet (Fleming et al., 1991) and more than 30 PR proteins have been identified in Norway spruce (*Picea abies*) (Sharma et al., 1993). Structure of these PR proteins varies widely. Several attempts have been made to classify them. PR proteins could not be classified based on their serological relationship. For example, the bean chitinases lack serological relatedness to tobacco basic class I and class II chitinases (PR-3 proteins) (Neuhaus, 1999). A PR-1 protein of tobacco, PR-1g, could not be detected by an antibody of another PR-1 protein of tobacco, PR-1a (Gordon-Weeks et al., 1997). Similarly, antisera raised to PR-1b from tobacco cultivar Samsun-NN do not detect the tobacco PR-1g (Niderman et al., 1995).

PR proteins also could not be classified based on their biological activities. PR-3, PR-4, PR-8, and PR-11 families strongly differ in sequence and substrate preference, but all of them show chitinase activity or chitin-binding activity (Ponstein et al., 1994a; Van Loon et al., 1994; Van Loon, 1999). A bean PR protein, Saxa PR-4, possesses chitinase activity; however, they show structural resemblance to tobacco PR-5 protein (Awade et al., 1989). Similarly, another Pinto bean PR protein, PR-4d, is structurally homologous with tobacco PR-5 proteins, but functionally different showing β-1,3-glucanase activity (Sehgal et al., 1991).

TABLE 6.1
Classification of PR Proteins

PR Proteins	Plants in Which PR Proteins Were Detected	Function
PR-1	Rice, barley, maize, tomato, tobacco, parsley	Involved in plant cell wall thickening
PR-2	Rice, wheat, barley, maize, potato, tomato, tobacco, pepper, bean, pea, chickpea, sugar beet, soybean, norway spruce, *Brassica napus*, *B. nigra*	β-1,3-Glucanase
PR-3	Rice, maize, tomato, pepper, sugar beet, rapeseed	Chitinase
PR-4	Tobacco, tomato, rubber tree	Chitinase
PR-5	Rice, wheat, barley, oats, sorghum, potato, tomato, tobacco	Alteration of fungal membrane permeability
PR-6	Barley, tomato, potato, tobacco	Proteinase inhibitor
PR-7	Tomato	Endoproteinase
PR-8	Cucumber	Chitinase
PR-9	Tomato, rice, tobacco, wheat	Peroxidase
PR-10	Parsley, potato, asparagus, pea, bean, rice, soybean	Ribonucleases
PR-11	Tobacco	Chitinase
PR-12	*Arabidopsis*, pea, radish	Defensin
PR-13	Barley	Thionin
PR-14		Lipid transfer proteins
PR-15	Barley	Germin-like oxalate oxidase
PR-16	Barley, wheat	Germin-like proteins without oxalate-oxidase activity
PR-17	Wheat, barley, tobacco	Peptidase

The PR-10 proteins have a ribonuclease-like structure; but a PR-10 protein from parsley, PR-1, did not display ribonuclease activity (Van Loon, 1999). Now, it is widely agreed to classify PR proteins based on their structure and sequence similarity. The isoelectric points of the PR proteins are used to classify some of the PR proteins into subclasses (Koiwa et al., 1994). PR proteins have been classified into 17 groups based on their structure, and not based on their functions (Table 6.1).

6.3 CLASSIFICATION OF PR PROTEINS

6.3.1 PR-1 Proteins

PR-1 proteins are the most common PR proteins detected in a large number of plant species. They have been detected in rice, wheat, barley, corn, tomato, tobacco, and in several other plant species belonging to Gramineae, Solanaceae, Amaranthaceae, and Chenopodiaceae (Ergon et al., 1998; Fidantsef et al., 1999; Vidhyasekaran, 2002, 2004, 2007; Cordelier et al., 2003; Seo et al., 2003; Ménard et al., 2004; Zabbai et al., 2004; Rodrigues et al., 2005; Makandar et al., 2006). These PR proteins remain soluble in acidic buffers (pH 3.0), whereas most other plant proteins are degraded under these conditions (Van Loon, 1976). Molecular weight of PR-1 proteins ranges from 14 to 16 kDa. Both acidic and basic isoforms of PR-1 proteins have been detected in plants infected by pathogens (Van Loon and Van Strien, 1999).

Several PR-1 proteins have been detected in different *Nicotiana* species. At least six acidic PR-1 proteins have been detected in different tobacco cultivars and species (Antoniw et al., 1980; Pfitzner and Goodman, 1987). PR-1a, PR-1b, and PR-1c have been detected in *Nicotiana tabacum* Samsun NN. PR-1d has been described in *N. tabacum* cv. White Burley (Antoniw and White, 1980). PR-1e has been detected in *N. sylvestris* (Ahl et al., 1985), whereas PR-1f has been found in *N. tomentosiformis* (Ahl et al., 1985). All of these acidic

PR proteins are serologically related and most of them have been thoroughly characterized both at protein and DNA levels. The mature PR-1a protein is 138 amino acids long and synthesized on membrane-bound ribosomes as higher molecular weight precursor containing an N-terminal 30 amino acid hydrophobic signal peptide, which is cleaved to yield a 15 kDa mature protein (Carr et al., 1985). The coding sequences of two cDNAs for PR-1a and PR-1b of tobacco share 93% homology and the deduced amino acid sequences of PR-1a and PR-1b precursors, which are synthesized as larger precursors containing signal peptides, are 91% homologous. The homology of mature PR-1a and PR-1b regions is higher than that of larger precursors, 94% in the nucleotide sequence and 93% in the amino acid sequence, whereas that of the signal peptide regions is 80% and 90%, respectively (Matsuoka et al., 1987). Tobacco Samsun NN contains at least eight genes encoding acidic PR-1 proteins, and at least six of them are expressed at the mRNA level (Matsuoka et al., 1987). Three basic forms of the PR-1 proteins, PRB-1b, PR-1g, and PR-1h, have also been detected in tobacco (Eyal et al., 1992; Niderman et al., 1995; Gordon-Weeks et al., 1997). At least four genes encoding these basic proteins have been identified (Cornelissen et al., 1987), and the gene encoding PRB-1b (*prb-1b*) has been cloned, sequenced, and characterized (Eyal et al., 1992).

Three acidic PR proteins similar to tobacco PR-1 proteins, P14a, P14b, and P14c, have been detected in tomato (Joosten et al., 1990; Niderman et al., 1995). Besides them, three basic PR proteins (designated P2, P4, and P6) of approximately 15 kDa have also been reported in tomato (Joosten et al., 1990; Fidantsef et al., 1999). P4 and P6 were serologically related to each other and to the PR-1 protein family of tobacco (Joosten et al., 1990). P6 has been renamed as PR-1b (Wubben et al., 1993).

PR-1 proteins serologically related to tobacco PR-1 have been detected in cowpea (Nassuth and Sanger, 1986), soybean (Vega-Sánchez et al., 2005), barley (Gjetting et al., 2004), and corn (Gillikin et al., 1991). A basic PR-1 protein has been identified in corn seeds (Casacuberta et al., 1991). *PR-1* expression was induced by *Fusarium graminearum* in wheat (Makandar et al., 2006). In parsley, the PR-1 protein is encoded by a gene family consisting of approximately 3–6 genes (Somssich et al., 1988). The gene encoding a PR-1 protein in *Arabidopsis* has been identified (Uknes et al., 1993b).

The exact function of PR-1 proteins in fungal pathogenesis is not yet known. However, Benhamou et al. (1991) suggested that PR-1 protein might be involved in cell wall thickening and may offer resistance to the spread of pathogens in the apoplast. Immunocytochemical studies revealed that the PR-1 protein accumulated on mesophyll cell walls in barley leaves infected by *Bipolaris sorokiniana* (Santén et al., 2005). PR-1 protein was also detected on the host cell appositions. Both infected and noninfected leaves showed immunogold labeling in chloroplasts. PR-1 was also detected on the outer cell wall layer and the cytoplasm of primary hyphae (Santén et al., 2005). Detection of the PR-1 protein on the outer cell wall layer and in the cytoplasm of invading hyphae of the pathogen suggests that it may have some inhibitory function on growth of the pathogen. Detection of the protein in plant cell wall appositions suggests that PR-1 protein may have a role in strengthening of host cell walls to prevent spread of the pathogen (Santén et al., 2005).

6.3.2 PR-2 Proteins

PR-2 proteins are commonly detected in several plants infected with various pathogens (Table 6.1; Dassi et al., 1998; Ham et al., 1991; Leubner-Metzger and Meins, 1999; Ménard et al., 2004). PR-2 proteins show β-1,3-glucanase activity. β-1,3-Glucanases are constitutively present in plants (Keefe et al., 1990; Meins et al., 1992; Lawrence et al., 1996). However, some of the β-1,3-glucanases are considered as PR proteins, the proteins that are newly induced in infected plants. Some proteins are expressed in leaves only during infection, whereas such proteins may be expressed constitutively in other parts of the plant. Such proteins are also

called PR proteins (Van Loon, 1999). Some PR proteins may be expressed only during pathogenesis in some varieties of a crop, whereas the same PR proteins may be constitutively expressed in other varieties of the same crop. Such proteins are also called PR proteins (Van Loon, 1999). Hence the β-1,3-glucanases, which are constitutively expressed in some plants, are considered as PR proteins when they are expressed in some organs of a plant or in some varieties during infection. In *Nicotiana* species, several PR-2 proteins have been reported, and they are classified into three structural classes based on amino acid sequence identity. The class I β-1,3-glucanases include 33 kDa PR-2e subgroup of tobacco (*N. tabacum*) PR proteins, and Gn1 and Gn2 of *Nicotiana plumbaginifolia*. These are basic proteins localized in the cell vacuole. The class II β-1,3-glucanases include PR-2a (PR-2), PR-2b (PR-N), and PR-2c (PR-O) of *N. tabacum*, and these PR proteins are acidic proteins secreted into the extracellular space. The class III β-1,3-glucanases are also acidic proteins secreted into the extracellular space. The PR-2d (PR-Q′) is the sole representative of tobacco class III β-1,3-glucanase and is also an acidic PR protein that differs in sequence by at least 43% from the class I and class II enzymes (Leubner-Metzger and Meins, 1999). At least eight genes encoding extracellular acidic β-1,3-glucanases and five or six genes encoding intracellular basic β-1,3-glucanases have been detected in *N. tabacum* (Linthorst et al., 1990a; Ohme-Takagi and Shinshi, 1990).

Three β-1,3-glucanases have been detected in tomato. These include one extracellular, slightly acidic 35 kDa protein, an acidic β-1,3-glucanase, and a basic 35 kDa protein with high homology to vacuolar, basic tobacco β-1,3-glucanase (Van Kan et al., 1992). Leubner-Metzger and Meins (1999) reported two class III β-1,3-glucanases in tomato. Tom PR-Q′a is an acidic isoform 86.7% identical to tobacco PR-Q′, and Tom-PR-Q′b is a basic isoform 78.7% identical to tobacco PR-Q′.

In rice, several induced PR-2 (29–36 kDa) have been reported (Schweizer et al., 1997). Approximately 22 electrophoretic forms of β-1,3-glucanases have been detected in infected wheat plants (Sock et al., 1990). In sugar beet, two acidic and one basic β-1,3-glucanases have been reported (Rousseau-Limouzin and Fritig, 1991). Many β-1,3-glucanases were induced in pepper (*Capsicum annuum*) stems after infection (Kim and Hwang, 1997). Several induced β-1,3-glucanases have been detected in potato plants (Rahimi et al., 1996). An acidic and a basic β-1,3-glucanase have been detected in bean leaves (Awade et al., 1989). Two β-1, 3-glucanases (PR-2 and PR-Q) have been detected in *Brassica napus* and *B. nigra* (Dixelius, 1994). Two β-1,3-glucanases have been identified in roots of Norway spruce (*Picea abies*) (Sharma et al., 1993).

6.3.3 PR-3 Proteins

PR-3 proteins are another important group of PR proteins and they show chitinase activity. Chitinases are endo β-1,4-glucosaminidases, which hydrolyze the β-glycosidic bond at the reducing end of glucosaminides found in chitin, chitosan, or peptidoglycan (Neuhaus, 1999). Based on sequence homology and the presence or absence of a chitin-binding domain (CBD), plant chitinases have been classified into seven classes. Class I contains CBD, whereas class II lacks the CBD of class I. Class III chitinases are structurally different from other chitinases. Class IV chitinases differ from class I by several internal deletions within both CBD and catalytic domains (loops 1, 3, and 4). Class V chitinases contain two CBDs. Class VI chitinases are characterized by the presence of a truncated CBD and a long proline-rich spacer. Class VII possesses a catalytic domain homologous to class IV, but lacks the CBD (Neuhaus, 1999). Besides these chitinases, a new type of chitinase with the most related sequences belonging to bacterial chitinases has been identified (Heitz et al., 1994; Melchers et al., 1994). Another class of chitinases constitutes hevein and Win proteins, which contain a CBD and PR-4 proteins that lack the CBD (Linthorst et al., 1991).

PR-3 proteins consist of chitinases belonging to class I to class VII except class III (Van Loon et al., 1994). Class I chitinases are basic proteins. Tobacco basic chitinase belongs to this class (Shinshi et al., 1990; Eyal and Fluhr, 1991). Four isoforms of chitinases, which belong to class I chitinases, have been identified in potato. The structural features of the potato chitinases included a hydrophobic signal peptide at the N-terminus, a hevein domain that is characteristic of class I chitinases, a proline- and glycine-rich linger region that varies among all potato chitinases, a catalytic domain, and a C-terminal extension (Beerhues and Kombrink, 1994). A basic 32 kDa chitinase has been detected in pepper (Kim and Hwang, 1997).

Class II chitinases include the acidic chitinases. They do not contain the hevein-like region but otherwise have high homology to the other main structural features of class I. CBD is absent in these acidic chitinases (Shinshi et al., 1990). Tobacco PR-P and PR-Q are type members belonging to this class (Neuhaus, 1999). Class IV chitinases include bean chitinases originally called PR-4 (de Tapia et al., 1987). These chitinases are not serologically related to class I and class II chitinases. They also show low homology to the class I and class II chitinases that prevented identification by DNA hybridization. A loop is missing in the CBD in the class IV chitinases, without affecting its sugar-binding properties. Within the catalytic domain, there are three deletions (Neuhaus, 1999). A maize chitinase (Huynh et al., 1992), the basic sugar beet Ch4, and rapeseed ChB4 (Nielsen et al., 1994b) also belong to this class.

The class V chitinase includes a single protein. When the cDNA for the precursor of stinging nettle (*Urtica dioica*) lectin was cloned, it was found that the precursor was synthesized as a chitinase homologue with two CBDs (Lerner and Raikhel, 1992). The only class VI chitinase known so far was isolated from sugar beet (Berglund et al., 1995). Its heavily truncated CBD lacks four out of eight cysteines and can at best form a single correct disulfide bond instead of four. This chitinase possesses by far the longest spacer sequence known, a stretch of more than 135 amino acids of which 90 are prolines (Neuhaus, 1999). The class VII chitinases include a rice chitinase without CBD but with a catalytic domain highly homologous to the domain of class IV chitinases (Neuhaus, 1999).

6.3.4 PR-4 Proteins

Two classes of PR-4 family have been recognized. Class I PR-4 proteins show similarity to hevein and WIN (wound-induced) proteins (Van Loon, 1999). Hevein is a small protein found in high concentration in the latex fluid of the rubber tree (*Hevea brasiliensis*). The three-dimensional structure of hevein is similar to each of the four domains of wheat-germ agglutinin (WGA) and other cereal lectins (Neuhaus, 1999). WIN proteins have been detected in potato, and these proteins include a CBD related to hevein, the cereal chitin-binding lectins, and the CBD of class I chitinases (Stanford et al., 1989). A class I PR-4 protein has been purified from tobacco (Ponstein et al., 1994b). Analysis of the protein and the corresponding cDNAs revealed that it contains a C-terminal domain showing homology to class II PR-4 proteins from tobacco. The protein was localized intracellularly (Ponstein et al., 1994b).

The class II PR-4 proteins show similarity to acidic chitinases. Four class II (acidic, extracellular, 13–14 kDa) PR-4 proteins have been reported in tobacco (Pierpoint, 1986; Van Loon et al., 1987; Fritig et al., 1989). One of the tobacco PR-4 proteins, CBP 20, was found to have chitinase activity (Ponstein et al., 1994a). In tomato, a PR-4 protein (P2, 15 kDa, pI 10.4), which is serologically related to PR-4 protein from tobacco, has been identified (Joosten et al., 1990). The tomato P2 protein showed homology with the potato *win1* and *win2* gene products and with pre-pro-hevein from *H. brasiliensis* (Linthorst et al., 1991). In barley, a PR-4 protein with chitin-binding ability has been identified in grains and stressed leaf (Hejgaard et al., 1992). In soybean, a WIN-like protein (PR-4 protein) has been identified (Graham et al., 2003).

6.3.5 PR-5 Proteins

The PR-5 group of proteins have close resemblance to a sweet-tasting protein, thaumatin, which occurs in the fruit of the West African shrub, *Thaumatococcus danielli* (Pierpoint et al., 1987). They have a similar sequence of amino acids with 8% cysteine content, a similar molecular weight, a similar circular dichroism spectrum, and have an immunological relationship with thaumatin (Cusack and Pierpoint, 1988), but they do not have a marked sweet taste (Pierpoint et al., 1987). The term thaumatin-like (TL) protein is used for this kind of proteins (Pierpoint and Shewry, 1987). Three subclasses of PR-5 proteins have been recognized in tobacco based on their isoelectric points (Woloshuk et al., 1991b): the basic forms (osmotins), neutral forms (osmotin-like proteins, OLPs), and acidic (PR-S) proteins (Koiwa et al., 1994). Acidic PR-5 proteins have been detected in leaf extracts of *N. tabacum*, *N. glutinosa*, *N. langsdorffii*, and *N. debneyi* plants in stress. *N. glutinosa* produces PR5a, whereas *N. debneyi* expresses PR5b. A hybrid from *N. glutinosa* × *N. debneyi* constitutively expresses both PR5a and PR5b (Pierpoint et al., 1992). An intercellular protein that resembles tobacco PR-5 has been purified from potato leaves (Pierpoint et al., 1990). A similar protein named Hv1 has been identified in barley (Bryngelsson and Green, 1989). Acidic PR-5 proteins with molecular weights near 21 kDa have been detected in soybean leaves (Graham et al., 1992) and Pinto beans (Sehgal et al., 1991). They are reciprocally immunoreactive with PR-5 protein from Samsun NN tobacco (Sehgal et al., 1991). Two PR-5 proteins of 23 and 17 kDa have been detected in white pine (*Pinus monticola*) (Piggott et al., 2004). When tobacco plants were inoculated with an incompatible pathogen, the leaf extracts showed the presence of a PR-5 protein called AP24 (Woloshuk et al., 1991b). Similar 24 kDa OLPs have been isolated from tomato, soybean, millet, carrot, cotton, potato, alfalfa, and bean (Singh et al., 1987; Dassi et al., 1998). Two PR-5 proteins have been detected in cotton and ethylene, and H_2O_2 induced the expression of these osmotin proteins (Wilkinson et al., 2005).

The function of PR-5 proteins is not yet known. PR-5 proteins alter permeability of fungal membranes (Vigers et al., 1992). The PR-5 protein of tobacco (PR-R = PR-S) has identity to a maize-trypsin/α-amylase inhibitor (Richardson et al., 1987). A PR-5 protein from bean cultivar Pinto, PR-4d, shows β-1,3-glucanase activity (Sehgal and Mohamed, 1990). Another PR protein from bean cultivar Saxa, which shows resemblance to tobacco PR-5 protein, Saxa PR-4d, possesses chitinase activity (Awade et al., 1989).

6.3.6 PR-6 Proteins

Several types of proteinase inhibitor proteins commonly occur in plants. Proteinase inhibitors are generally categorized according to the class of proteinases that they inhibit (Koiwa et al., 1997). Four types of proteinases, such as serine proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases, have been recognized. Serine proteinase inhibitors that inhibit trypsin and chymotrypsin contain several families: Kunitz family (Soybean trypsin inhibitor family), Bowman-Birk family, Barley trypsin inhibitor family, Potato inhibitor I family, Potato inhibitor II family, Squash inhibitor family, Ragi I-2/maize trypsin inhibitor family, and Serpin family. Cysteine proteinase inhibitors (phytocystatins) inhibit cysteine proteinases. Aspartic proteinase inhibitors inhibit cathepsin D and metalloproteinase inhibitors inhibit papain, cathepsin B, H, L (Koiwa et al., 1997). Although most of these proteinase inhibitors are constitutively expressed, some of the serine proteinase inhibitors are induced because of infection by pathogens and they are considered as PR-6 proteins (Heitz et al., 1999). Tomato inhibitor I (belonging to Potato inhibitor I family) is the type member of the PR-6 protein family (Green and Ryan, 1972). Tomato inhibitor II is also commonly induced in tomato because of stresses (Doares et al., 1995). Cordero et al. (1994) have shown that mRNA of a maize proteinase inhibitor (MPI) accumulates in germinating maize embryos

upon fungal infection. A Kunitz trypsin inhibitor (PR-6 protein) was induced in soybean by an elicitor isolated from the cell wall of the oomycete *Phytophthora sojae* (Graham et al., 2003).

6.3.7 PR-7 Proteins

PR-7 proteins show endoproteinase activity. One of the tomato PR proteins, PR-69, has been identified as an alkaline proteinase with a molecular weight of 69,000–70,000 Da (Vera and Conejero, 1988). It was identified as P70 protein by Christ and Mosinger (1989). It accumulates in tomato leaves after inoculation with races of *Cladosporium fulvum* (De Wit et al., 1986). This protein was found in two compartments, namely vacuoles and intercellular spaces in a leaf tissue, and it was found to be a potential weapon against attacking pathogens (Vera and Conejero, 1988, 1989). Tornero et al. (1997) identified P69B, a second member of the family of plant proteinases induced during the response of tomato plants to pathogen attack. P69B represents a new plant subtilisin-like proteinase based on amino acid sequence conservation and structural organization that is highly related to the previously identified PR-69 proteinase (Tornero et al., 1997). The subtilisin-like P69B is induced by *Phytophthora infestans*, citrus exocortis viroid, and *Pseudomonas syringae* (Zhao et al., 2003; Tian et al., 2004, 2005). Rcr3 is an apoplastic papain-like cysteine protease detected in tomato and it has been shown to be required for resistance to *Cladosporium fulvum* (Kruger et al., 2002).

6.3.8 PR-8 Proteins

PR-8 proteins include class III chitinases. These chitinases strongly differ from other chitinases in sequence and substrate preference (Van Loon et al., 1994). They lack the cysteine-rich domain and have structural homologies to a bifunctional lysozyme/chitinase from *Parthenocissus quinquifolia* (Vogelsang and Barz, 1993; Lawton et al., 1994a). Both acidic and basic forms of PR-8 proteins have been reported. Acidic PR-8 proteins have been detected in cucumber, tobacco, and chickpea (Vogelsang and Barz, 1993; Lawton et al., 1994a). Basic forms have been described in tobacco and *Arabidopsis* (Neuhaus, 1999).

6.3.9 PR-9 Proteins

Several peroxidases have been shown to be constitutively expressed in many plants. However, some of the peroxidases are induced during pathogen stress, and these peroxidases are considered as PR-9 proteins. A lignin-forming peroxidase from tobacco is the type member of the PR-9 protein family (Lagrimini et al., 1987). Vale et al. (1994) reported induction of a peroxidase in *Drechslera graminea*-infected wheat rootlets and considered the induced peroxidase as a PR protein. In wheat, six peroxidase genes have been detected and two of them, *pox2* and *pox3*, were induced only during pathogen stress (Rebmann et al., 1991; Reimann et al., 1992; Baga et al., 1995). These two peroxidases are considered as PR proteins. Two peroxidase genes (*POX 8.1* and *POX 22.3*) were found to be induced during pathogenesis in rice (Chittoor et al., 1997). Two peroxidase genes, *sphx6a* and *sphx6b*, were shown to be induced by methyl jasmonate in *Stylosanthes humilis* (Curtis et al., 1997). In azuki bean (*Vigna angularis*), an intracellular basic glycoprotein (AZ42) has been shown to accumulate because of ethylene signal (Ishige et al., 1991). A cDNA encoding the glycoprotein has been cloned and its complete nucleotide sequence has been determined. An open reading frame (1071 base pairs) in the cDNA encoded a protein of 357 amino acids with a molecular mass of 39.3 kDa. The purified AZ42 had specific peroxidase activity (Ishige et al., 1993). The mRNA for the basic peroxidase was not present at a detectable level in leaves that had not been treated with ethylene. Hence, it appears that ethylene specifically regulates transcription of the gene for the basic isozyme of peroxidase. The basic peroxidase gene was also expressed in

response to wounding of tissue and treatment with salicylate (Ishige et al., 1993). These characteristics of the cationic peroxidase suggest that it may be a PR protein.

Acidic peroxidases have also been considered as PR proteins (Schweizer et al., 1989; Mohan and Kolattukudy, 1990; Ye et al., 1990). Parent and Asselin (1987) showed that the PR proteins of potato include peroxidases. Two tomato anionic peroxidase genes, *tap1* and *tap2*, were expressed in response to elicitor treatment (Mohan and Kolattukudy, 1990) and fungal attack (Robb et al., 1991; Mohan et al., 1993a,b). They were not constitutively expressed. The two *tap* genes were located in tandem on the chromosome. They were highly homologous (about 96%) to each other (Roberts and Kolattukudy, 1989). All these induced peroxidases are considered as PR-9 proteins.

6.3.10 PR-10 Proteins

PR-10 proteins are all acidic proteins that are shown to be intracellular (Somssich et al., 1988; Warner et al., 1992). In other cases, only basic PR proteins have been shown to be intracellular. The acidic PR-1 proteins are known to be synthesized as precursor proteins with an N-terminal extension characteristic of a signal sequence, which is cleaved during import into the endomembrane system before secretion into extracellular spaces. In contrast, all acidic PR-10 proteins did not contain the characteristic N-terminal extension signal peptide and they were found to be located inside the cells, not in the apoplast (Somssich et al., 1988). The PR-10 family includes parsley PoPR1-I (Somssich et al., 1986, 1988), rice PBZ1 (Midoh and Iwata, 1996; Nishizawa et al., 2003), JIOs PR-10 (Jwa et al., 2001), and OsPR10 (Agrawal et al., 2002), potato pSTH2, pSTH21, and StPR-10 (Constabel and Brisson, 1992; Constabel et al., 1993; Matton et al., 1993; Halim et al., 2004), bean pvPR1 and pvPR2 (Walter et al., 1990; Sharma et al., 1992), pea pI49 (Fristensky et al., 1988; Chiang and Hadwiger, 1990), and asparagus AoPR1 (Warner et al., 1994). Soybean SAM22 protein and pea ABR17 protein were also identified as PR-10 proteins (Midoh and Iwata, 1996). PR-10 proteins have been identified in soybean (Graham et al., 2003) and sorghum (Lo et al., 1999). A cDNA, *GaPR-10*, encoding a PR-10 protein has been isolated from cotton (*Gossypium arboreum*) (Zhou et al., 2002). These proteins were induced by pathogens (Constabel and Brisson, 1992; Midoh and Iwata, 1996). A high sequence similarity between a ribonuclease from ginseng and parsley PR-10 protein has been observed (Moiseyev et al., 1994). The cotton PR-10 (GaPR-10) exhibited ribonuclease activity *in vitro* (Zhou et al., 2002). The PR-10 isolated from hot pepper functions as ribonuclease in an antiviral pathway (Park et al., 2004). These observations suggest that PR-10 gene family may encode ribonucleases.

6.3.11 PR-11 Proteins

PR-11 protein has been detected in tobacco and it was induced by various stresses including virus infection (Heitz et al., 1994; Melchers et al., 1994). It shows no sequence similarity to the previously identified plant chitinases (Melchers et al., 1994). It shows similarity to some bacterial exochitinases. However, unlike the bacterial chitinases, the plant enzyme was endochitinase lacking detectable exochitinase activity (Ohl et al., 1994). The PR-11 protein in tobacco was encoded by a small multigene family (Ohl et al., 1994).

6.3.12 PR-12 Proteins

PR-12 proteins include defensins, which show the structural and functional similarities with insect defensins (Terras et al., 1995; Van Loon and Van Strien, 1999). Defensins are a family of small (about 5 kDa), usually basic, peptides that are rich in disulfide-linked cysteine residues (Conceicao and Broekaert, 1999). They are also called γ-thionins (Colilla et al., 1990), but the γ-thionins do not belong to the classical thionin protein family

(Bohlmann, 1994). All defensins (γ-thionins) are less than 50 amino acids in length and contain 8 cysteine residues.

Several defensins have been shown to be constitutively expressed; however, some of them are induced exclusively during pathogenesis, and the induced defensins are considered as PR-12 proteins. For example, in *Arabidopsis*, five different defensin genes (*PDF1.1*, *PDF1.2*, *PDF2.1*, *PDF2.2*, *PDF2.3*) have been identified. In healthy *Arabidopsis* plants, *PDF1.1* is expressed exclusively in seeds, *PDF2.1* in roots and seeds, *PDF2.2* in all organs except stems and seeds, and *PDF2.3* in all organs except roots (Conceicao and Broekaert, 1999). The gene *PDF1.2*, on the other hand, is not expressed constitutively but is strongly induced in leaves upon attack by fungal pathogens (Penninckx et al., 1996). Hence, *PDF1.2* alone can be called a PR protein.

Two PR-12 proteins have been detected in radish. The proteins Rs-AFP3 and Rs-AFP4 were barely detectable in healthy uninfected leaves of radish, but accumulated systemically at high levels after localized fungal infection (Terras et al., 1995). These proteins are typical members of defensins family and are considered as PR-12 proteins. PR-12 proteins also have been detected during pathogenesis in tobacco (Gu et al., 1992), pea (Chiang and Hadwiger, 1991), and *Arabidopsis thaliana* (Penninckx et al., 1996).

6.3.13 PR-13 Proteins

Thionins are small (5 kDa), basic, cysteine-rich proteins commonly found in plants. Although thionins are constitutively expressed in seeds and roots, some of the leaf thionins were found to be induced during pathogenesis (Bohlmann et al., 1988). Those induced thionins are considered as PR-13 proteins (Van Loon, 1999). Induction of leaf thionins was demonstrated at the mRNA and protein levels after infection with *Blumeria graminis* f. sp. *hordei* in barley (Bohlmann et al., 1988). mRNAs encoding thionin accumulated in barley roots after inoculation with *Drechslera graminea* (Vale et al., 1994). Methyl jasmonate leads to accumulation of thionin transcripts and the mature proteins in barley leaves (Andresen et al., 1992). Jasmonic acid and ethylene induced a thionin THI 2.1 in *Arabidopsis* (Epple et al., 1995, 1997). Such thionins are considered as PR-13 proteins.

6.3.14 PR-14 Proteins

PR-14 proteins include lipid transfer proteins (LTPs) (Van Loon and Van Strien, 1999). LTPs are small proteins (~8.7 kDa) of approximately 90 aminoacids stabilized by four disulfide bonds with a central tunnel-like hydrophobic cavity (Selitrennikoff, 2001; Ge et al., 2003). LTPs in different plants share common characteristics such as a signal peptide in the N-terminus, eight conserved cysteine residues engaged in forming four disulfide bonds, a basic isoelectric point, and a low molecular weight (<10 kDa) (Ge et al., 2003). LTPs stimulate the transfer of a broad range of lipids between membranes *in vitro* (Yamada, 1992). They facilitate intracellular lipid transfer *in vivo* (Kader, 1996). Some of the LTPs are induced in plants because of pathogen infection (Molina et al., 1993; Molina and Garcia-Olmedo, 1993, 1994; Garcia-Olmedo et al., 1995; Park et al., 2002) and they are considered as PR-14 proteins. In rice, a lipid transfer protein, LTP1, was detected and the *Ltp1* transcripts were found to strongly accumulate in response to inoculation with *Magnaporthe grisea* (Guiderdoni et al., 2002).

6.3.15 PR-15 Proteins

A germin-like oxalate oxidase has been considered as a PR-15 protein (Zhang et al., 1995). Germin is a 130 kDa pentameric glycoprotein and shows oxalate oxidase activity (Schweizer et al., 1999). Germin produces hydrogen peroxide, which is involved in cell-wall cross-linking

(Carter and Thornburg, 1998). The PR-15 protein accumulated in barley leaves infected by *Blumeria graminis* f. sp. *hordei*, and germin-like oxalate oxidase activity increased during the fungal infection (Dumas et al., 1995; Zhang et al., 1995; Hurkman and Tanaka, 1996; Zhou et al., 1998).

6.3.16 PR-16 Proteins

A PR protein, other than PR-15, appeared in barley leaves inoculated with *Blumeria graminis* f. sp. *hordei*. This protein was also a germin-like protein, but it was without oxalate oxidase activity (Wei et al., 1998). This barley oxalate oxidase-like protein was classified as PR-16 protein (Wei et al., 1998). Similar PR-16 protein, TaGLP4 (formerly referred to as TaGLP2a), was detected in *B. graminis*-attacked epidermis tissue of wheat leaves (Thordal-Christensen et al., 2004). The TaGLP4 protein specifically accumulated in resistant cells (Gjetting et al., 2004). The PR-16 protein may generate H_2O_2, necessary for cross-linking of cell wall components during formation of papillae, utilizing substrates other than oxalate (Wei et al., 1998).

6.3.17 PR-17 Proteins

The PR-17 family includes proteins that show similarity to the active site and to the peptide-binding groove of the exopeptidase aminopeptidase N from eukaryotes and the endo-peptidase thermolysin from bacteria (Christensen et al., 2002). The PR proteins WCI-5 from wheat (Görlach et al., 1996), HvPR-17a and HvPR-17b from barley (Christensen et al., 2002), and NtPRp27 from tobacco (Okushima et al., 2000) are the important members of PR-17 protein family.

6.3.18 Chitosanases

Besides these 17 families, some unclassified PR proteins have also been described. Grenier and Asselin (1990) have identified chitosanases as pathogenesis-related proteins in barley, cucumber, and tomato leaves. Chitosanases (EC 3.2.1.99) (acting on chitosan without activity on chitin) are distinguished from chitinases (acting on chitin without activity on chitosan) by their molecular weight and substrate specificity. Chitosan [poly (1–4)-β-D-glucosamine], the deacetylated chitin, is the substrate for the enzyme. Chitosan is found in various fungal cell walls. Elicitors and viral pathogens induced the chitosanases in barley, cucumber, and tomato (Grenier and Asselin, 1990). In barley, six chitosanases were detected; four of them were acidic proteins (three at 22 kDa, one at 19 kDa), whereas the other two were basic ones (one at 22 kDa, one at 19 kDa). Four acidic chitosanases (one at 10 kDa, one at 12 kDa, and two at 14 kDa) were observed in cucumber, whereas one basic chitosanase (24 kDa) alone was detected in tomato. All these chitosanases were detected in intercellular fluids (Grenier and Asselin, 1990). Three chitosonase activities have been detected in spruce roots infected with *Pythium* sp. (Sharma et al., 1993).

6.4 INDUCTION OF PR PROTEINS DURING FUNGAL PATHOGENESIS

When the fungal pathogen invades host tissues, several PR proteins accumulate both locally and systemically. The accumulation of PR proteins occurs in both susceptible and resistant interactions (Benhamou et al., 1991; Van Kan et al., 1992). PR-1 mRNA accumulated in potato after inoculation with both incompatible and compatible races of *Phytophthora infestans* (Taylor et al., 1990). Four isoforms of β-1,3-glucanases have been detected in tobacco leaves inoculated with *Peronospora tabacina* (Pan et al., 1991a). Several chitinases

(PR-3 proteins) have been detected in diseased potato plants. Six chitinases (PR-3 proteins) with molecular weights 38,000, 38,700, 32,200, 32,600, 34,300, and 33,200 have been identified in potato leaves infected with *P. infestans* (Kombrink et al., 1988). Two different molecular forms of chitinase, Ch1 and Ch2, were detected in pea pods inoculated with *Fusarium solani* f. sp. *pisi* (Mauch et al., 1988). Ten chitinase isoforms and three chitosanase activities have been shown to accumulate in roots of Norway spruce (*Picia abies*) infected by *Pythium* sp. (Sharma et al., 1993).

Four chitinase isozymes have been detected in tomato genotypes infected with *Alternaria solani* (Lawrence et al., 1996). Infection with *Colletotrichum lindemuthianum* resulted in accumulation of chitinase transcripts in bean (Hedrick et al., 1988). An acidic chitinase has been found to accumulate in sugar beet leaves infected with *Cercospora beticola* (Nielsen et al., 1993). PR-5 proteins accumulate in barley leaves infected by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Bryngelsson and Green, 1989).

PR-6 protein activity increased in melon plants infected by *Colletotrichum lagenarium* (Roby et al., 1987). In maize germinating embryos, mRNA of a proteinase inhibitor of the potato I family accumulates in response to infection by *Fusarium moniliforme* (Cordero et al., 1994). Accumulation of PR-7 protein, PR-69, has been reported in tomato leaves inoculated with *Cladosporium fulvum* (De Wit et al., 1986). PR-9 proteins accumulated in *Stylosanthes humilis* inoculated with *Colletotrichum gloeosporioides* (Harrison et al., 1995). The PR-10 protein PBZ1 was found to accumulate in *Magnaporthe grisea*-infected rice leaves (Midoh and Iwata, 1996). The PR-12 protein PDF1.2 was strongly induced in leaves upon attack by *Alternaria brassicicola* in *Arabidopsis thaliana* (Penninckz et al., 1996). The mRNA of a PR-13 protein accumulated in barley roots inoculated with *Drechslera graminea* (Vale et al., 1994). PR-13 (Bohlmann et al., 1988), PR-15 (Zhang et al., 1995), and PR-16 (Thordal-Christensen et al., 2004) proteins accumulate in barley leaves inoculated with *B. graminis* f. sp. *hordei*.

6.5 GENES ENCODING PR PROTEINS

PR proteins are newly induced proteins under pathological conditions. It suggests that the transcription and translation of PR genes would have been induced by some signals generated by the pathogen attack. Genes encoding PR proteins have been identified in different plants and they are almost silent in healthy plants (Cornelissen et al., 1987; Sharma et al., 1992; Gordon-Weeks et al., 1997). Most PR protein-encoding genes belong to multigene family (Sharma et al., 1992). However, not all genes are expressed in plants suggesting that there may also be pseudogenes (Van Kan et al., 1992).

Sixteen genes encoding PR-1 proteins have been detected in tobacco plants (Cornelissen et al., 1987; Pfitzner et al., 1988; Eyal et al., 1992). Two PR-1 genes, PR-1 and PR-1a, have been cloned from rice plants (Kim et al., 2001). Both rice *PR-1* (basic protein) and *PR-1a* (acidic protein) genes were found to exist as small gene families (Kim et al., 2001). Tobacco contains more than 13 genes encoding *PR-2* proteins (Ohme-Takagi and Shinshi, 1990, 1995). The class I PR-2 genes, *Glb* (Livne et al., 1997) and *ggl50* (Van de Rhee et al., 1993) and class II *PR-2b* and *PR-2d* genes (Henning et al., 1993; Van de Rhee et al., 1993; Shah and Klessig, 1996) have been cloned from tobacco. A gene encoding β-1,3-glucanase, *gn1*, has been cloned from *Nicotiana plumbaginifolia* (Castresana et al., 1990). Several genes encoding acidic and basic chitinases have been identified in tobacco (Hooft van Huijsduijnen et al., 1987). A small gene family encodes the tobacco and barley PR-5 proteins (Melchers et al., 1993; Vale et al., 1994). Four genes encoding PR-5 proteins, *tlp1*, *tlp2*, *tlp3*, and *tlp4*, have been cloned from oat (Lin et al., 1996). A gene encoding a PR-5 protein has been cloned from *Arabidopsis* (Hu and Reddy, 1995). A PR-6 protein gene (MPI) has been detected in maize (Cordero et al., 1994).

Genes encoding PR-9 have been identified in barley (Vale et al., 1994). The bean PR-10 protein PvPR3 is encoded by approximately 15 genes (Sharma et al., 1992), whereas at least 12 genes have been identified to encode the bean PR-10 proteins, PvPR 1 and PvPR 2 (Walter et al., 1990). Genes encoding PR-10 protein have been detected in bean, pea, and parsley (Fristensky et al., 1988; Somssich et al., 1988; Walter et al., 1990), soybean (Crowell et al., 1992), and asparagus (Warner et al., 1992). PR-10 gene, *PBZ1*, has been identified in rice (Midoh and Iwata, 1996). Three PR-10 genes, *RPR-10a*, *RPR10b*, and *RPR10c*, have been identified in rice (McGee et al., 2001). *RPR-10a* and *RPR-10b* encode predicted proteins of 158 and 160 amino acids, respectively. *PR-10c* appears to be a nonfunctional pseudogene (McGee et al., 2001). A rice gene homologous to a sorghum PR-10 protein gene has been cloned and named as *JIOs PR-10* (jasmonate inducible) (Jwa et al., 2001). *JIOPR-10* encoded a 160 amino acid polypeptide with a predicted molecular mass of 17,173 Da and a pI of 5.84 (Jwa et al., 2001). The PR-11 proteins in tobacco are encoded by a small multigene family (Ohl et al., 1994). The PR-12 gene, *PDF1.2*, has been cloned from *Arabidopsis* (Penninckz et al., 1996). A family of genes encoding PR-13 protein has been reported in barley (Vale et al., 1994). The PR-16 proteins are encoded by a gene family. Genes of these germin-like proteins subfamily 4 of barley (*HvGLP4*, formerly referred to as *HvOxOLP*) and the wheat orthologue *TaGLP4* (formerly referred to as *TaGLP2a*) were expressed in pathogen-attacked epidermis tissue of barley and wheat leaves, respectively (Thordal-Christensen et al., 2004).

6.6 TRANSCRIPTION OF PR GENES

The PR genes that are quiescent in healthy plants may be induced either at the level of transcription or at the translation stage. Van Loon (1985) and Carr et al. (1982) provided evidences that induction of PR protein synthesis was only at the level of translation stage. It suggests that mRNA of the PR proteins exists constitutively in healthy plant tissues and it is translated into proteins during pathogenesis. Van Loon (1985) showed that inhibitors of DNA-dependent RNA-synthesis, such as actinomycin D or cordycepin, did not inhibit the induction of PR proteins in tobacco. It suggests that no transcription is required for the expression of PR proteins. When polyA-mRNA from healthy leaves were translated *in vitro*, as much PR proteins were synthesized as from mRNA from infected leaves (Carr et al., 1982). These studies suggest that PR-mRNA is constitutively present, but not translated in nonstimulated leaves (Van Loon, 1985). Matsuoka and Ohashi (1986) demonstrated the putative regulation of PR proteins at the translational level with *in vivo* pulse-labeling experiments using antibiotics. The synthesis of PR proteins in tobacco leaf discs treated with potassium salicylate for 1 day was inhibited completely by cycloheximide, an inhibitor of protein synthesis. Actinomycin and -amanitin are the well-known inhibitors of transcription and both of them did not inhibit synthesis of PR proteins. It suggests that transcription is not required for induction of PR proteins. These studies indicate that untranslatable mRNAs of PR proteins are already present in healthy leaves and are changed to translatable forms by certain stresses like pathogens or salicylic acid treatment (Matsuoka and Ohashi, 1986). In contrast, others have found that translatable mRNAs for PR-1 proteins are present only in infected leaves but not in healthy leaves (Carr et al., 1985; Hooft van Huijsduijnen et al., 1985; Matsuoka et al., 1985). The induction kinetics of the PR-1 protein synthesis in tobacco leaves treated with salicylate treatment was studied. There were no detectable mRNAs for PR-1 proteins in healthy leaves, and an almost linear increase in PR-1 protein synthesis was observed after a lag phase of about 10 h until 48 h. The induction kinetics of mRNAs was similar to PR-1 protein synthesis and it suggests that the synthesis of PR-1 proteins is not regulated at a translational step but at a transcriptional step (Matsuoka et al., 1988).

Regulation of β-1,3-glucanase has been shown to occur at the mRNA level in barley (Jutidamrongphan et al., 1991), bean (Vogeli et al., 1988), and tobacco (Mohnen et al., 1985). Thiamine treatment induced PR-2, N and O proteins, and these β-1,3-glucanases accumulated to high levels in a manner consistent with the observed rise in steady-state levels of β-1,3-glucanase mRNA. The parallel accumulation of PR-2, N and O proteins, and mRNA suggests that the β-1,3-glucanases gene expression is regulated at the level of mRNA accumulation (Cote et al., 1991).

The induction of chitinase gene has been found to occur at the level of mRNA accumulation in cucumber (Metraux et al., 1989). When bean leaves were treated with ethylene, chitinase and β-1,3-glucanase activities doubled within 2 h and continued to increase over a period of 48 h. The mRNA activity for both enzymes increased rapidly for the first 2 h after ethylene treatment, and thereafter remained at a high level (Vogeli et al., 1988). The results suggest that chitinase and β-1,3-glucanase are regulated coordinately at the level of mRNA. De novo synthesis of mRNA of PR proteins has been demonstrated in two cultivars of tobacco (Cornelissen et al., 1986; Hooft van Huijsduijnen et al., 1987) and parsley (Somssich et al., 1986). Accumulation of proteinase inhibitors I and II is accompanied by the appearance of translatable mRNAs that code for each (Nelson and Ryan, 1980). All these studies suggest that PR proteins are transcriptionally regulated.

6.7 SIGNALS INVOLVED IN TRANSCRIPTIONAL INDUCTION OF PR GENES

6.7.1 Induction of PR Genes by Elicitors

Fungal pathogens produce several elicitors and these elicitors may serve as primary signal molecules in triggering PR genes. *Cis*-acting elicitor-responsive elements (EREs) have been identified in promoters of many PR proteins. A *cis*-acting ERE has been identified in promoter of the class I chitinase gene, *CHN50*, of tobacco (Fukuda, 1997). Both expression and responsiveness to the elicitor disappeared when the region of the promoter that included the ERE sequence had been deleted. The nuclear factors bound specifically to the sequence motif, ^{534}GGTCANNNAGTC^{523} (Fukuda, 1997).

Binding of the elicitor message to the *cis*-acting elements in the promoter of PR gene appears to be important for transcription of the gene. Several second messengers may be involved in this process. Treatment with calcium channel blockers, calmodulin antagonist, phospholipase inhibitor, and proteinase inhibitors before an elicitor treatment suppressed PR gene activation in potato (Furuse et al., 1999). An elicitor (arachidonic acid from *Phytophthora infestans*) induced activation of the potato PR gene, *PR-10a*. This activation was positively controlled by a protein kinase that affected the binding of the nuclear factors PBF-1 and PBF-2 to an elicitor response element in the promoter of the gene (Subramaniam et al., 1997). Treatment of potato tuber discs with specific inhibitors of protein kinase abolished elicitor-induced binding of PBF-2 to the ERE. This correlated with a reduction in the accumulation of the PR-10a protein. In contrast, treatment of potato tuber discs with 12-*O*-tetradecanoyl-phorbol 13-acetate, an activator of protein kinase, led to an increase in binding of PBF-2 to the ERE and a corresponding increase in the level of the PR-10a protein (Subramaniam et al., 1997). Analysis of the promoter region of the *PR-10a* gene in transgenic potato plants indicates that a 1015 bp 5′ upstream region of the gene is sufficient to confer elicitor-inducibility of the β-glucuronidase (GUS) reporter gene in potato tuber discs (Matton et al., 1993). *STH-2* gene family (PR-10 protein gene) in potato is induced by elicitor treatment (Constabel and Brisson, 1992). About 1 kb of 5′ flanking DNA was sufficient to direct elicitor induction of the *STH-2* gene (Matton et al., 1993). Deletion analysis of the *STH-2* gene 5′ flanking region suggested that 135 bp of upstream region may be sufficient for full promoter activity (Matton et al., 1993). An important regulatory sequence is localized

between nucleotides −135 and −105 (Matton et al., 1993). Deletion analysis of the 5′ region of the proteinase inhibitor II gene (PR-6 protein gene) revealed that the region from −136 to −165 and from −520 to −620 may contain a complex of *cis* elements that interact with elicitor-inducible *trans*-acting factors to regulate PR genes (Ryan, 1992).

6.7.2 Induction of PR Genes by Salicylic Acid

Salicylic acid accumulates during fungal pathogenesis, and the accumulated salicylic acid triggers transcription of PR genes. Accumulation of salicylic acid induces expression of PR genes, and inhibition of accumulation of salicylic acid inhibits expression of PR genes. Increased levels of salicylic acid induced PR genes in various plants. Several *Arabidopsis* mutants such as *acd2*, *lsd1*, *lsd2*, *lsd3*, *lsd4*, *lsd5*, *lsd6*, *lsd7*, *cep1*, *cpr1*, and *cim3* constitutively express elevated levels of salicylic acid and all of them show constitutively high *PR* gene expression (Dangl et al., 1996; Ryals et al., 1996). Transgenic tobacco plants expressing *Halobacterium* bacteriopsin gene with proton pump function show elevated levels of salicylic acid and show high expression of *PR* genes (Dangl et al., 1996). When tobacco plants that are deficient in catalase are transformed with tobacco catalase gene, elevated levels of salicylic acid were observed in those transgenic plants. These transgenic plants showed constitutively high expression of PR genes (Chamnongpol et al., 1996; Takahashi et al., 1997). When Xanthi-nc tobacco plants were incubated at 32°C instead of 24°C, salicylic acid accumulation was inhibited, and it was followed by the disappearance of PR proteins (Yalpani et al., 1991). *Pseudomonas putida* produces an enzyme, salicylate hydroxylase, which degrades salicylic acid to catechols (Lawton et al., 1994b). Transgenic plants, engineered to express salicylate hydroxylase (*NahG*) gene from *P. putida*, could not accumulate salicylic acid following pathogen infection (Gaffney et al., 1993). Transgenic *Arabidopsis* plants expressing *NahG* did not accumulate salicylic acid and also mRNAs encoding PR proteins suggesting the importance of salicylic acid in induction of PR proteins (Lawton et al., 1994b). Salicylic acid induced mRNAs that encode nine different PR proteins in tobacco. They are acidic form of PR-1, PR-2, PR-3, PR-4, PR-5, basic form of PR-1, basic class III chitinase structurally unrelated to PR-3, acidic class III chitinase, and PR-Q′ (Ward et al., 1991). Exogenous application of salicylic acid also induces PR genes in different plants (Uknes et al., 1992). Treatment of tobacco plants with salicylic acid strongly induced accumulation of mRNAs of class II and class III β-1,3-glucanases and certain other PR proteins (Ward et al., 1991; Niki et al., 1998). Promoter activity of the class II PR-2b and PR-2d genes was induced in tobacco in response to salicylic acid (Eyal et al., 1992; Van de Rhee et al., 1993). Spraying of the leaves of young potato plants with salicylic acid induced the appearance of eight intercellular acidic PR proteins including β-1,3-glucanase, chitinase, and PR-5 (Pierpoint et al., 1990). All these results suggest that salicylic acid is the key signal in triggering transcription of PR genes. Several signals for induction of salicylic acid have been identified, and H_2O_2 appears to be one of the important signals for salicylic acid accumulation. High levels of H_2O_2 stimulate salicylic acid biosynthesis (Leon et al., 1995). When catalase expression leading to accumulation of H_2O_2 was suppressed in leaves of transgenic tobacco plants through sense cosuppression or antisense suppression, most plants failed to show constitutive PR gene expression (Takahashi et al., 1997). H_2O_2 and H_2O_2-inducing chemicals were unable to induce PR gene expression in transgenic plants expressing *NahG* (salicylate hydroxylase from *Pseudomonas putida*, which degrades salicylic acid), although they could activate *PR-1* genes in wild-type tobacco (Neuenschwander et al., 1995). These results suggest that salicylic acid may act downstream of H_2O_2 in inducing PR genes.

G-proteins, which modulate phosphorylation, also induce salicylic acid. Transgenic tobacco plants expressing the cholera toxin gene, which modulates signaling system induced by G-proteins, constitutively accumulate high levels of salicylic acid and express PR genes

(Beffa et al., 1995). Salicylic acid may also act as signal molecule in inducing other signals for induction of PR genes. Salicylic acid binds to catalase, inhibits its activity, and thereby increases the intracellular concentration of H_2O_2, which might then serve as a second messenger for the induction of a defense response (Chen et al., 1993). Lipid peroxides are potent signaling molecules in animals (Durner et al., 1997). Salicylic acid induces lipid peroxidation in tobacco suspension cells, and the lipid peroxides activate *PR-1* genes in these cells. Salicylic acid binds with a protein (SABP2) (Du and Klessig, 1997), which triggers induction of PR proteins (Görlach et al., 1996; Du and Klessig, 1997).

Although salicylic acid induces synthesis of several PR proteins, some of them are not induced by salicylic acid. Many basic PR proteins such as class I PR-2 proteins Ggl50 and Glb are not induced by salicylic acid in tobacco (Castresana et al., 1990). Transcripts of tobacco class I β-1,3-glucanase and chitinases are not induced in response to salicylic acid (Beffa et al., 1995; Vidal et al., 1997; Niki et al., 1998). Although PR-1, PR-2, and PR-5 genes require salicylic acid signaling for activation, the plant defensin gene *PDF1.2* along with *PR-3* and *PR-4* genes are induced by jasmonic acid signal pathway in *Arabidopsis* (Thomma et al., 1998).

Certain *cis*-acting elements and *trans*-acting factors may be involved in the induction of PR gene expression by salicylic acid. A deletion analysis of the promoter region of a gene encoding an acidic PR-1 protein revealed that salicylic acid-responsive elements might reside between nucleotides 625 and 902 upstream of the transcription site (Van de Rhee et al., 1990). Uknes et al. (1993a) have reported that 661 bp of 5′ flanking sequence is sufficient for fivefold induction of *PR-1* gene by salicylic acid. It has been shown that the *cis*-acting elements for regulating gene expression of the acidic PR-1a protein gene might exist in 0.2 kb of the 5′-flanking region just upstream of the TATA box of active PR-1 protein genes (PR-1a, PR-1b, and PR-1c) (Oshima et al., 1990). A *PR-1a* promoter fragment containing nucleotides −902 to +29 was able to confer high levels of salicylic acid-induced expression to the GUS reporter gene in transgenic tobacco (Van de Rhee et al., 1990).

PR-1a promoter has been reported to contain four regulatory elements, located between nucleotides −902 and −691 (element 1), −689 and −643 (element 2), −643 and −287 (element 3), and −287 and +29 (element 4) (Van de Rhee and Bol, 1993). All four elements were required for PR gene expression by salicylic acid. Elements 1 to 3 positively regulate the *PR-1a* promoter, whereas element 4 appears to be important for a correct spacing between the other three elements and the transcription site (Van de Rhee and Bol, 1993). *PR-1a* promoter consists of at least two functional domains. One is located upstream of position −335 and contains a strong positive regulatory element. The other domain resides within the region between −71 and +28 of the *PR-1a* gene (Beilmann et al., 1991).

Several *trans*-acting factors binding nuclear sites in promoters of acidic *PR-1* genes have been reported in tobacco (Buchel et al., 1996). These may include GT-1 like proteins (Buchel et al., 1996) and Myb1 proteins (Martin and Paz-Ares, 1997). TGA1 is a transcription factor from tobacco that specifically binds to the salicylic acid-responsive motifs *as-1* and *ocs* (Qin et al., 1994; Ulmasov et al., 1994). Buchel and Linthorst (1999) suggested that salicylic acid might induce the *PR-1a* gene, at least partly by mediating through binding of TGA1a to the *as-1*-like element present in the promoter. The *as-1*-like element in the tobacco PR-1a promoter upstream region has been shown to be involved in the salicylic acid signal transduction (Grüner et al., 2003). *Cis*-acting elements inducing *PR-2* genes by salicylic acid have also been detected in tobacco (Shah and Klessig, 1996).

6.7.3 Induction of PR Genes by Ethylene

Several studies have indicated that ethylene accumulates during fungal pathogenesis (Toppan et al., 1982; Toppan and Esquerre-Tugaye, 1984). The fungal elicitors would have triggered

the accumulation of ethylene in the infected tissues. Treatment of melon petioles with an elicitor from *Colletotrichum lindemuthianum* induced ethylene within 6 h after treatment (Roby et al., 1985). Ethylene acts as a signal molecule inducing various PR protein genes (Xu et al., 1994). Generally, ethylene induces basic rather than acidic PR proteins (Del-Campillo and Lewis, 1992; Koiwa et al., 1994; Knoester et al., 1998). It induced seven basic PR proteins in bean, including two isoforms of β-1,3-glucanase, multiple isoforms of chitinase, a PR-5 protein, and a 15 kDa polypeptide serologically related to PRP1 (P14) from tomato (Del-Campillo and Lewis, 1992). Ethylene induced basic PR-1 (Eyal et al., 1992), two basic class I β-1,3-glucanases such as GLB and gglb50 (Van de Rhee et al., 1993), and basic and neutral PR-5 proteins (Koiwa et al., 1994; Knoester et al., 1998) in tobacco. It also induced a thionin named THI 2.1 (Epple et al., 1997) and the defensins Pdf1.2 in *Arabidopsis* (Penninckz et al., 1996).

The importance of ethylene in signaling induction of PR proteins has been demonstrated by developing ethylene-insensitive plants. Tobacco plants were transformed with the mutant *etr1–1* gene from *Arabidopsis*, which confers ethylene insensitivity (Knoester et al., 1998). These transformants did not express the basic PR proteins, PR-1g and PR-5c, whereas wild tobacco plants expressed them (Knoester et al., 1998). Ethylene did not induce the synthesis of defensin PDF1.2 in the ethylene-insensitive *Arabidopsis* mutants *ein2* and *etr1*, whereas it induced the defensin in wild plants (Penninckz et al., 1996). These results suggest the importance of ethylene in induction of PR proteins. A cellulase elicitor induced *PR-5* gene in tobacco seedlings. Accumulation of *PR-5* mRNA induced by the elicitor was reversed by norbornadiene, an ethylene action inhibitor (Chang et al., 1995). It suggests that ethylene is involved in the induction of the PR-5 gene.

Ethylene-responsive *cis* elements (EREs) have been detected in the promoters of several PR genes. AGCCGCC sequences are highly conserved in the promoters of several PR genes and may constitute EREs (Hart et al., 1993). AGCCGCC sequences, which are found at −46 to −52 and −161 to −167 in the promoter region, were found to regulate ethylene-responsive expression of a neutral PR-5 gene in tobacco (Sato et al., 1996). The promoter sequence −248 to −108 bp has been shown to be required for osmotin gene induction by ethylene in tobacco and it contains the AGCCGCC motif (Eyal et al., 1993). The region from −1452 to −1193 of the promoter of the β-1,3-glucanase gene *GLB* promoter contains two copies of the heptanucleotide AGCCGCC, and this region is important for ethylene-regulated expression of the gene in tobacco leaves (Vogeli-Lange et al., 1994). In another tobacco class I β-1, 3-glucanase gene *ggl50*, which is also induced by ethylene, elements for responsiveness to ethylene are present in the −0.45 to −1.5 kb region of the 1.5 kb tobacco *ggl50* promoter. Two copies of the AGC box have been detected in 1030 bp segment of the ggl50 (Van de Rhee et al., 1993). Several other studies have demonstrated the importance of AGCGCC sequences present in the promoters of several ethylene-responsive genes (Buttner and Singh, 1997). Transgenic tobacco plants with mutated AGCGCC promoter did not induce expression of PR-5 gene when treated with ethylene, whereas wild plants with the complete AGCGCC sequence induced the PR-5 gene in response to ethylene (Sato et al., 1996). It suggests that the AGCGCC sequence is the important *cis* element in triggering induction of ethylene-responsive PR gene.

Several *trans*-acting factors called ethylene-responsive element binding proteins (EREBPs), which regulate the ethylene-induced expression of PR genes, have been identified (Ohme-Takagi and Shinshi, 1995). Four EREBPs, EREBP-1, EREBP-2, EREBP-3, and EREBP-4, have been detected in tobacco and they specifically bind the ERE AGC box (Ohme-Takagi and Shinshi, 1995). Ethylene induces mRNAs for these EREBPs in tobacco leaves (Zhou et al., 1997). The EREBP2 was found to bind to the AGCCGCC sequences of tobacco PR-5 gene (Sato et al., 1996). EREBPs may be transcription factors important for ethylene-dependent transcription of PR genes.

A homologue of the tobacco EREBP has been detected in *Arabidopsis thaliana* and it was called AtEBP. It binds specifically to TAAGAGCCGCC, an AGC box containing sequence and confers ethylene responsiveness to promoters of genes encoding PR proteins (Buttner and Singh, 1997). AtERF1 (AtEBP) gene was found to be induced by ethylene in wild-type *Arabidopsis* but not in *ein3* mutant. The EIN3 protein was capable of binding to the promoter sequence of AtERF1, suggesting that EIN3 functions as transcription regulator of AtERF1. The AtERF1 protein has been shown to bind to the GCC box and activate a defensin gene (PDF1.2) and a basic chitinase gene (Solano et al., 1998).

6.7.4 Induction of PR Genes by Jasmonic Acid/Jasmonate

Methyl jasmonate/jasmonic acid is another signal that systemically induces accumulation of PR proteins in plants during fungal pathogenesis. Jasmonic acid induced PR-1, PR-3, PR-5, and PR-9 proteins/mRNAs in rice (Schweizer et al., 1997). Jasmonic acid induced PR-1 proteins in tobacco leaves following infection by a virulent pathogen (Green and Fluhr, 1995). The *Arabidopsis acd2* mutant plants, which show increased jasmonic acid content, accumulate high levels of the PR-1 transcript (Penninckz et al., 1996). Jasmonate or methyl jasmonate induced expression of PR-6 genes in a variety of plant species (Farmer and Ryan, 1992; Wasternack and Partheir, 1997). The transcription of *GaPR-10* gene was inducible by jasmonate, but not by salicylic acid and ethylene (Zhou et al., 2002). Methyl jasmonate strongly induced the expression of the defensin gene *PDF1.2* in *Arabidopsis* (Penninckz et al., 1996). Jasmonic acid induced the thionin THI2.1 in *Arabidopsis* (Epple et al., 1997).

The importance of jasmonic acid/methyl jasmonate in inducing PR proteins has been demonstrated by showing correlation between their content and induction of PR proteins. When the concentration of endogenous jasmonates increased, the PR protein synthesis was activated (Lehmann et al., 1995; Penninckz et al., 1996). An *Arabidopsis thaliana* mutant, *acd2*, with high jasmonic acid content was identified, and in this mutant, mRNAs of the defensin PDF1.2 accumulated much higher than those detected in wild-type plants (Penninckz et al., 1996). In *Arabidopsis* mutants insensitive to methyl jasmonate (*coi* plants), PDF1.2 was not induced (Feys et al., 1994). When the synthesis of PR proteins was prevented by using specific inhibitors, the accumulation of PR proteins was suppressed. Inhibitors of the jasmonate biosynthesis pathway, such as tetracyclasis, ibuprofen, urosolic acid, or mefenamic acid, prevented an increase of endogenous jasmonates and induction of PR proteins (Xu et al., 1994; Lehmann et al., 1995). Tetracyclasis inhibits jasmonic acid synthesis. It inhibits PR-1 accumulation in rice, and the PR-1 induction in these plants could be rescued by exogenous application of jasmonic acid (Schweizer et al., 1997). These observations suggest the importance of jasmonate acting as a molecule in induction of PR proteins.

The *cis*-acting element in promoting jasmonate-induced PR protein synthesis has been identified as a G box sequence (CACGTGG) in the region of −574 nucleotides in the promoter in the potato *pin2* (proteinase inhibitor, PR-6 protein) gene (Kim et al., 1992). The putative promoter elements associated with jasmonate-responsive expression of *PDF1.2* gene of *Arabidopsis* encoding a plant defensin (PR-12) were studied (Brown et al., 2003). When the GCC box region in the promoter was deleted, a substantially lower response to jasmonate was observed. In addition, point mutations introduced into the core GCC box sequence substantially reduced jasmonate responsiveness, whereas addition of a 20 nucleotide-long promoter element carrying the core GCC box and flanking nucleotides provided jasmonate responsiveness to a 35S minimal promoter. These results indicate that the GCC box plays a key role in conferring jasmonate responsiveness to the *PDF1.2* promoter (Brown et al., 2003). Other promoter elements lying downstream from the GCC box region may also contribute to jasmonate responsiveness. Ethylene response factors also appear to

play important roles in regulating jasmonate-responsive gene expression via interaction with the GCC box (Brown et al., 2003).

A palindrome TGACG element within the *LOX1* (lipoxygenase) promoter was found to be essential for jasmonate inducibility (Rouster et al., 1997). This element has been identified as a binding site for bZIP *trans*-acting factors (Rouster et al., 1997). Methyl jasmonate enhanced the activity of promoters containing *as-1* type elements and it had the same effect on induction of PR-1 genes. It suggests that methyl jasmonate would have induced the expression of PR-1 genes by binding TGA1a to the *as-1*-like element present in the promoter of PR-1 proteins (Buchel and Linthorst, 1999).

Jasmonic acid/methyl jasmonate may not induce all types of PR proteins and only specific proteins may be induced. Methyl jasmonate did not induce PR-1, PR-2, PR-5, and PR-6 proteins in tobacco when this compound was administered alone (Heitz et al., 1999). Jasmonic acid induced class I and class IV but not class II chitinase in pine (*Pinus* sp.) (Davis et al., 2002). Jasmonate stimulated a proteinase inhibitor (*MPI*) gene but not PR-1 mRNA in maize (Cordeo et al., 1994). Salicylic acid may act as an antagonist of jasmonic acid in inducing PR proteins and it may negatively regulate the function of jasmonates (Wasternack and Parthier, 1997). Methyl jasmonate induced expression of a 23 kDa PR-5 protein in white pine, whereas salicylic acid treatment did not (Piggott et al., 2004).

6.7.5 Induction of PR Proteins May Require Different Signal Transduction Systems

PR proteins may be induced by different signal transduction systems rather than by a single transduction system. The PR protein genes *PR-1*, *PR-2*, and *PR-5* are induced by salicylic acid, whereas the *PR-3*, *PR-4*, and *PR-12* genes are induced by jasmonic acid in *Arabidopsis thaliana* (Thomma et al., 1998; Kunkel and Brooks, 2002). Ethylene and not salicylic acid induces class I chitinase in tobacco (Penninckz et al., 1996). Although salicylic acid induced both basic and acidic type *PR-1* transcript accumulation in tobacco, ethylene induced only acidic type PR-1 transcript (Eyal et al., 1992). Salicylic acid induces accumulation of class II and class III PR-2 proteins, and not class I PR-2 and PR-3 in tobacco (Ward et al., 1991; Van de Rhee et al., 1993; Vidal et al., 1997; Niki et al., 1998). By contrast, ethylene induces class I PR-2 proteins in tobacco (Ecker, 1995; Penninckz et al., 1996). Salicylic acid could not induce accumulation of PR-5 protein (Xu et al., 1994), whereas ethylene induced PR-5 transcripts in tobacco (Raz and Fluhr, 1993; Xu et al., 1994).

Jasmonic acid induced PR-6 protein, and not the PR protein P4 in tomato (Fidantsef et al., 1999). In maize embryos infected with *Fusarium moniliforme*, mRNAs of a proteinase inhibitor protein (MPI) and another PR-protein (PRms) accumulated (Cordero et al., 1994). Although treatment with fungal elicitor induced an increase in the PRms mRNA level, it did not induce accumulation of the *MPI* mRNA level. Treatment with abscisic acid or methyl jasmonate did not induce PRm mRNA synthesis, whereas these compounds were effective in induction of the accumulation of the MPI mRNA (Cordero et al., 1994).

Methyl jasmonate induced proteinase inhibitor (PR-6 protein), but not PR-1, PR-2, and PR-5 proteins in tobacco (Heitz et al., 1999). Methyl jasmonate or ethylene induced the expression of defensin (PR-12) gene *PDF1.2*, whereas salicylic acid did not induce the gene in *Arabidopsis* (Penninckz et al., 1996; Epple et al., 1997). Jasmonic acid and ethylene induced the *Arabidopsis* thionin (PR-13) *THI2.1* gene, whereas salicylic acid could not induce it (Epple et al., 1995, 1997).

Salicylic acid induces several PR-10 proteins such as AoPR1 (asparagus PR protein) and SAM22 (soybean PR protein) (Warner et al., 1994), but not rice PR-10 protein PBZ1 (Midoh and Iwata, 1996). Salicylic acid does not induce PR proteins in rice, whereas jasmonic acid induces them (Schweizer et al., 1997). These results suggest that distinctly different signal transduction pathways may exist in triggering induction of PR proteins in plants.

6.7.6 Synergistic Effect of Different Signals

Different signals may induce accumulation of PR proteins in different manners. It has been shown that in some cases, there may be synergistic effect of these signals in triggering PR protein synthesis. Synergistic effect of ethylene and methyl jasmonate in induction of PR proteins has been reported. When methyl jasmonate was applied in combination with ethylene, the activity of the promoter of PR-5 (osmotin) was induced dramatically beyond that seen with either ethylene or methyl jasmonate in tobacco seedlings (La Rosa et al., 1992; Xu et al., 1994). Salicylic acid substantially induced the accumulation of PR-1b protein in Wisconsin 38 tobacco. The combination of salicylic acid and methyl jasmonate induced PR-1b protein to accumulate severalfold more (Xu et al., 1994).

6.7.7 Antagonistic Effect of Different Signals

There may also be antagonistic effects between different signals in inducing PR proteins. Accumulation of PR protein P4 and PR-6 protein in tomato induced by jasmonic acid signaling system has been shown to be suppressed by benzothiadiazole treatment, which induces the salicylic acid signaling system (Fidantsef et al., 1999). Salicylic acid inhibited synthesis of PR-6 protein in tomato leaves induced by systemin and jasmonic acid (Doares et al., 1995; Wasternack and Partheir, 1997). Salicylic acid suppressed expression of a PR-12 gene, which is induced by ethylene and jasmonic acid in *Arabidopsis* (Ellis and Turner, 2001; Ellis et al., 2002). Jasmonic acid induced PmPR-10 protein accumulation in western white pine (*Pinus monticola*), whereas the induction of the PR-10 protein was suppressed by salicylic acid and abscisic acid (Liu et al., 2003). These results suggest that there may be antagonism between signals.

6.8 PR PROTEINS ARE SYNTHESIZED AS LARGER PRECURSORS

PR proteins may be synthesized as larger precursors. When polyA^+ RNA from tobacco leaves treated with potassium salicylate were translated in the rabbit reticulocyte lysate, two translation products were observed and they were immunologically identical to PR-1a. However, in the SDS-polyacrylamide gel electrophoresis, these two products showed slightly lower mobilities than the radioactive PR-1 group produced in tobacco leaf discs. The two translation products were further subjected to two-dimensional gel electrophoresis. These spots separated into at least four bands in the gel electrophoresis, all showing higher isoelectric points. These results suggest that PR-1 proteins are synthesized as larger precursors and their shift to higher isoelectric points reflects the involvement of a signal peptide, which generally has a basic charge. The *in vitro* products were translated in the presence of dog pancreatic microsome membrane. The *in vitro* products were cleaved by the membrane to the same size as mature PR-1 proteins. These results clearly show that PR-1 proteins are synthesized as larger precursors containing signal peptides (Matsuoka et al., 1988).

Carr et al. (1985) also provided evidences that PR-1 proteins are synthesized as large precursors. The PR-1 synthesized *in vivo* in Xanthi-nc tobacco leaves migrated faster in the gel electrophoresis than the *in vitro* translation product made in the wheat germ cell-free system, suggesting a difference in size of approximately 1 kDa. Hooft van Huijsduijnen et al. (1985) showed that PR-1 proteins are synthesized as large precursors containing signal peptides, which are composed of 30 amino acid residues. The molecular weight of precursors of PR-1a and PR-1b were calculated to be 15,300 and 15,100, respectively.

Both tobacco and bean β-1,3-glucanases are postulated to be synthesized as larger precursors with an N-terminal signal peptide (Shinshi et al., 1988; Vogeli et al., 1988). β-1,3-Glucanase is produced as a 359 residue preproenzyme with an N-terminal hydrophobic signal peptide of 21 residues and a C-terminal extension of 22 residues containing a putative

N-glycosylation site. The prepro-β-1,3-glucanase is processed sequentially. First, the N-terminal signal peptide is removed and an oligosaccharide side chain is added. Later, the oligosaccharide and C-terminal extension are removed to give the mature enzyme. The distinctive feature of tobacco β-1,3-glucanase processing is that the proenzyme undergoes a loss of an oligosaccharide side chain and the C-terminal extension. Mature β-1,3-glucanase does not contain *N*-acetyl glucosamine, which is a constituent of plant *N*-glycans. This observation and the fact that the only putative N-glycosylation site in the molecules is in the C-terminal extension suggest that processing to the mature form results from the loss of an N-glycopeptide (Shinshi et al., 1988).

Pea β-1,3-glucanase is processed from a larger precursor with an N-terminal signal peptide that is cleaved between Ala-1 and Glu-2. There is a potential intron 3′-splice acceptor site located at just 5′ of Ala-1 (Chang et al., 1992). Pea β-1,3-glucanase is further processed by cleavage of a glycosylated 21 amino acid peptide from its C-terminus (Chang et al., 1992). The enzyme has a single putative glycosylation site, Asn–Xaa–Ser–Thr, starting at Asn-330 in the C-terminal extension, suggesting that processing of the pea β-1,3-glucanase may involve glycosylation. There is a glycine adjacent to a phenylalanine at the C-terminus and processing may involve cleaving its glycosylated C-terminus (Chang et al., 1992). Processing of pea β-1,3-glucanase is similar to that of tobacco basic β-1,3-glucanase. The location of the 3′-splice acceptor site at 5′ of Ala-1 is similar in both the β-1,3-glucanases (Castresana et al., 1990; Ohme-Takagi and Shinshi, 1990). Pea β-1,3-glucanase has significant amino acid homology with the C-terminal regions of tobacco β-1,3-glucanase (Chang et al., 1992). The presence of a glycine adjacent to a phenylalanine at the C-terminus is also common to both the glucanases (Chang et al., 1992).

Class I chitinases (PR-3 proteins) of tobacco are synthesized as precursors with an N-terminal signal sequence, which directs them to the secretory pathway. At the N-terminal end of the enzyme, a cysteine-rich domain is found. Most class I chitinases also have a C-terminal polypeptide, which is required for their targeting to the vacuole. Within the mature protein, the N-terminal CBD is linked to the catalytic domain by a spacer, variable in length and sequence, but usually rich in proline or glycine (Neuhaus, 1999). The complete nucleotide sequence of a bean chitinase gene has been determined. The structural gene contained a single open reading frame comprising 981 bp that encode the 301 amino acids of the mature protein and a 26 amino acid signal peptide (Broglie et al., 1989).

Tomato proteinase inhibitor I cDNA encodes a precursor of 111 amino acids that contain an NH_2-terminal propeptide that is removed before maturation of the protein. The propeptide may be 19 amino acids in length (Graham et al., 1985). The tomato proteinase inhibitor I processing shows three domains. These domains are the signal sequences, which span amino acids 1 through 23, the propeptide spans residues 24 through 42, and the mature protein that begins at residue 43 (Osteryoung et al., 1992). Translation of the inhibitor I precursor results in cleavage of the signal sequence. The signal sequence is cleaved between Ala 23 and Arg 24 of the precursor protein (Osteryoung et al., 1992). The propeptide is retained following cotranslational removal of the signal sequence. Proteolytic process of the inhibitor I propeptide occurs resulting in mature protein (Osteryoung et al., 1992). Processing of tobacco AP24 protein includes the removal of both an N-terminal signal peptide of 25 amino acids and a C-terminal propeptide of 18 amino acids (Melchers et al., 1993). Thus, several studies have shown that PR proteins are synthesized as larger precursors.

6.9 SECRETION OF PR PROTEINS

6.9.1 Secretory Pathways

PR proteins accumulate in the apoplast (Bol et al., 1990) and the vacuole (Keefe et al., 1990). They are synthesized principally in membrane-bound polysomes, and mRNAs for PR-1

proteins have been found to be associated with membrane-bound polysomes (Carr et al., 1985). It suggests that the PR proteins are secreted from the membranes. The secretory system of plant cells delivers proteins to vacuole, tonoplast, plasma membrane, and the cell wall/extracellular space. In addition, proteins that enter the secretory system may be retained in the endoplastic reticulum (ER) or in various compartments of the Golgi complex. The first step common to the transport of all these proteins is translocation across the ER membrane (Walter and Lingappa, 1986). Once inside the lumen of the ER, transport depends on two types of information: informational domains that contain specific targeting or retention information and transport competence. Proteins that lack transport competence may be broken down in the secretory system (Pelham, 1989; Klausner and Sitia, 1990). Proteins that have entered the ER and that lack targeting or retention information are secreted via the bulk-flow or default pathway. When chimeric constructs of genes encoding various foreign proteins with the nucleotide sequence for a signal peptide are expressed in plant cells, the resulting proteins are secreted (Lund et al., 1989). The nature of the bulk-flow or default pathway followed by proteins that enter the secretory system by virtue of the presence of a signal peptide suggests that signal peptide of the protein is both necessary and sufficient for efficient secretion (Hunt and Chrispeels, 1991). Entry into the secretory pathway is accompanied by glycosylation in Golgi apparatus (Hunt and Chrispeels, 1991). Glycans help to stabilize protein conformation and protect proteins against breakdown (Faye and Chrispeels, 1989). The glycosylated protein may have a somewhat greater transport competence and stability (Hunt and Chrispeels, 1991). However, glycosylation of chitinase is not required for its efficient secretion from plant cells (Lund and Dunsmuir, 1992). Nonglycosylated forms of the protein are secreted as efficiently as are the glycosylated forms (Sidman, 1981; Kukuruzinska et al., 1987).

6.9.2 Site of Accumulation of PR Proteins

PR proteins accumulate in apoplast or vacuole of plant cells. Acidic PR proteins normally accumulate in the apoplast, whereas basic proteins are localized in the vacuole with many exceptions. PR proteins belonging to the acidic PR-1 family have been localized in the apoplast (Dumas et al., 1988; Hosokawa and Ohashi, 1988). The acidic PR protein PvPR1 was localized primarily in the extracellular space in bean leaves (de Tapia et al., 1986). The acidic chitinases, β-1,3-glucanases, and thaumatin-like proteins were found to accumulate in extracellular pocket-like vesicles in tobacco infected with a virus (Dore et al., 1991). Several basic PR proteins accumulate in vacuoles of cells in the epidermis of plants (Ohashi and Matsuoka, 1987; Mauch et al., 1992). A basic chitinase was found localized in vacuole in bean leaves (Broglie et al., 1986). Basic chitinases and β-1,3-glucanases accumulate in vacuoles of cells of the lower epidermis and in cells adjacent to the vascular tissue in tobacco (Mauch et al., 1992). The basic PR-5 protein accumulated in vacuoles in potato (Liu et al., 1996) and tobacco (Melchers et al., 1993).

There are many exceptions in this general statement that acidic proteins accumulate in apoplast (extracellularly) and basic proteins accumulate in vacuole (intracellularly). Some basic PR proteins have been shown to be secreted into the apoplast. Two basic PR proteins of barley, Hv PR-1a and Hv PR-1b, were detected in the intercellular fluid of barley leaves (Bryngelsson et al., 1994). Some acidic proteins have been localized intracellularly. The acidic bean PR proteins, PvPR2 and PvPR3, were localized to the cytoplasm (de Tapia et al., 1986; Sharma et al., 1992). Intracellular location of the acidic PR proteins, STH-2 in potato, SAM22 in soybean, PoPR1 in parsley, and AoPR1 in asparagus, has also been reported (Somssich et al., 1986; Walter et al., 1990; Warner et al., 1994; Midoh and Iwata, 1996).

A C-terminus 7-amino acid peptide was identified to be required and sufficient for vacuolar targeting of a basic chitinase in tobacco (Neuhaus et al., 1991b; Melchers et al.,

1993). Further analysis of this C-terminal peptide indicated that deletion of the C-terminal methionine did not affect the intracellular location, but deletion of even a single internal amino acid caused predominantly extracellular secretion of chitinase (Neuhaus et al., 1994). A vacuolar targeting signal has been detected at the C-terminal end of basic PR-1, which contains a C-terminal extension compared with its acidic counterpart (Cornelissen et al., 1986). A 22 amino acid C-terminal extension was identified to be the vacuolar targeting signal of β-1,3-glucanase in tobacco (Melchers et al., 1993). A PR-5 protein AP24 was shown to contain a C-terminal sequence required for intracellular retention in tobacco (Melchers et al., 1993).

Comparative sequence analysis between intracellular and extracellular isoforms of chitinases, β-1,3-glucanases, and PR-5 proteins have shown that in general intracellular proteins contain a C-terminal extension compared with their extracellular homologue (Cornelissen et al., 1986; Van den Bulcke et al., 1989; Linthorst et al., 1990a,b). Deletion of C-terminal regions of 20, 7, and 22 amino acids resulted in the secretion of basic PR-5 protein AP24, basic chitinase, and basic β-1,3-glucanase, respectively, in tobacco (Melchers et al., 1993). It seems that the C-terminal propeptide of AP24, basic chitinase, and basic β-1,3-glucanase is necessary for efficient sorting of these proteins to vacuole.

It appears that all vacuolar PR proteins may contain a C-terminal propeptide that is necessary for proper targeting of these proteins. A precursor to the basic chitinase of tobacco that is deposited in the vacuole contains a C-terminal extension of seven amino acids (Nakamura and Matsuoka, 1993). The C-terminal extension is absent in mature proteins as well as in the homologous precursor to the acidic chitinase deposited in the cell wall. Deletion of this C-terminal extension missorts chitinase, causing it to be secreted into the intercellular space, and fusion of the C-terminal extension to the C terminus of the cucumber secretory form of chitinase through a 3-amino acid linker redirects the fusion protein to the vacuole (Neuhaus et al., 1991b). It suggests that C-terminal extension of tobacco chitinase is necessary and sufficient for vacuolar sorting (Neuhaus et al., 1991b). Some basic proteins of barley, HvPR-1a and HvPR-1b, have been shown to be secreted into the apoplast and they lack C-terminal sequences (Bryngelsson et al., 1994).

The role of C-terminal extension in vacuolar targeting of β-1,3-glucanase has also been reported. After removal of the N-terminal signal peptide, the C-terminal sequence of 22 residues of the basic tobacco β-1,3-glucanase becomes glycosylated and is subsequently cleaved off during vacuolar targeting of the protein (Shinshi et al., 1988). An extracellular acidic β-1,3-glucanase of tobacco lacks this C-terminal extension (Linthorst et al., 1990a). A terminal extension with a potential glycosylation site, which is involved in vacuolar targeting, is also present in the C-terminal part of the precursor protein of the basic 35 kDa β-1,3-glucanase of tomato (Van Kan et al., 1992).

6.10 PR PROTEINS MAY BE INVOLVED IN INHIBITION OF PATHOGEN DEVELOPMENT

6.10.1 Inhibition of Fungal Growth by PR Proteins *In Vitro*

PR proteins accumulate in the infected tissues; however, the exact function of these proteins is not known. These proteins accumulate in both susceptible and resistant interactions (Benhamou et al., 1991; Pan et al., 1991a; Newman et al., 1994; O'Garro and Charlemange, 1994; Punja, 2001). In many instances, they accumulate more in resistant interactions (Tuzun et al., 1989; Rasmussen et al., 1992). However, there are also reports indicating that PR proteins accumulate more in susceptible interactions. In fact, some proteins are exclusively induced during disease development, and such proteins are not induced in resistant

interactions (Baga et al., 1995). Van Loon (1999) did not consider these proteins as PR proteins. According to him, PR proteins should have a role in disease resistance. The role of PR proteins in inhibiting fungal disease development has been demonstrated by both *in vitro* and *in vivo* studies.

The PR-protein fractions extracted from infected tomato leaves inhibited both discharge of zoospores from sporangia and the germination of encysted zoospores of the oomycete *Phytophthora infestans* (Enkerli et al., 1993). β-1,3-Glucanases (PR-2 proteins) isolated from pea pods inhibited *Fusarium solani* f. sp. *pisi in vitro* when they were tested at the concentrations of 250 μg/mL (Mauch et al., 1988). The purified *Arabidopsis* chitinase (PR-3 protein) effectively inhibited the growth of *Trichoderma reesei* even at as low as 0.5 μg/disc. However, growth of several fungi including *Alternaria solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, and *Gaeumannomyces graminis* and the oomycete *Phytophthora megasperma* was not inhibited, even when as much as 8 μg of purified protein was applied per disc (Verburg and Huynh, 1991). Chitinases purified from thorn-apple, tobacco, and wheat were effective inhibitors of spore germination and hyphal growth of *Trichoderma hamatum* and *Phycomyces blakesleeanus* (Broekaert et al., 1988).

Chitinase isolated from bean leaves showed antifungal activity against *Rhizoctonia solani*. The fungal hyphae were markedly reduced in size and lysis of apical zone was seen within 3 h after treatment with the chitinase. Swelling of the hyphal tips and hyphal distortions were also observed. Disruption of chitin macromolecules in the fungal cell wall preceded cell wall breakdown and protoplasm alteration (Benhamou et al., 1993). Chitinase purified from bean inhibited growth of *Trichoderma viride*, the test fungus, even at 2 μg mL (Schlumbaum et al., 1986). Toyoda et al. (1991) reported that haustoria of *Blumeria graminis* were effectively lysed in the barley epidermal cells by chitinase. In bean seedlings inoculated with *Uromyces phaseoli*, the rust pathogen, chitinase was found to be induced about 10-fold in response to infection (Schlumbaum et al., 1986). Crude extracts from bean leaves infected by the rust pathogen *U. phaseoli* produced larger inhibition zones than did extracts from uninfected control leaves when *T. viride* was used as the test fungus. Adding the antiserum against bean chitinase prevented the formation of an inhibition zone (Schlumbaum et al., 1986). It shows that the chitinase has antifungal action.

PR-4 class I protein from tobacco exhibits antifungal activity toward *T. viride* and *F. solani* by causing lysis of the germ tubes and growth inhibition (Ponstein et al., 1994b). PR-5 group of proteins contain many antifungal proteins (Salzman et al., 2004). The tobacco PR protein AP 24 shows high antifungal activity (Woloshuk et al., 1991b). NP 24, an OLP from tomato, inhibits hyphal growth of the oomycete *Phytophthora infestans* at concentrations greater than 400 nM (Woloshuk et al., 1991b). The PR-6 proteins (proteinase inhibitors I and II) from tomato show antifungal action (Walker-Simmons and Ryan, 1977). Defensins (PR-12 proteins) isolated from spruce (*Picia glauca*) were found to cause extensive growth inhibition of *Cylindrocladium floridanum*, *Fusarium oxysporum*, and *Nectria galligena* at 2.5 μM (Pervieux et al., 2004). Thionins (PR-13 proteins) have been shown to be toxic to fungal pathogens (Apel et al., 1990; Bohlmann and Apel, 1991). They have been shown to permeate fungal cell membranes and inhibit DNA, RNA, and protein synthesis (Garcia-Olmedo et al., 1989). The PR-14 proteins (LTPs) have been shown to be fungitoxic. They may insert themselves into the fungal cell membrane and the central hydrophobic cavity may form a pore, allowing efflux of intracellular ions, and thus leading to fungal cell death (Selitrennikoff, 2001). The basic chitosanases isolated from stressed barley leaves induced lysis of spores of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Verticillium albo-atrum*, and *Ophiostoma ulmi* (Grenier and Asselin, 1990).

Some of the PR proteins act synergistically with other PR proteins in inhibiting growth of fungi. Tobacco class I chitinase acts synergistically with tobacco class I PR-4 protein inhibiting *Fusarium solani* germlings (Ponstein et al., 1994a,b). Similarly, tobacco class I PR-protein

acts synergistically with a tobacco class I β-1,3-glucanase against *F. solani* germlings (Ponstein et al., 1994a,b). Tobacco class V chitinases act synergistically, with class I β-1,3-glucanase inhibiting growth of *T. viride* and *Alternaria radicina* (Ohl et al., 1994; Ponstein et al., 1994b). A combination of chitinase and ribosome-inactivating protein from barley inhibit fungal growth more efficiently than do either protein alone (Leah et al., 1991). Probably, chitinase shows more antifungal action when it is combined with other PR proteins.

Although several PR proteins have been shown to be inhibitory against pathogens, there are also other reports showing that some PR proteins may not have any inhibitory action against fungal pathogens. The purified PR-1 protein did not show any effect on axenically growing *Chalara elegans* when tested at concentrations ranging from 10 to 300 μg (Tahiri-Alaoui et al., 1993). In general, although basic (class I) chitinases and β-1,3-glucanases show antifungal activity, acidic forms of these PR proteins do not possess antifungal activity (Sela-Buurlage et al., 1993; Ji and Kuc, 1996; Moravčikova et al., 2004). PR-8 proteins from cucumber do not have antifungal activity (Moravčikova et al., 2004). Class III chitinases (PR-8 proteins) seem to lack antifungal activity (Vogelsang and Barz, 1993).

6.10.2 Inhibition of Fungal Growth by PR Proteins *In Vivo*

Inhibitory action of some PR proteins against fungal pathogens has been demonstrated in the infected tissue itself. In eggplant (*Solanum melongena*) infected with *Verticillium albo-atrum*, a purified β-1,3-glucanase was used to study its role in lysis of fungal structures in the infected plant tissues by means of postembedding immunogold labeling (Benhamou et al., 1989). In the infected tissues, intense gold deposition occurred on the fungal cell surface. The diffusion of gold particles toward the inside of fungal cells was observed indicating that the structural integrity of fungal cell walls was generally altered. It suggests that β-1,3-glucanase lyses the fungal cell wall (Benhamou et al., 1989). Chitinase has also been shown to lyse the cell walls of the oomycete *Phytophthora capsici* in the infected pepper plants (Lee et al., 2000b). Immunogold labeling data showed specific labeling of chitinase on the cell wall of *P. capsici* with predominant accumulation over areas showing signs of degradation (Lee et al., 2000b).

Benhamou et al. (1991) found that the PR-1 protein (PR-P14) accumulated over host cell wall papillae in tomato roots infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici* and suggested that the PR protein may restrict the development of the pathogen by host cell wall modifications. PR-1 proteins were found to be associated with the host cell wall outgrowths and papillae produced by tobacco roots in response to *Chalara elegans* infection. These proteins increase mechanical strength of these defense-related structures and inhibit the fungal development (Tahiri-Alaoui et al., 1993). Broglie et al. (1991) developed *Brassica napa* plants that constitutively expressed a bean vacuolar chitinase gene under the control of the strong 35S promoter of the cauliflower mosaic virus. When the transgenic *B. napa* plants were inoculated with *Rhizoctonia solani*, significant reduction in fungal growth and delay in disease development were observed. It suggests the antifungal action of bean chitinase *in vivo*.

Transgenic wheat plants expressing the barley chitinase gene showed resistance to the powdery mildew pathogen *Blumeria graminis* f. sp. *tritici* and the leaf rust pathogen *Puccinia triticina*. In the infected tissues, the colony formation of the two pathogens was reduced and it resulted in inhibition of spread of these two diseases (Oldach et al., 2001). Transgenic *Arabidopsis* plants constitutively overexpressing the thionin *Thi2.1* (*PR-13*) gene showed resistance to *Fusarium oxysporum* f. sp. *matthiolae* (Epple et al., 1997). Cotyledons of transgenic lines had less fungal growth and more hyphae with growth anomalies, including hyperbranching, than on cotyledons of susceptible plants (Epple et al., 1997). These observations strongly demonstrate the antifungal activity of PR proteins *in vivo*.

6.10.3 Some PR Proteins May Be Involved in Release of Elicitor Molecules in Planta

The function of some PR proteins may be to release elicitor molecules from the host and pathogen cell wall surface, and these elicitors may stimulate biosynthesis of other antifungal PR proteins, phytoalexins, and fungitoxic phenolics (Hammerschmidt, 1999). Yoshikawa et al. (1993) developed transgenic tobacco plants expressing a nonfungitoxic β-1,3-glucanase from soybean. These transgenic plants showed resistance to fungal pathogens. Phenylalanine ammonia-lyase transcripts increased earlier in the transgenic plants than in control plants (Yoshikawa et al., 1993). It suggests that the increased resistance involved the release of active elicitor molecules and not the direct action of the PR-2 protein on the pathogen. Some chitinases may release signal molecules (specific oligosaccharides) from the plant cell walls and trigger host defense mechanisms (Van Loon, 1999).

6.10.4 Some PR Proteins May Be Involved in Reinforcement of Cell Wall Structures

PR-1 proteins have not shown any fungitoxicity. However, Benhamou et al. (1991) suggested that they may be involved in cell wall thickening and may offer resistance to the spread of pathogens in the apoplast. The PR protein PR-9 (peroxidase) is involved in lignification. Peroxidases are also involved in cross-linking of extensin monomers, polysaccharide cross-linking, and suberization. The peroxidases may enhance resistance by the construction of a cell wall barrier that may retard the fungal penetration and development in host cells (Venere et al., 1993). The PR-15 and PR-16 proteins have been suggested to release H_2O_2, necessary for cross-linking of cell wall components during formation of papillae (Carter and Thornburg, 1998; Wei et al., 1998).

6.11 PR PROTEINS MAY BE INVOLVED IN TRIGGERING DISEASE RESISTANCE

6.11.1 Demonstration of the Role of PR Proteins in Disease Resistance Using Chemical or Biological Elicitors

PR proteins were found to be associated with induction of disease resistance. Resistance could be induced by both biotic and chemical elicitors. Two forms of induced resistance in plants, systemic acquired resistance (SAR) and induced systemic resistance (ISR), have been recognized. SAR refers to a distinct signal transduction pathway that is mediated by salicylic acid and activates defense genes (Dong and Beer, 2000). Salicylic acid and several synthesized analogues of salicylic acid, such as 2,6-dichloroisonicotinic acid (INA), are known to activate SAR. In contrast to SAR, which is mediated by salicylic acid, ISR is mediated by jasmonic acid and ethylene (Dong and Beer, 2000). ISR appears to be induced by biocontrol bacteria, whereas SAR is induced by infection by pathogens and chemical inducers. There are many exceptions to these definitions. For example, riboflavin, a chemical, activates defense mechanisms and induces resistance; however, the activation is not through salicylic acid pathway (Dong and Beer, 2000). Several authors prefer to call this type of resistance induced by chemicals as ISR (Kuc, 2001; Oostendorp et al., 2001). Such chemically induced ISR has been related to induction of PR proteins. Thiamine protected rice plants against *Magnaporthe grisea* and cucumber plants against *Colletotrichum lagenarium* and *Sphaerotheca fuliginea* (Ahn et al., 2005). Thiamine treatment induced three rice PR genes, *PR-1*, *PR-9* (*POX22.3*, a gene encoding peroxidase), and *PR-10* (*PBZ1*), and induction of these PR proteins resulted in disease resistance. Similarly, thiamine treatment induced *POX* (a gene encoding acidic peroxidase) and conferred resistance against *C. lagenarium* in cucumber (Ahn et al., 2005). Acibenzolar-*S*-methyl induced PR proteins, PR-1, chitinase, and PR-10, in *Venturia nashicola*-inoculated pear and it induced resistance

against the pathogen (Faize et al., 2004). Benzothiadiazole (BTH, Acibenzolar-*S*-methyl) root treatment induced elevated chitinase and β-1,3-glucanase activities and expression of two members of the PR-1 gene family and a PR-5 gene in papaya leaves. This treatment also increased resistance to the oomycete *Phytophthora palmivora* (Zhu et al., 2003; Qiu et al., 2004). Salicylic acid treatment induced PR-1 proteins and conferred resistance against *Botrytis cinerea* in tobacco (Murphy et al., 2000).

Arabidopsis mutants deficient in ascorbic acid (vitamin C) were more resistant to the oomycete *Peronospora parasitica* than the wild-type plants, and in these mutants, induction of PR-1 and PR-5 proteins were higher than that in the wild-type plants (Barth et al., 2004). When ascorbic acid was fed to the ascorbic acid-deficient mutants, the expression of PR-1 gene was reversed (Pastori et al., 2003), and the plants without ascorbic acid deficiency become susceptible to pathogens (Barth et al., 2004). These results suggest that PR proteins may play an important role in disease resistance.

The role of PR proteins in disease resistance was demonstrated by inducing SAR by biological inducers. SAR was induced in tobacco following inoculation of the oomycete *Peronospora tabacina* sporangia into the stems of the plants highly susceptible to the pathogen. Upregulation of the PR-2 gene, *PR-2d*, was observed in the induced resistant plants. The induction of PR-2 proteins was associated with the induced resistance (Funnell et al., 2004). It suggests that the PR protein may be involved in conferring resistance.

6.11.2 Demonstration of Role of PR Proteins in Disease Resistance by Inducing Mutation

Some mutants overexpressing PR proteins show resistance to fungal diseases. An *Arabidopsis* mutant iop1 (induced overexpression of PDF1.2) showed induced overexpression of PR genes *PR-3*, *PR-4*, and *PR-12*, and the mutant showed resistance to a number of necrotrophic pathogens (Penninckx et al., 2003). Another *Arabidopsis* mutant, cpr1 (constitutive expressor of PR genes), has elevated expression of the endogenous PR genes, *BGL2* (*PR-2*), *PR-1*, and *PR-5*. This mutant showed enhanced resistance to the oomycete *Peronospora parasitica* (Bowling et al., 1994). The *Arabidopsis* mutant cep (constitutive expression of PR-1 genes) showed constitutive expression of the *PR-1*, *PR-2*, and *PR-5* genes, and this mutant was resistant to virulent fungal pathogens (Silva et al., 1999). These results suggest that these PR proteins are involved in conferring resistance to pathogens.

6.11.3 Demonstration of Role of PR Proteins in Disease Resistance by Developing Transgenic Plants

To demonstrate the role of PR proteins in inducing resistance, transgenic plants overexpressing genes encoding specific PR proteins have been developed. Transgenic plants expressing high levels of PR-1a show resistance against the oomycete pathogens *Peronospora tabacina* and *Phytophthora parasitica* (Alexander et al., 1993). Transgenic alfalfa plants overexpressing β-1,3-glucanase were resistant to the oomycete *Phytophthora megasperma* f. sp. *medicaginis* (Masoud et al., 1996). Transgenic tobacco plants expressing barley β-1,3-glucanase transgene showed high resistance to *Rhizoctonia solani* (O'Brein et al., 2001). Transgenic flax (*Linum usitatissimum*) plants expressing β-1,3-glucanase showed enhanced resistance to *Fusarium oxysporum* f. sp. *lini* (Figure 6.1; Wróbel-Kwiatkowska et al., 2004).

Transgenic rice plants expressing a chitinase gene constitutively showed resistance to the rice sheath blight pathogen *R. solani* (Lin et al., 1995; Datta et al., 2000, 2001) and to the blast pathogen *Magnaporthe grisea* (Nishizawa et al., 1999). The transgenic tobacco plants constitutively expressing a bean vacuolar chitinase gene showed resistance to *Rhizoctonia solani*, the soil-borne pathogen infecting numerous plant species (Broglie et al., 1991). Transgenic

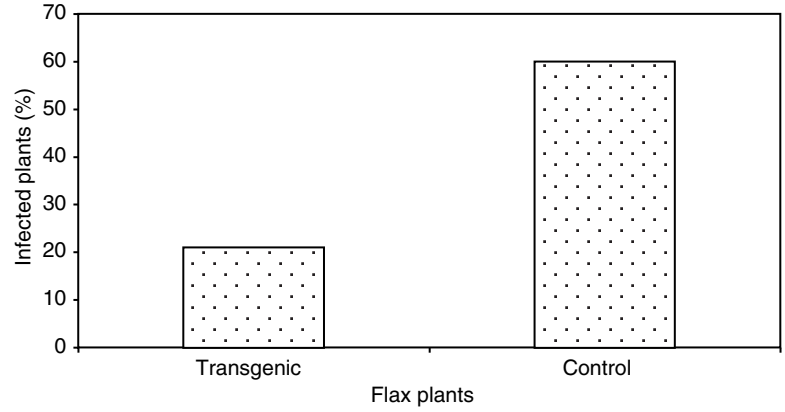

FIGURE 6.1 Resistance of transgenic flax plants expressing β-1,3-glucanase to *Fusarium oxysporum* f. sp. *lini*. (Adapted from Wróbel-Kwiatkowska, M., Lorenc-Kukula, K., Starzycki, M., Oszmiański, J., Kepczyńska, E., and Szopa, J., *Physiol. Mol. Plant Pathol.*, 65, 245, 2004.)

tobacco plants expressing chitinase genes have been reported to afford protection against *R. solani* by several other workers (Jach et al., 1992; Oppenheim and Chet, 1992; Vierheilig et al., 1993). Transgenic rose (*Rosa hybrida*) plants overexpressing chitinase showed resistance against *Diplocarpon rosae* (Marchant et al., 1998). Transgenic apple plants overexpressing chitinase gene showed enhanced resistance to *Venturia inaequalis* (Wong et al., 1999). The transgenic wheat plants expressing barley chitinase showed increased resistance to the powdery mildew pathogen *Blumeria graminis* (Bliffeld et al., 1999). Grapevine plants expressing a class I chitinase from rice showed enhanced resistance against *Uncinula necator* and *Elsinoe ampelina* (Yamamoto et al., 2000). The transgenic tomato plants expressing an acidic chitinase gene from *Lycopersicon chilense* showed enhanced resistance to *Verticillium dahliae* (Tabaeizadeh et al., 1999). Transgenic cucumber plants harboring a rice chitinase gene exhibited enhanced resistance to *Botrytis cinerea* (Tabei et al., 1998). Transgenic chrysanthemum (*Dendrathema grandiflorum*) expressing a rice chitinase gene showed enhanced resistance to *B. cinerea* (Takatsu et al., 1999). Transgenic silver birch plants expressing the sugar beet chitinase gene showed resistance to *Pyrenopeziza betulicola* (Pappinen et al., 2002).

Transgenic rice plants overexpressing the rice PR-5 gene showed resistance to *R. solani* (Datta et al., 1999). Transgenic tomato plants constitutively expressing the PR-5 gene showed enhanced resistance to several pathogens (Veronese et al., 1999). Transgenic wheat plants expressing the barley PR-5 gene showed increased resistance against *Alternaria triticina* (Pellegrineschi et al., 2001), whereas transgenic wheat plants expressing the rice PR-5 gene plants showed enhanced resistance to wheat scab caused by *Fusarium graminearum* (Chen et al., 1999). Transgenic carrot plants expressing rice *PR-5* gene showed enhanced resistance to *Botrytis cinerea* or *Sclerotinia sclerotiorum* (Chen and Punja, 2002). Transgenic potatoes containing pea *PR-10* gene, showed resistance against *Verticillium dahliae* (Chang et al., 1993). Transgenic rice plants with constitutive expression of PR genes such as *PR-1* and *PR-10* significantly enhanced resistance to *Magnaporthe grisea* (Xiong and Yang, 2003). Constitutive expression of radish defensin (PR-12 protein) protected transgenic tobacco from *Alternaria longipes* (Terras et al., 1995). Sunflower plants were transformed with the wheat oxalate oxidase (*PR-15*) gene and the transgenic plants showed resistance against *Sclerotinia sclerotiorum* (Bazzalo et al., 2000). Transgenic soybean plants expressing wheat PR-15 gene showed resistance to stem rot caused by *S. sclerotiorum* (Donaldson et al., 2001;

Cober et al., 2003). Poplar plants expressing a wheat PR-15 gene expressed increased resistance against *Septoria musiva* (Liang et al., 2001). Transgenic wheat and barley plants overexpressing PR-16 genes showed enhanced resistance against the powdery mildew fungus *Blumeria graminis* (Thordal-Christensen et al., 2004).

All these observations suggest that accumulation of PR proteins may restrict the pathogen development. However, all the PR proteins need not be involved in disease resistance. There are also reports that transgenic plants overexpressing some PR proteins do not show resistance to the pathogens. The transgenic *Nicotiana sylvestris* plants overexpressing tobacco chitinase did not show resistance against the frog-eyespot pathogen *Cercospora nicotianae* (Neuhaus et al., 1991a). Transgenic tobacco plants overexpressing *PR-5* gene did not show resistance against the oomycete *Phytophthora parasitica* (Liu et al., 1994). Transgenic potato plants overexpressing the *PR-10* gene (STH-2) did not show any enhanced resistance against *P. infestans* (Constabel et al., 1993). Transgenic canola plants expressing pea *PR-10* gene did not confer any resistance against *Leptosphaeria maculans* (Wang et al., 1999). Expression of a cucumber class III chitinase (*PR-8*) and *Nicotiana plumbaginifolia* class I β-1,3-glucanase (*PR-2*) genes in transgenic potato plants did not confer resistance against *Rhizoctonia solani* (Moravčikova et al., 2004).

6.11.4 Demonstration of the Role of PR Proteins by Developing Transgenic Plants with Antisense Suppression of PR Genes

The role of PR proteins in conferring disease resistance has been demonstrated by suppressing the expression of PR genes by antisense construction of the gene. Resistance to the oomycete pathogens *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* in the tobacco leaves was closely associated with high levels of β-1,3-glucanase activity. Introduction of the antisense cDNA in one of the antisense regenerated lines was associated with lower levels of β-1,3-glucanase activity and decreased resistance to both the pathogens (Lusso and Kuc, 1996).

6.12 HOW DO PATHOGENS OVERCOME FUNGITOXIC PR PROTEINS OF THE HOST?

6.12.1 Slower Accumulation of PR Proteins May Enable Pathogens to Escape the Antifungal Action of PR Proteins

When the pathogen invades the host tissues, PR proteins accumulate almost immediately. However, pathogens try to avoid the antifungal PR proteins by several methods. One such method is that the virulent pathogen delays the accumulation of the PR proteins in the host. When tomato roots of a susceptible variety were inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*, root colonization started to appear in the epidemis within 48 h after inoculation and progressed rapidly to reach the vascular stele by 120 h after inoculation (Brammall and Higgins, 1988). Treatment of sections with the tomato PR-1 (PR P14) protein antiserum and gold antibodies resulted in the deposition of gold particles on host cell walls and intercellular spaces. A considerable increase in wall labeling was noticeable 72 h after inoculation. In contrast, treatment of sections with the PR P14 protein antiserum and gold antibodies resulted in the accumulation of gold particles on host cell walls at 48 h after inoculation in the resistant variety (Figure 6.2; Benhamou et al., 1991). These histological observations suggest that the accumulation of PR P14 protein in the resistant variety was detected at 48 h itself, whereas similar accumulation was detected only at 72 h after inoculation in the susceptible variety. By the time accumulation of PR protein was observed,

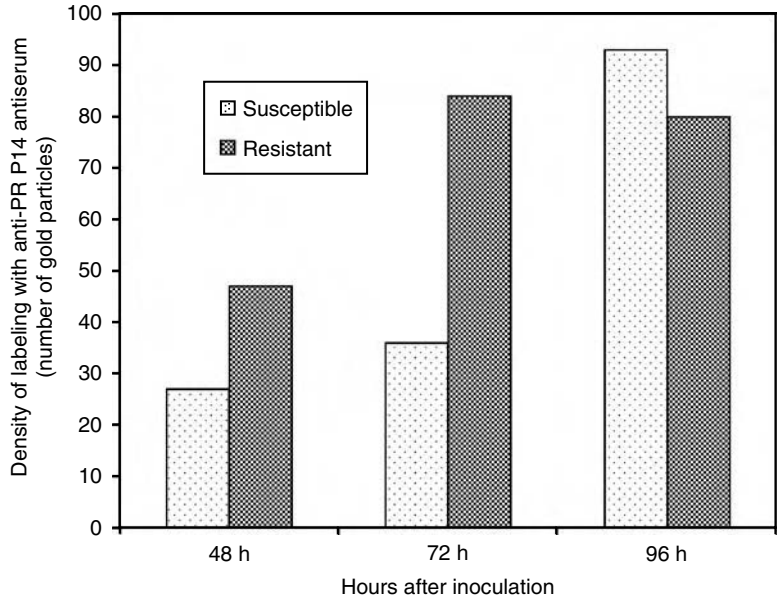

FIGURE 6.2 Accumulation of PR P14 protein in susceptible and resistant tomato cultivars inoculated with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. (Adapted from Benhamou, N., Grenier, J., and Asselin, A., *Physiol. Mol. Plant Pathol.*, 38, 237, 1991.)

the pathogen had already colonized the root tissues in the susceptible variety. Thus, slower accumulation of the PR protein would have helped the pathogen to avoid the defense-related PR protein and favored the pathogenesis (Benhamou et al., 1991).

Cladosporium fulvum induced synthesis of PR-1 proteins in tomato. When the compatible race was inoculated, these PR proteins were detectable 8–10 days after inoculation. The PR proteins, however, appeared even 6 days after inoculation when an incompatible race was inoculated (Christ and Mosinger, 1989). De Wit et al. (1986) have also shown delayed accumulation of PR-1 protein (P14) as an indication of susceptibility in tomato against *C. fulvum*.

In the compatible *Cladosporium fulvum*–tomato interaction (the tomato genotype Cf5 and fungal race 5), mRNAs encoding the PR-1 proteins could be detected only at 6 days after inoculation and they slowly increased to reach a maximum at 12 days after inoculation. In the incompatible interaction, the mRNA levels increased rapidly between 4 and 6 days after inoculation, reaching a maximum 8 days after inoculation. On the fourth day when PR protein accumulated, inhibition of the fungal growth was observed in the incompatible interaction, whereas the fungus continued to grow well in the compatible interaction, because no PR protein accumulated on the fourth day after inoculation in the compatible interaction (Van Kan et al., 1992).

Taylor et al. (1990) identified a PR protein, PR-1, in potato. The PR-1 mRNA started accumulating in about 5 h after inoculation with incompatible race of the oomycete *Phytophthora infestans*. Similar increase was observed in the compatible interaction only after about 12 h of inoculation (Taylor et al., 1990). The mRNA accumulated more in compatible interaction than in the incompatible one at later time points. The delayed accumulation of PR-1 mRNA in compatible interaction might have favored pathogenesis (Taylor et al., 1990). In rice, two PR-1 proteins have been detected. The expression of both PR-1 genes (*PR-1* and *PR-1a*) was higher and more rapidly induced in an incompatible

interaction in the rice–*Magnaporthe grisea* interactions (Kim et al., 2001). In tobacco, β-1,3-glucanase has been shown to be involved in the resistance mechanism to the oomycete *Peronospora tabacina*. Tobacco plants stem-injected with *P. tabacina* were protected against the disease after challenge with the fungus. Protection was greater than 95% in immunized plants 21 days after stem injection, compared with controls, as determined by leaf area with lesions and by sporulation. β-1,3-Glucanase activity increased in immunized plants before challenge, 15 and 21 days after stem injection with *P. tabacina*. These immunized plants were challenged by leaf inoculation 21 days following stem injection. Enzyme activity continued to increase in immunized tobacco 2 and 6 days after challenge. In contrast, β-1,3-glucanase activity was not detected in extracts of control plants before challenge. Very low activity was observed in control plants 2 days following challenge. The activity increased by 6 days after inoculation with *P. tabacina*, and by that time disease symptoms were already apparent (Tuzun et al., 1988, 1989). The results suggest that although β-1,3-glucanase may suppress the disease development, in successful pathogenesis, the PR protein is induced slowly and high enzyme activity is seen only after enough damage has been done by the pathogen (Tuzun et al., 1989).

Changes in isoforms of β-1,3-glucanase in the immunized tobacco plants were also studied (Pan et al., 1991a). Four major isoforms of β-1,3-glucanase (G1, G2, G3, and G4) were detected in the challenged tobacco leaves. The activities of G1 and G2 were higher in leaves of systemically protected plants than in those of the controls during early stages after challenge. G3 was not associated with protection, whereas G4 was of pathogen (*P. tabacina*) origin. In the susceptible control plants, symptoms began to appear 4 days after inoculation with the pathogen and G2 began to accumulate in the control, but its activity was still lower compared with the protected (resistant) plants. The control plants were severely diseased 6 days after inoculation, when G1 was detected and the activity of G2 also increased markedly in the susceptible control plants. The high activities of β-1,3-glucanase in the susceptible controls at 6 days after inoculation may be too late to prevent development of *P. tabacina*, formation of lesions, and sporulation of the oomycete (Pan et al., 1991a). Much faster and higher induction of mRNAs encoding PR-1 (*pr1*) and chitinase (*chi*) genes was observed in pear variety resistant to the scab pathogen *Venturia nashicola*, and in the susceptible variety the increases were weak and delayed to 5–7 days after inoculation with the pathogen (Faize et al., 2004).

The induction of β-1,3-glucanases gl_2 and gl_4 in barley leaves inoculated with *Blumeria graminis* f. sp. *hordei* was much slower in the susceptible variety than in the near-isogenic resistant lines (Jutidamrongphan et al., 1991). In the compatible tomato–*Cladosporium fulvum* interaction (Cf5/race 5), β-1,3-glucanase activity increased slowly compared with the incompatible interaction (Cf4/race 5) (Figure 6.3; Joosten and De Wit, 1989). This type of slower increase in β-1,3-glucanase in the compatible interaction was reflected in slower increase of β-1,3-glucanase mRNAs (Van Kan et al., 1992). β-1,3-Glucanase gene was induced 2–4 days earlier in incompatible tomato–*C. fulvum* interactions than in compatible interactions (Ashfield et al., 1994).

An antiserum raised against a purified tobacco β-1,3-glucanase was used to study the subcellular localization of β-1,3-glucanase in *Fusarium oxysporum* f. sp. *radicis-lycopersici*-infected tomato plant tissue by means of postembedding immunogold labeling (Benhamou et al., 1989). In the susceptible reaction, gold labeling accumulated over host wall areas in the immediate vicinity of fungal cells accompanied by a heavy deposition of gold particles along the fungus pathway as well as over host wall areas neighboring the channel of penetration. In the resistant variety, although uninvaded by the pathogen, cells of the endodermis, pericycle, and paratracheal parenchyma displayed an intense labeling of their walls. Similarly, secondary thickenings of uncolonized xylem vessels were also heavily labeled. This contrasted sharply with susceptible root tissues, where labeling occurred only over walls of invaded cells. The slow accumulation of

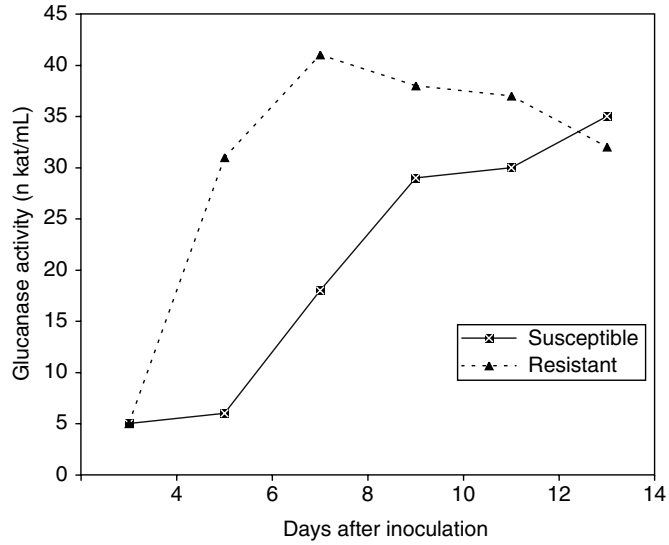

FIGURE 6.3 β-1,3-Glucanase activity in susceptible and resistant tomato cultivars inoculated with *Cladosporium fulvum*. (Adapted from Joosten, M.H.A.J. and De Wit, P.J.G.M., *Plant Physiol.*, 89, 945, 1989.)

β-1,3-glucanase that is also only in the fungus-invaded cells in the susceptible variety is in sharp contrast to that observed in the resistant variety in which a rapid induction of β-1,3-glucanase even in the uninvaded inner cells far ahead of fungal penetration was observed (Benhamou et al., 1989). These observations suggest that accumulation of PR-2 proteins is delayed in susceptible interactions, which permit growth of pathogen in the infected plant tissues.

When underside of leaf 1 of cucumber plants was sprayed with 50 mM K_2HPO_4, chitinase activity increased approximately 3.5-fold within 24 h and 8-fold by 40 h and the leaf became resistant to *Colletotrichum lagenarium*. This treatment induced systemic resistance also and leaf 2 showed about 61% reduced disease intensity when challenge inoculated with the pathogen. The leaf 2 from induced resistant plant showed higher chitinase activity even at the time of inoculation with the pathogen. The increased activity compared with control susceptible leaf was seen up to 2 days after inoculation, and on the fourth day the chitinase activity increased even in the susceptible control leaf as high as in the induced resistant leaf (Irving and Kuc, 1990). The results suggest that although chitinase activity increased to a great extent in the susceptible leaf, the increase was observed only after 2 days of inoculation. Delayed increase in the chitinase activity would have made the plant susceptible (Irving and Kuc, 1990).

When *Colletotrichum lindemuthianum* spores were inoculated on bean leaves, appressoria were observed after about 30 h. Intracellular primary hyphae emerging from infection vesicles were detected about 60 h after inoculation. Dark brown limited lesion appeared mainly on the veins 92 h after inoculation. Extensive development of primary mycelium was established within 100 h after inoculation. Simultaneously, narrow secondary hyphae branching out of the primary hyphae were visible in brown-colored cells. By 116 h after inoculation, secondary hyphae were well developed in the host cells (Mahe et al., 1992). The chitinase mRNA induction started at 84 h after inoculation and rapidly increased to reach a maximum level at 116 h. The results suggest that the chitinase mRNA is more in the necrotrophic phase, and early induction is not observed in the compatible susceptible interaction (Mahe et al., 1992). In the incompatible interaction (host resistant), there was an early increase in chitinase mRNA activity in tissue immediately underlying the site of the spore inoculation and it

TABLE 6.2
Time and Intensity of Induction of Chitinase (CAChi2) mRNA in Compatible and Incompatible Pepper–*Phytophthora capsici* Interactions

Type of Pepper–*P. capsici* Interaction	Accumulation of Chitinase mRNA in Pepper Stem Tissues at Different Hours after Inoculation (h)			
	6	12	24	36
Compatible interaction	−[a]	+[b]	++[b]	+++[b]
Incompatible interaction	++[b]	++[b]	+++[b]	+++[b]

Source: Adapted from Lee, Y.K., Hippe-Sanwald, S., Jung, H.W., Hong, J.K., Hause, B., and Hwang, B.K., *Physiol. Mol. Plant Pathol.*, 57, 111, 2000b.

[a] − indicates no accumulation.
[b] +, ++, and +++ indicate increasing degree of accumulation.

was correlated with the onset of flecking and expression of hypersensitive resistance (Mahe et al., 1992). In pepper (*Capsicum annuum*) inoculated with the oomycete *Phytophthora capsici*, the chitinase, *CAChi2* mRNA began to accumulate as early as 6 h after inoculation in the incompatible interaction (Lee et al., 2000b). In contrast, the compatible interaction exhibited a lower accumulation level of the *CAChi2* mRNA than did the incompatible interaction. *Cercospora beticola* induced class III acidic, class IV acidic, and class IV basic chitinases and a basic β-1,3-glucanase in sugar beet leaves. The major difference between susceptible and partially resistant plants was a stronger early transient expression in the latter (Nielsen, et al., 1994a). The induction of *CAChi2* mRNA started at 12 h after inoculation and the accumulation was about 2–3 times lower than that in the incompatible interaction (Table 6.2; Lee et al., 2000b).

Phoma lingam infection results in a strong induction of chitinase mRNA in rapeseed. In the resistant cultivar, the level of chitinase mRNA was threefold higher than that in the susceptible cultivar 1 day after inoculation. The chitinase transcript increased approximately 50-fold and 38-fold in resistant and susceptible cultivars 8 days after inoculation (Rasmussen et al., 1992). When barley cultivars were inoculated with *Blumeria graminis* f. sp. *hordei*, chitinase genes were expressed much earlier in incompatible interactions (Boyd et al., 1994).

Stem injection with *Peronospora tabacina* induced resistance against challenge inoculation with the same pathogen in tobacco. Increased chitinase activity was evident in both immunized and control plants 2 and 6 days after challenge, but lesser enzyme activity was detected in susceptible plants than in immunized plants 2 days after challenge (Figure 6.4; Tuzun et al., 1989). The results suggest that delayed increase in chitinase activity allows the pathogen to overcome the defense mechanism (Tuzun et al., 1989).

When *Cladosporium fulvum* was inoculated on a susceptible tomato variety, chitinase activity increased slowly, whereas in the resistant variety the fungus induced rapid induction of chitinase activity (Joosten and De Wit, 1989). The slower accumulation of chitinase would have resulted in susceptibility in Cf5/race 5 interaction. The slower rate of accumulation may be due to the presence of race-specific elicitors in the apoplastic fluids, which would not have activated the defense mechanisms in the susceptible interaction (De Wit and Spikman, 1982; De Wit et al., 1986). Greater and earlier PR-4 and PR-5 transcript accumulation was observed in resistant wheat genotype compared with a susceptible genotype during *Fusarium graminearum* infection (Pritsch et al., 2000). Induction of the genes encoding PR-2, PR-3, and PR-9 proteins (*pr-2*, *pr-3*, and *pr-9*) occurred earlier in resistant cultivar than in susceptible cultivar in response to *Phytophthora infestans* in potato (Wang et al., 2005). The expression of the OLP

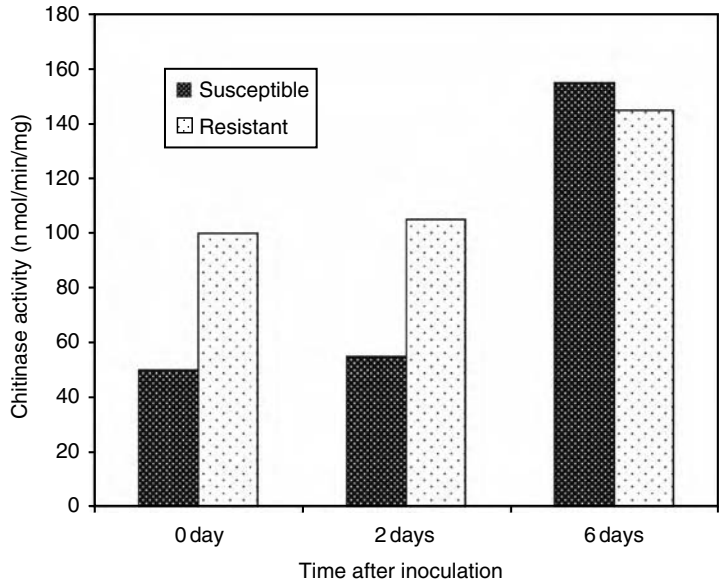

FIGURE 6.4 Chitinase activity in susceptible and resistant (immunized) tobacco plants inoculated with *Peronospora tabacina*. (Adapted from Tuzun, S., Rao, M.N., Vogeli, U., Schardl, C.I., and Kuc, J., *Phytopathology*, 79, 979, 1989.)

gene, *CAOSM1*, was higher and earlier in the incompatible than in the compatible interactions in pepper leaves infected by *Colletotrichum coccodes* (Table 6.3; Hong et al., 2004).

Brassica napus is susceptible to *Phoma lingam,* whereas *B. nigra* is resistant. *B. nigra* showed a very rapid production of PR-2, PR-Q (PR-3 protein), and PR-S (PR-5 protein) because of infection with *P. lingam*. In contrast, in *B. napus* stressed by the fungus, the production of the three PR proteins was delayed or absent (Dixelius, 1994). Resistance to the blast pathogen *Magnaporthe grisea* in resistant rice plants was related to earlier activation of PR-1 and PBZ1 proteins than in susceptible plants (Nishizawa et al., 2003).

TABLE 6.3
Time Courses of the Expression of CAOSM1 mRNA in Pepper Plants during Compatible and Incompatible Interactions with *Colletotrichum coccodes*

Hours after Inoculation with *C. coccodes*	Intensity of Expression of *CAOSM1* Gene	
	Compatible Interaction	Incompatible Interaction
0	−	−
12	−	+
24	+	+
36	++	+++
48	++	++++
72	+++	+++++

Source: Adapted from Hong, J.K., Jung, H.W., Lee, B.K., Lee, S.C., Lee, Y.K., and Hwang, B.K., *Physiol. Mol. Plant Pathol.*, 64, 301, 2004.

Note: −indicates no expression; +, ++, +++, ++++, and +++++ indicate increasing intensity of expression of CAOSMI gene.

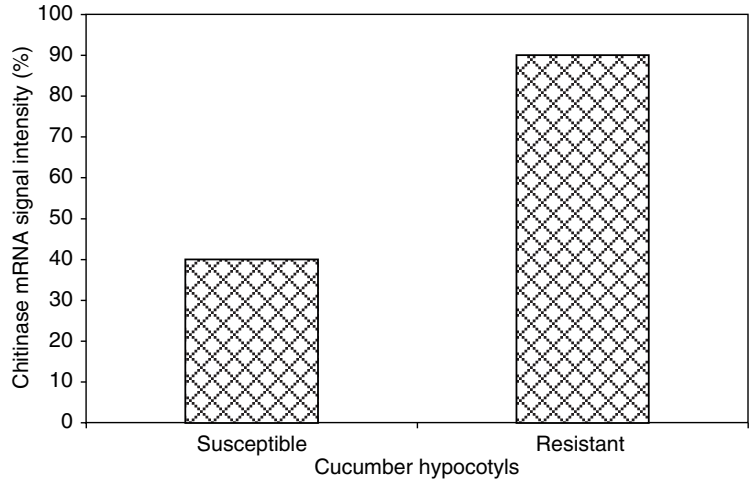

FIGURE 6.5 Induction of chitinase mRNA by fungal elicitor in cucumber hypocotyls showing susceptibility or resistance. (Adapted from Kastner, B., Tenhaken, R., and Kauss, H., *Plant J*., 13, 447, 1998.)

All these observations suggest that slower accumulation of PR proteins in the plants may allow the pathogens to escape the antifungal proteins at the critical stage of penetration and establishment in the host. The PR proteins are induced by elicitors present in the fungal cell wall.

The major causes for slower accumulation of PR proteins in the susceptible hosts may be due to delayed release of elicitors from the cell wall of fungal pathogens into host tissues. When the melon plants were inoculated with *Colletotrichum lagenarium*, there was slow increase in chitinase activity. However, when the plants were treated with a fungal elicitor and inoculated with *C. lagenarium* 4 days later, chitinase activity increased at a faster rate than in the plants inoculated with the fungus alone (Roby et al., 1988). The elicitor-treated plants contained up to 42% less chitin than the untreated plants. Chitin content indicates the mycelial content in the infected leaves and hence the results suggest that the early increase in chitinase activity inhibits the fungal development in host tissues (Roby et al., 1988). The results also suggest that there may be slow release of elicitor from the cell wall of the pathogen. By providing elicitor earlier the chitinase activity would have increased faster.

The other mechanisms involved may be inaction of elicitors in the susceptible hosts, or presence of host-specific elicitors that do not act on susceptible hosts, or pathogens may have suppressors to suppress action of elicitors, or the susceptible host may have suppressor to suppress action of fungal elicitors or degradation of elicitors by host enzymes, as described in Chapter 2.

The fungal elicitors may be less efficient in inducing accumulation of PR proteins in susceptible varieties. Induction of chitinase mRNA by an elicitor from *Phytophthora sojae* in cucumber hypocotyls was studied. The elicitor induced only less amount of chitinase mRNA in the susceptible plants compared with that in resistant plants (Figure 6.5; Kastner et al., 1998). The absence or reduced action of some elicitors in the susceptible cultivars may be due to absence or reduced presence of receptor molecules for binding the available elicitor molecules in those susceptible cultivars (Hanania and Avni, 1997; Montesano et al., 2003).

6.12.2 Pathogens May Shed Away from Their Cell Wall the Substrate for the PR Proteins of Enzymatic Nature and Avoid Their Lytic Enzyme Action

Chitin is an important structural component in the cell walls of many plant pathogenic fungi. It is the substrate for PR-3, PR-8, and PR-11 proteins with chitinase enzyme activities, which cause lysis of hyphal tips. Chitin and chitin oligomers are active elicitors of chitinase activity

(see Chapter 2). The precise location of the chitin in the infection structures of *Colletotrichum lindemuthianum* during penetration and colonization of bean tissues was studied (O'Connell and Ride, 1990). WGA binds strongly to chitin and chitin oligomers and hence WGA, conjugated with colloidal gold, was used as cytochemical probe to detect and localize chitin within the walls of *C. lindemuthianum*. Chitin was detected in *C. lindemuthianum* cell walls and it was absent in the fungal cytoplasm. The lectins, WGAs, and pokeweed mitogen are known to bind with chitin, and they bound to conidia, conidial germ tubes, hyaline appressoria, and appressorial germ tubes (O'Connell, 1991). When the fungus was inoculated on the susceptible bean cultivar, WGA gold bound to the cell walls of germinated conidia indicating the presence of chitin. The cell walls of mature, melanized appressoria also showed the presence of chitin. An electron lucent growth from the appressorial wall, termed the appressorial cone, surrounded the ventral germ pore of the appressoria. Both the appressorial cone and the infection peg were not labeled by WGA gold, and they remained unlabeled at all stages of infection. The cell walls of young, small-diameter intracellular hyphae were also unlabeled (O'Connell and Ride, 1990).

The development of *C. lindemuthianum* was studied on the surface of Formvar membranes instead of host surface. The walls of conidial germ tubes were strongly labeled by WGA gold. The appressorial germ tubes penetrated Formvar membranes and developed in the water beneath as narrow filamentous hyphae. WGA was found to bind to the walls of young appressorial germ tubes unlike the infection pegs and young intracellular hyphae produced during infection. Exclusion of chitin from the hyphal walls of infection peg and young intracellular hyphae appears to be important in pathogenesis, as appressorial germ tubes at an equivalent early stage of development *in vitro* were labeled by WGA gold indicating presence of chitin. The absence of chitin from the fungal wall at the site of initial contact with the host plasma membrane may be important in establishment of infection by intracellular biotrophs. By excluding chitin from its wall, the fungus may not only resist lysis by host chitinases but also avoid triggering other host defense mechanisms as chitin acts as an elicitor of plant defense reaction also (O'Connell and Ride, 1990).

Similar observations were made in various host–rust interactions. The walls of uredospore-derived substomatal vesicles and basidiospore-derived intraepidermal vesicles contain less chitin than those of appressoria and germ tubes (Kapooria and Mendgen, 1985; Mendgen et al., 1985). The walls of uredospore-derived penetration pegs, haustorial necks, and young haustorial bodies appear to lack chitin, whereas the walls of haustorial mother cells and older haustorial bodies contain chitin (Chong et al., 1986). The observations suggest that the first fungal structures that contact host cells following intercellular or intracellular penetration lack chitin, and this exclusion is important in pathogenesis (O'Connell and Ride, 1990).

Another supporting evidence for this theory has come from *Rigidoporus lignosus*–rubber tree interaction. *R. lignosus* causes white rot of rubber tree. It attacks roots by means of fast-growing rhizomorphs that contain both thick- and thin-walled mycelium. *R. lignosus* rhizomorphs have been demonstrated to produce specialized infection hyphae (Nicole et al., 1987). Only thin-walled hyphae penetrate the outer root tissue. WGA in conjunction with colloidal gold was used for labeling the chitin oligomers. WGA labeling was substantially reduced over the walls of root-penetrating hyphae as compared with that of rhizomorphs. Similarly, reduced labeling was found over walls of branching hyphae responsible for host cell wall penetration. Modification or release of chitin from fungal cell walls may thus be related to the establishment of root infection, and to colonization (Nicole and Benhamou, 1991; Nicole et al., 1991).

6.12.3 Pathogens May Produce Enzymes That Protect Them from Fungitoxic Action of PR-3 Proteins

Chitosan is present along with chitin in cell wall of fungal pathogens. Chitosan arises mainly by deacetylation of nascent chitin, which is formed by chitin synthase, before the polymer

chains aggregate to form fibrils. This mode of synthesis suggests that chitin and chitosan in hyphal walls are not a mixture of homopolymers but represent chitin chains deacetylated to varying degrees (Davis and Bartinicki-Garcia, 1984).

Chitin deacetylase is produced *in vitro* by *C. lindemuthianum* (Kauss et al., 1983) and *C. lagenarium* (Kauss and Bauch, 1988). When cucumber plants were inoculated with *C. lagenarium*, both chitinase and chitin deacetylase activity increased (Siegrist and Kauss, 1990). The striking increase in chitin deacetylase occurred during lesion development (Siegrist and Kauss, 1990), a period in which secondary hyphae presumably spread from cell to cell and through the tissue (Xuei et al., 1988). The chitin deacetylase activity in the infected plant tissue was correlated with hyphal growth. This observation was strengthened by the findings that when cucumber plants were inoculated with tobacco necrosis virus on the first leaves, chitinase activity increased systemically in the second leaves, and this increase was associated with reduced lesion formation and chitin deacetylase production by *C. lagenarium*. In other words, chitin deacetylase activity was less in leaf tissues containing less mycelia of the fungus. Chitin deacetylase is known to be secreted in the fungal culture medium. Hence, the enzyme may accumulate on the leaf surface, and after penetration it may accumulate in the apoplast within the leaf. The chitinase of cucumber is known to be localized in the apoplast (Boller and Metraux, 1988) and so both the enzymes are likely to be present together in the intercellular spaces where the infecting hyphae of *Colletotrichum* are located and first come into contact with the host plasma membrane. The tips of the penetration hypha may synthesize chitin on their outer surfaces. The nascent chitin is a preferred substrate for both chitinase and chitin deacetylase. If deacetylation occurs rapidly, the polymer chains would become less accessible to chitinase and remain intact in the form of partially N-acetylated chitosan polymers. Such a chitosan is likely to remain bound to the hypha and thus sustain the rigidity of the fungal wall. N-acetylation of chitin might, therefore, be a way by which the fungal wall could be partially protected against the fungitoxic action of plant chitinase (Siegrist and Kauss, 1990).

6.12.4 Pathogens May Produce Enzymes to Inhibit Activity of Some PR Proteins

PR-7 proteins show serine protease activity. PR-7 proteins have been shown to confer disease resistance (Jorda et al., 1999, 2000; Kruger et al., 2002; Xia et al., 2004; Tian et al., 2005). It has been suggested that virulent pathogens may inhibit the plant apoplastic proteases and cause the disease in susceptible interactions. Several oomycete pathogens, such as *Phytophthora infestans*, *P. sojae*, *P. brassicae*, and *Plasmopara halstedii*, secrete serine protease inhibitors (Tian et al., 2004). Two protease inhibitors, EPI1 and EPI10, have been shown to be secreted by *P. infestans* and they inhibit tomato P69B protease (Tian et al., 2004). The *epi10* gene was found to be upregulated during infection of tomato, suggesting a potential role during pathogenesis (Tian et al., 2005). To prove that EPI10 inhibits the tomato proteinase P69B, the P69B was expressed in the apoplast of *Nicotiana benthamiana* plants by agroinfiltration technique. The intercellular fluids were extracted from infiltrated leaves of *N. benthamiana* and the presence of the P69b protein was established. The intercellular fluids containing the P69B were incubated with EPI110. It was observed that EPI10 completely inhibited the protease activity of P69b (Tian et al., 2005). These results suggest that the inhibitory function of PR-7 protein may be suppressed by the virulent pathogen in the susceptible plants.

6.12.5 Less Elicitor Is Released from Pathogen's Cell Wall to Activate Synthesis of PR Proteins

Chitosan treatment induced chitinase activity in celery roots. This chitinase liberated chitin oligosaccharide, *N*-acetylglucosamine (GlcNAc), from isolated cell walls of compatible race 2

and incompatible race 1 of *Fusarium oxysporum* f. sp. *apii*. Under identical conditions, more GlcNAc was released from race 1 cell walls than from race 2 (Krebs and Grumet, 1993). The results suggest that the ability of the virulent race to invade and infect the host may reside in the cell wall structure that is less accessible to chitinase, the mechanism through which elicitor is released.

6.12.6 PR Proteins Are Degraded Quickly in the Susceptible Host Tissues

When the pathogen establishes infection, the PR proteins may be degraded. Pea pods were inoculated with a compatible pathogen, *Fusarium solani* f. sp. *pisi*, or with an incompatible pathogen, *F. solani* f. sp. *phaseoli*. There was initially little difference in mRNA accumulation between the compatible and the incompatible treatments. However, a significant decrease in β-1,3-glucanase mRNA expression was observed in the compatible pathogen *F. solani* f. sp. *pisi* inoculated pods between 24 and 48 h after inoculation. In the incompatible reaction, mRNA accumulation remained high for 48 h (Chang et al., 1992). The decrease in mRNA in compatible pathogen inoculated tissues may be due to the severe deterioration of the tissue and the breakdown of plant defense system (Chang et al., 1992).

In the susceptible-infected tobacco plants, the isoform of β-1,3-glucanase, G3, decreased when symptoms appeared and subsequently disappeared 6 days after inoculation with the oomycete *Peronospora tabacina*. The loss of G3 may be significant in pathogenesis (Pan et al., 1991b). Tomato PR proteins are degraded by an aspartyl proteinase, a host enzyme that is constitutively present in healthy and infected leaves at similar levels. However, an acidic pH is required for the activity of this proteinase. Fungal infection leads to acidification of the apoplast and activation of the enzyme activity (Rodrigo et al., 1989). Tobacco leaves were also found to contain an extracellular proteinase that endoproteolytically cleaves tobacco PR proteins. This proteinase was partially purified from tobacco leaves and characterized as an aspartyl proteinase with a pH optimum around pH 3 and a molecular mass of 36–40 kDa. Purified tobacco PR proteins, PR-1a, PR-1b, and PR-1c, were degraded upon incubation with tobacco intercellular fluids at acidic pH values, remaining practically unattacked at pH values above 3.5. The purified tobacco aspartyl proteinase was able to cleave endoproteolytically tobacco PR-1a, PR-1b, and PR-1c and tomato P1 (p14) at 37°C in citrate–phosphate buffer (pH 2.8). Progressively, smaller proteolytic fragments appeared upon prolonged incubation. The degradation products differed in size between the four PR proteins tested. The tomato and tobacco aspartyl proteinase had similar substrate specificities as the pattern of degradation of tomato P1 (p14) by tobacco aspartyl proteinase closely resembled that obtained upon incubation with the tomato 37 kDa aspartyl proteinase (Rodrigo et al., 1991). The results indicate that these two proteinases are homologous and probably conserved among Solanaceae (Rodrigo et al., 1991). Acidification of the apoplast appears to be important in degradation of PR proteins. It is known that *Sclerotinia sclerotiorum* infection leads to production of oxalic acid in white beans (Tu, 1985). These results suggest that PR proteins are degraded in the infected tissues, particularly in susceptible interactions.

6.12.7 Site of Accumulation of Some PR Proteins May Determine Susceptibility or Resistance

The central vacuole is considered as a defense arsenal (Boller, 1982). Many defense-related proteins such as chitinases (Boller and Vogeli, 1984), glucanases (Legrand et al., 1987), OLPs (Woloshuk et al., 1991b), proteinase inhibitors (Walker-Simmons and Ryan, 1977), proteinases (Vera and Conejero, 1988), and thionins (Bohlmann et al., 1988) are compartmentalized within the vacuole and all of them are basic in nature.

Generally, intracellular (vacuolar) PR proteins show antifungal activity. Basic chitinases and glucanases that are found intracellularly show antifungal effect, whereas those occurring extracellularly (acidic forms) do not have appreciable antifungal activity (Woloshuk et al., 1991b). Specific enzymatic activity of both basic chitinases and basic β-1,3-glucanases is much higher than that of the acidic isoforms (Kauffmann et al., 1987; Legrand et al., 1987). The OLP AP20, which occurs intracellularly in tobacco, shows antifungal activity, whereas PR-S, which is very much homologous to OLPs, is an extracellular protein and does not have any antifungal activity. The tomato PR protein NP24, a basic OLP, shows antifungal activity (Woloshuk et al., 1991b). PR-1 proteins also fit in this picture (Woloshuk et al., 1991b). The fungal pathogens, particularly biotrophs, initially develop in the intercellular space and subsequently they develop intracellularly in necrotrophic phase. Since only intracellular proteins are fungitoxic, the PR proteins may not have any important role in the early stage of infection process.

mRNA encoding the basic 35 kDa β-1,3-glucanase accumulated very rapidly in both the compatible and the incompatible *Cladosporium fulvum*–tomato interactions. The expression of intracellular β-1,3-glucanase in the compatible interaction almost similar to incompatible interaction suggests that it is not harmful to the fungus (Van Kan et al., 1992). The sites in which intracellular chitinases and β-1,3-glucanases accumulate in mesophyll cells of tomato leaves infected by *Cladosporium fulvum* were not significantly different between compatible and incompatible interactions. Chitinase and β-1,3-glucanase accumulated in the cytoplasm of mesophyll cells adjacent to fungal hyphae in both compatible and incompatible interactions (Wubben, 1992). *C. fulvum* grows exclusively in the intercellular space without penetrating tomato cells, and hence intracellular β-1,3-glucanase or chitinase may not interact with fungal hyphae (Van Kan et al., 1992; Wubben, 1992). Transgenic rice plants expressing a modified ribosome-inactivating protein and rice basic chitinase genes did not show any significant symptom reduction in case of blast (*Magnaporthe grisea*) and brown spot (*Bipolaris oryzae*) disease incidence (Kim et al., 2003). It suggests that basic PR proteins may not be useful in conferring resistance against some pathogens.

The fact that basic vacuolar glucanases may not help plants to defend against fungal pathogens has been demonstrated using antisense transformants (Neuhaus et al., 1992). The chimeric antisense β-1,3-glucanase gene was introduced into *Nicotiana sylvestris*. The 33 kDa basic β-1,3-glucanase was not found in extracts prepared from leaves of antisense plants, but all the four acidic β-1,3-glucanases were detected in both untransformed and antisense leaves. It indicates that antisense transformation primarily blocked expression of a class I 33 kDa β-1,3-glucanase corresponding to the polypeptide encoded by the tobacco gene used to make the antisense construction (Neuhaus et al., 1992). The antisense transformed plants and untransformed plants were inoculated with *Cercospora nicotianae*, the frog-eyespot pathogen. There was no consistent difference in the time course of infection between untransformed and antisense transformed plants (Neuhaus et al., 1992). The results suggest that the vacuolar basic β-1,3-glucanases inhibited in the antisense transformant are not important in the successful defense of *N. sylvestris* plants against *C. nicotianae* (Neuhaus et al., 1992).

These studies suggest that if the vacuolar basic proteins are targeted into the apoplast, they may induce resistance. It was demonstrated by Woloshuk et al. (1991a), who developed two types of transgenic tobacco plants. In one set of transgenic tobacco plants, the basic OLP, AP20, was expressed only in the vacuole, and in the other set of plants, the basic protein was targeted to the apoplast. The transgenic tobacco plants with AP20 targeted to the extracellular space showed more resistance to the oomycete *Phytophthora nicotianae* compared with those with AP20 in vacuole (Table 6.4; Woloshuk et al., 1991a).

Similar results were obtained with transgenic potato plants. The transgenic plants expressing AP20 with targeting of apoplast showed consistent resistance even 14 days after

TABLE 6.4
Effect of Targeting Basic PR-5 Protein into Vacuole or Apoplast in Tobacco on Induction of Resistance or Susceptibility to *Phytophthora nicotianae*

Plant Line	Leaf Area Infected by *Phytophthora infestans*
Non-transgenic	8 discs 100%, 2 discs 60%
Transgenic-vacuolar targeting	7 discs 100%, 3 discs <25%
Transgeic-apoplast targeting	1 disc 100%, 2 discs 60%, 7 discs <25%

Source: Adapted from Woloshuk, C.P., Melchers, L.S., Cornelissen, B.J.C., Meulenhoff, E.J.S., Sela-Buurlage, M.B., and Van Den Elzen, P.J.M., International Patent Application, PCT/NL 91/0089, p. 44, 1991a.

inoculation with the oomycete *Phytophthora infestans*. The transgenic plants with intracellular target showed only delayed symptom development (Woloshuk et al., 1991a).

PR proteins found intracellularly may be secreted into the intercellular space, and by that process they may inhibit the fungal growth. Treatment of Samsun NN tobacco leaves with salicylic acid caused an increase of PR-1 proteins. The increase started 1 or 2 days after treatment and continued linearly. PR proteins in the free spaces increased on the ninth day after salicylic acid treatment, whereas those in the remaining tissue ceased increasing from the sixth day. PR proteins detected in free spaces at the ninth day came to about threefold of those in the remaining leaf discs, suggesting their secretion into the free spaces (Ohashi and Matsuoka, 1987). Although secretion of intracellular PR proteins is possible, the secretion occurs slowly and 9 days after elicitor treatment. The delayed accumulation would favor the pathogen to escape the defense arsenal.

Although antifungal basic PR proteins occur in vacuoles, decompartmentalization of plant cells has been shown to be a response to pathogen attack (Wilson, 1973; Boller, 1987). Disruption of vacuole will lead to liberation of its hydrolytic constituents to the intracellular spaces. However, disruption of vacuole occurs only in late necrotrophic phase; by that time, enough damage would have been caused.

There are many PR proteins that may not have any fungitoxic action, but may induce disease resistance by releasing elicitors to activate host defense mechanisms, or by strengthening cell wall barriers. They are mostly released in the apoplast and these apoplast PR proteins may also induce resistance (Kastner et al., 1998; Lawrence et al., 2000; Wróbel-Kwiatkowska et al., 2004). Transgenic wheat plants constitutively expressing a barley class II chitinases were developed. The transgene was localized in the apoplast of the transgenic lines, and these transgenic wheat plants showed resistance to the powdery mildew fungus *Blumeria graminis* f. sp. *tritici* (Bliffeld et al., 1999). It suggests that acidic PR proteins which are secreted into the apoplast may also confer resistance against pathogens.

6.12.8 Adaptation of Pathogens to PR Proteins

Either slower accumulation or accumulation in lesser amount may be the major reason for the pathogens to overcome the antifungal PR proteins in the host. However, it has been shown that even overexpression of chitinases constitutively did not lead to increased resistance of transgenic plants (Neuhaus et al., 1991a). A tobacco (*Nicotiana tabacum*) basic vacuolar chitinase gene under the control of the CaMV 35S promoter was transferred to the closely related species, *Nicotiana sylvestris*. The transgenic plants were shown to accumulate high levels of chitinase in the leaves. However, they were found to show only slight increase in

resistance to the frog-eyespot pathogen *Cercospora nicotianae* (Neuhaus et al., 1991a). It is possible that expression of chitinases does not lead to fungal resistance because the fungus has adapted to the defense mechanisms of its host. Basic chitinases and β-1,3-glucanases from tomato showed high antifungal activity; but *Cladosporium fulvum*, the tomato pathogen, was insensitive to these PR proteins (Joosten et al., 1995).

Chitinases from unrelated species in transgenic plants (new chitinases) could not be overcome by the invading fungus (Lamb et al., 1992). Tobacco plants expressing bean vacuolar chitinase gene (Roby et al., 1990) show resistance to *Rhizoctonia solani* (Broglie et al., 1991). Potato plants expressing tobacco osmotin gene show delayed development of disease symptoms caused by *Phytophthora infestans*. In contrast, transgenic tobacco plants expressing constitutively its own osmotin gene do not show any increased resistance against *Phytophthora parasitica* var. *nicotianae* (Liu et al., 1994). The chitinase gene (Chi A) from the bacterium *Serratia marcescens* was transferred to tobacco, and the transgenic tobacco plants showed resistance to *R. solani* (Jach et al., 1992). These results suggest that pathogens may adapt to their host's PR proteins.

6.12.9 Some PR Proteins May Not Be Involved in Disease Resistance

There are many reports to indicate that PR proteins are only stress proteins induced because of infection and they may not be involved in host defense mechanism. In tomato, expression levels of PR protein encoding genes were correlated to gray mold disease (*Botrytis cinerea*) severity (Diaz et al., 2002). Thus, PR proteins truly acted as PR proteins and not as defense-related proteins in tomato infected by *B. cinerea* (Diaz et al., 2002). Nine isoforms of chitinases have been detected in pepper stem tissues after inoculation with the oomycete *Phytophthora capsici*. All of them except a basic isoform (which also occurred only in a low level in the incompatible interaction) accumulated both in compatible and incompatible interactions (Kim and Hwang, 1994). Several isoforms of β-1,3-glucanases accumulated in both the compatible and the incompatible interactions in pepper inoculated with *P. capsici* (Kim and Hwang, 1994). Lee et al. (2000a) showed that transcripts of the thionin gene CATHION1 were more induced in the compatible *Phytophthora capsici*–pepper plant interactions than in the incompatible interactions. Transgenic wheat line coexpressing a chitinase and β-1,3-glucanase gene combination and another wheat line expressing a PR-5 gene were developed and both of them did not show resistance to *Fusarium graminearum* under field conditions (Anand et al., 2003). Thus, some PR proteins may not have any role in disease resistance.

6.13 CONCLUSION

The most important event occurring during fungal pathogenesis in plants is induction and accumulation of new readily detectable proteins called PR proteins. At least 17 structurally different classes of PR proteins have been reported in the plant kingdom. Each plant may synthesize numerous PR proteins during fungal pathogenesis; even 33 PR proteins have been detected in tobacco. What is the function of these newly induced proteins? Are they involved in helping the plants to resist the pathogens or aiding the pathogens to invade the host tissues? Are they resistance factors or pathogenicity factors? These questions are still to be answered. However, it is reported that genes encoding PR proteins could be isolated even from healthy plants, and transcription of these genes alone is induced during pathogenesis. Another interesting observation is that transcription of PR genes could be induced not only by elicitors, toxins, and enzymes isolated from fungal and oomycete pathogens, but also by bacteria, viruses, viroids, nematodes, and insects. Several plant hormones, salicylic acid, and any chemical that can activate signal transduction systems in plants could induce transcription of PR genes. It suggests that some specific signals generated from pathogens and infected plants

are involved in induction of accumulation of PR proteins. It appears that specific signals are needed to activate the synthesis of each type of PR proteins. Different elicitors of fungus and host origin may induce different sets of PR proteins. Salicylic acid, jasmonic acid, and ethylene may activate transcription of different sets of PR proteins. Thus, it is well established that different signal transduction systems are involved in induction of PR proteins. Rate and amount of release of elicitors in the infection court may differ in susceptible and resistant interactions and this may determine the time and the amount of accumulation of PR proteins.

Although direct evidences are lacking to prove that these PR proteins may be involved in triggering disease resistance, several indirect evidences have been presented to show that the induction of these PR proteins is an attempt by host plants to restrict the spread of pathogens in host tissues. Several PR proteins have been shown to have antifungal activity *in vitro*. Equally, several other PR proteins do not have any toxic action against some pathogens. Some PR proteins may be involved in reinforcement of host plant cell wall and induction of disease resistance. Many transgenic plants overexpressing individual or combination of PR proteins have been developed, and these plants showed enhanced disease resistance or at least a delay in disease development. However, there are also reports that some of these transgenic plants overexpressing PR proteins do not show any enhanced disease resistance. Some of the PR proteins accumulate more in susceptible interactions. These observations suggest that not all, but some specific PR proteins may be involved in conferring or triggering disease resistance. Most of the studies have been conducted with only individual or a combination of two PR proteins. However, in nature, several PR proteins appear simultaneously, and synergism between these PR proteins has also been reported. Hence, studies should be undertaken to examine the role of the bunch of PR proteins, and not that of the individual PR proteins. Studies on PR proteins are still in the rudimentary stage, and intensive studies are needed to utilize these PR proteins as a tool for management of crop diseases.

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7 Evasion and Detoxification of Secondary Metabolites

7.1 INTRODUCTION

Plants produce several secondary metabolites that are distinct from the components of intermediary (primary) metabolism, in that they are generally nonessential for the basic metabolic processes of the plant (Dixon, 2001). Most of the secondary metabolites show antifungal action. There are two types of antifungal secondary metabolites: phytoalexins (inducible secondary metabolites) and phytoanticipins (constitutive secondary metabolites) (VanEtten et al., 1994). Phytoalexins are defined as the compounds that are synthesized denovo in response to infection, accumulating to antimicrobial concentrations in the area of infection (Dixon and Harrison, 1990; VanEtten et al., 1995), whereas phytoanticipins are defined as the compounds that are preformed infectional inhibitors (VanEtten et al., 1994). However, sometimes it is difficult to differentiate phytoalexin from phytoanticipin based on these definitions. Some compounds may be phytoalexins in one plant species and phytoanticipins in others. For example, sakuranetin is inducible in rice leaves, but accumulates constitutively in leaf glands of blackcurrant (Kodama et al., 1988). Some phytoanticipins may be produced in larger amounts after infection. If their constitutive concentrations were sufficient to be antimicrobial, they are called phytoanticipins, otherwise they should be called phytoalexins (Dixon, 2001).

Both phytoalexins and phytoanticipins are recognized based on their *in vivo* antimicrobial activity. However, it is difficult to measure the concentrations of these compounds in the cells that are in direct contact with the invading pathogen. An attempt has been made by Pierce et al. (1996) to measure the cellular concentrations of sesquiterpenoid phytoalexins in cotton leaves by inoculating a bacterial pathogen *Xanthomonas campestris* pv. *malvacearum*. They showed that phytoalexin levels in and around the challenged cells were significantly higher than that required to effectively inhibit growth of the pathogen *in vitro*.

Phytoalexins and phytoanticipins may belong to the same chemical classes, such as phenylpropanoids, terpenoids, and aliphatic acid derivatives (Dixon, 2001). Dakora and Phillips (1996) have reported that in many cases, phytoalexins and phytoanticipins are identical isoflavonoid molecules. Both phytoalexins and phytoanticipins accumulate because of infection almost in a similar way (Vidhyasekaran, 1988, 2002, 2004, 2007). Both of them have been shown to be involved in disease resistance (Lo et al., 1999; Brader et al., 2006), although they have been detected in both resistant and susceptible interactions (Dixon, 2001; Brader et al., 2006). Induction and accumulation of secondary metabolites, and evasion and detoxification processes in various plant–pathogen interactions during fungal pathogenesis are described in this chapter.

7.2 CHEMICAL STRUCTURAL CLASSES OF PHYTOALEXINS

More than 300 phytoalexins have been identified and characterized so far (Dixon et al., 1983a; Monde et al., 1992; Grayer and Harborne, 1994; Kokubun and Harbone, 1995; Smith, 1996; Lo et al., 1999; Dixon, 2001; Buzi et al., 2003; Landini et al., 2003; Vidhyasekaran, 2007). Phytoalexins constitute a chemically heterogenous group of substances. Several phytoalexins belong to phenylpropanoid structural class. Flavanone, isoflavone, stilbene, biphenyl, bibenzyl, coumarin, aurone, anthocyanidin, and anthranilic acid groups of phytoalexins have been reported from infected plants (Table 7.1). Another major group of phytoalexins belongs to terpenoid class. Sesquiterpene, diterpene, and triterpene phytoalexins have been reported in rice, potato, tobacco, and cocoa (Table 7.2). Indole compounds constitute another group of phytoalexins (Table 7.3). Some of the phytoalexins are alkaloids (Table 7.3), whereas some others are nitrogen-containing compounds (Table 7.3). Some phytoalexins belong to fatty acid derivative compounds (Table 7.4).

Taxonomically related plant species may produce similar types of phytoalexin compounds. Isoflavonoids are common in the Leguminosae (Dixon, 2001). Sesquiterpenoids are common in the Solanaceae, and coumarins are the major phytoalexins in plants belonging to the Umbelliferae (Vidhyasekaran, 2002). Sulfur-containing indole phytoalexins are unique to the Cruciferae (Dixon, 2001). There are some exceptions to these generalizations. Stilbene phytoalexins have been detected in diverse plant species such as grapevine (Vitaceae) (Thomzik et al., 1997), peanuts (Leguminosae) (Steffens et al., 1989), and sugarcane (Poaceae) (Brinker and Seigler, 1991). In some cases, different kinds of phytoalexins can be detected in one and the same plant. For example, the polyacetylene phytoalexin falcarinol and falcarindiol and the sesquiterpene rishitin have been detected in tomato (DeWit and Kodde, 1981). In broad

TABLE 7.1
Phenylpropanoid Phytoalexins

Chemical Structure of Phytoalexins	Trivial Name of Phytoalexin	Host Plant	References
Flavanone	Sakuranetin	Rice	Kodama et al. (1992)
Isoflavone	Phaseollin, phaseollidin, kievitone, genistein, phaseollinisoflavan, coumestrol	Bean	Dixon and Harrison (1990)
	Glyceollins	Soybean	Morris et al. (1991)
	Pisatin	Pea	Sweigard et al. (1986)
	Maackiain	Chickpea	Enkerli et al. (1998)
	Medicarpin	Alfalfa	He and Dixon (2000)
Coumarin	Marmesin, psoralen	Parsley	Scheel et al. (1986); Jahnen and Hahlbrock (1988)
	Scopoletin	Sunflower, rubber	Tal and Robeson (1986)
	Angelicin	*Pastinaca sativa*	Desjardins et al. (1989a)
Aurone	—	*Cephalocereus senilis*	Dixon (2001)
Stilbene	Resveratrol	Grapevine, peanut	Calderon et al. (1994); Hipskind and Palva (2000)
	Pinosylvin	*Pinus sylvestris*	Gorham (1989)
Biphenyl	Aucuparin	Apple	Dixon (2001)
Bibenzyl	Dihydropinosylvin	*Dioscorea batatas*	Takasugi et al. (1987)
Anthocyanidin	Apigeninidin, luteolinidin	*Sorghum* spp.	Yamaoka et al. (1990); Lo et al. (1999)
Anthranilic acid	Avenalumins	Oat	Niemann (1993)
	HDIBOA glucoside	Wheat	Niemann (1993)

TABLE 7.2
Terpenoid Phytoalexins

Chemical Structure of Phytoalexin	Trivial Name of Phytoalexin	Host Plant	References
Sesquiterpene	Rishitin, lubimin, solavetivone	Potato	Komaraiah et al. (2003)
	Rishitin, phytuberin, phytuberol, capsidiol	Tobacco	Sato et al. (1985); Klarzynski et al. (2000)
Diterpene	Momilactone A	Rice	Kodama et al. (1988)
Triterpene	Arjunolic acid	Cocoa	Dixon (2001)

TABLE 7.3
Nitrogen- and Sulfur-Containing Compounds

Chemical Structure of Phytoalexin	Trivial Name of Phytoalexin	Host Plant	References
Indole-based sulfur compound	Brassilexin, cyclobrassinin spirobrassinin, cyclobrassinin, oxymethoxybrassinin, brassinin, dioxybrassenin, brassicanols	*Brassica juncea* *Brassica campestris*	Devys et al. (1990) Monde et al. (1990)
	Methoxybrassinin	*Brassica napus*	Dahiya and Rimmer (1988)
	Camalexin	*Arabidopsis thaliana*, *Camelina sativa*	Browne et al. (1991)
Alkaloid—benzophenanthridine	Sanguinarine	*Papaver bracteatum*	Cline and Coscia (1988)
Alkaloid—benzyl isoquinoline	Berberine	*Berberis* spp.	Freille et al. (2003)
Anthranilamide	Dianthramide	*Dianthus caryophyllus*	Yang et al. (1997)

TABLE 7.4
Fatty Acid Derivative Phytoalexins

Chemical Structure of Phytoalexin	Trivial Name of Phytoalexin	Host Plant	References
Acetylene	Safynol, dehydrosafynol	Sunflower	Brooks and Watson (1985)
Furanoacetylene	Wyerone, wyerone acid	Broad bean	Buzi et al. (2003)
Polyacetylene	Falcarindiol	Tomato	Tietjen and Matern (1984)

bean, the furanoacetylene wyerone acid and the isoflavonoid medicarpin have been detected (Hargreaves et al., 1977).

7.3 BIOSYNTHESIS OF ISOFLAVONOID PHYTOALEXINS

7.3.1 Phaseollin and Related Compounds

Several isoflavonoid phytoalexins have been detected in bean (*Phaseolus vulgaris*) and biosynthetic pathways of phytoalexins in bean have been studied in detail. Phaseollin, phaseollidin, and kievitone are the important phytoalexins in bean. Phaseollinisoflavan, coumestrol, and genistein are the other phytoalexins reported in bean. Increases in the activity of a number of biosynthetic enzymes and concomitant accumulation of phytoalexins have been reported in bean (Robbins and Dixon, 1984; Cramer et al., 1985a). Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway. Cinnamic acid 4-hydroxylase (Ca4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) are the key enzymes in biosynthesis of phaseollin (Figure 7.1; Collinge and Slusarenko, 1987; Dixon and Harrison, 1990).

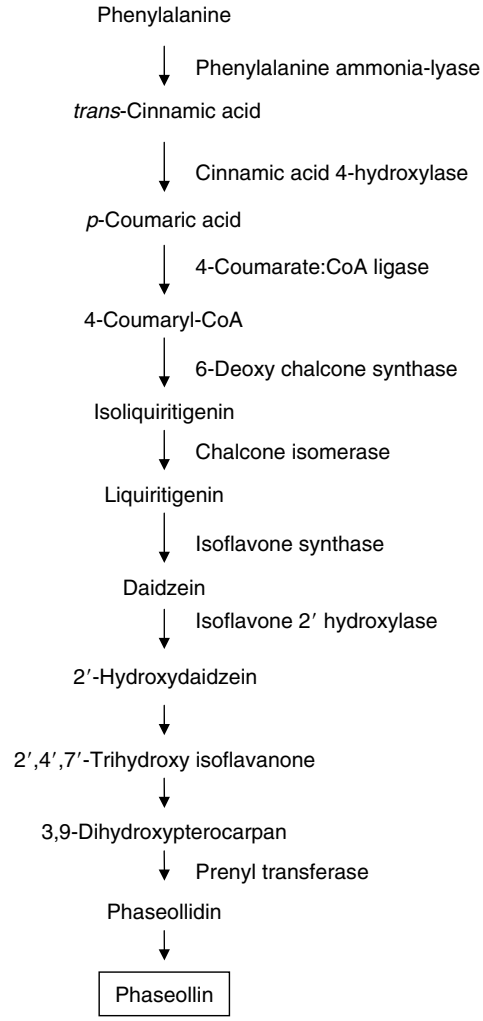

FIGURE 7.1 Biosynthesis of phaseollin in bean.

The first enzyme in phenylpropanoid metabolism, PAL, is present constitutively in all stages of plant development (Hahlbrock and Scheel, 1989). The enzyme is a tetramer and it contains two active sites per molecule (Bolwell et al., 1985a). Multiple tetrameric forms differing in K_m, size, and pI of the subunits have been obtained from purified PAL preparations (Bolwell et al., 1985a). Multiple mRNA species for the enzyme have been reported (Cramer et al., 1989). PAL is encoded in bean by a family of divergent classes of genes (Mavandad et al., 1990). Several PAL polypeptides have been observed following *in vitro* translation of mRNA isolated from elicited bean cell cultures (Bolwell et al., 1985a). Three divergent classes of PAL gene have been cloned in bean (Cramer et al., 1989). The nucleotide sequences of gPAL2 and gPAL3 have been determined. These genes contain open reading frames encoding polypeptides of 712 and 710 amino acids, respectively. Single introns in the two genes (1720 bp in gPAL2, 447 bp in gPAL3) occur in identical positions. They show 59% nucleotide sequence similarity in exon I, 74% similarity in exon II, and extensive divergence in the intron and 5′- and 3′-flanking sequences (Cramer et al., 1985b; 1989). The full sequence of PAL1 gene has not yet been obtained (Dixon and Harrison, 1990).

Fungal infection results in de novo synthesis of PAL (Lawton et al., 1983a,b). PAL is rapidly synthesized de novo and accumulated in bean cell cultures because of elicitor treatment also (Cramer et al., 1985b). Transcriptional activation of the genes encoding PAL has been observed within 5 min of elicitor treatment (Lamb et al., 1986). PAL mRNA was almost completely absent from untreated bean cells but rapidly accumulated following *Colletotrichum lindemuthianum* elicitor treatment. Increases in PAL mRNA could be observed 30 min after elicitation, and maximal accumulation occurred 3–4 h after elicitor treatment. Subsequently, the mRNA decayed rapidly to relatively low levels. PAL mRNA did not accumulate in unelicited control cells. Accumulation and decay of PAL mRNA in total and polysomal RNA fractions exhibited very similar kinetics. There was a close correlation between changes in hybridizable mRNA, translatable mRNA activity, and PAL synthesis *in vivo*. Maximal stimulation coincided with the period of most rapid increase in PAL enzyme activity (Cramer et al., 1985b; Edwards et al., 1985). Increased mRNA activity encoding PAL has been observed in the total RNA, polysomal RNA, and poly A^+ RNA-fractions (Lawton et al., 1983b). cDNA clones to bean PAL have been obtained (Edwards et al., 1985).

The genes of *gPAL1* and *gPAL2* are activated by fungal elicitor in cell cultures of bean cultivar Canadian Wonder, whereas *gPAL3* is not activated (Cramer et al., 1989; Liang et al., 1989a). In suspension cultures of bean cultivar Immuna, all three PAL genes are elicitor inducible and particularly *PAL3* transcripts were strongly induced (Ellis et al., 1989). Transgenic tobacco plants expressing *PAL2* gene have been developed (Bevan et al., 1989; Liang et al., 1989b). In these plants, the gene was developmentally expressed in vascular tissue (Liang et al., 1989b). Thus the PAL genes appear to express variably in different bean cultivars or in different plants.

Cinnamic acid 4-hydroxylase (CA4H) activity was induced before the accumulation of phytoalexin in bean cell cultures treated with fungal elicitor (Bolwell et al., 1985b). 4-Coumarate: CoA ligase (4CL) activity increased in cell suspension cultures of French bean treated with elicitor before the accumulation of phaseollin (Dixon and Bendall, 1978). mRNA of 4CL was induced before the increase in the enzyme activity was observed (Hahlbrock et al., 1983).

The first committed step in the biosynthesis of isoflavonoids is catalyzed by CHS (Heller and Forkmann, 1988). Substrates of the CHS reaction are the major product of general phenylpropanoid metabolism, 4-coumaryl-CoA, and the product of the acetyl-CoA carboxylase reaction, malonyl-CoA. Three acetate units from malonyl-CoA are converted into the C_{15} intermediate 2′,4,4′,6′-tetrahydroxy chalcone (Hahlbrock and Scheel, 1989). CHS enzyme is necessary for formation of phytoalexins in French bean tissues (Dixon et al., 1981).

CHS mRNA was almost completely absent in control bean cells but was rapidly induced following *Colletotrichum lindemuthianum* elicitor treatment. Increases in CHS mRNA could be observed 30 min after elicitation, and maximal induction occurred about 3 h after elicitor treatment. Subsequently, the mRNA rapidly decayed to relatively low levels. There was a close correlation between the kinetics of induction of translatable mRNA activity and CHS synthesis *in vivo* (Ryder et al., 1984).

Induction of CHS transcription in response to fungal elicitor was observed even within 5–10 min (Lawton and Lamb, 1987). It suggests that an efficient signal pathway may operate elicitor perception by putative receptors at the plasma membrane. The increase in CHS mRNA levels was because of de novo synthesis (Cramer et al., 1985b). Increased levels of mRNA are followed by the accumulation of isoflavonoid-derived phytoalexins in elicitor-treated bean cells and infected plants (Ebel, 1986).

Several CHS genes have been detected in bean genome (Ryder et al., 1987). In bean *CHS1*, *CHS8*, and *CHS15* are induced by elicitors to produce phytoalexins. *CHS4*, *CHS14*, and *CHS17* are not induced by fungal elicitors. *CHS1* may be specifically involved in phaseollin biosynthesis (Ellis et al., 1989), whereas another *CHS* may be involved in biosynthesis of kievitone in bean (Dixon, 1986).

Bean *CHS15* is an elicitor-inducible gene (Dixon et al., 1988). A number of deletions of the bean CHS 15 promoter with 5′ end points of −326, −173, −130, −72, and −19 were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene and introduced into soybean protoplasts by electroporation. The −326 construct was strongly induced by an elicitor from *Colletotrichum lindemuthianum* (Dron et al., 1988). The construct was only weakly expressed in the soybean protoplasts without elicitor treatment (Dron et al., 1988). Deletion to −173 results in a 2.5-fold increase in expression in response to elicitor. Deletion of the bean CHS promoter to −72 almost totally abolished CAT expression, whereas the −130 deletion gives expression similar to that of the −326 construct (Dron et al., 1988). The results suggest that the region between −130 and TATA box-containing sequences is essential for transcriptional activation (Dixon and Harrison, 1990).

The 336 bp upstream of the transcription start site in *CHS15* gene has been found to be sufficient to confer tissue-specific and elicitor responsiveness to the GUS reporter gene in transgenic tobacco (Stermer et al., 1990). When it was fused to the bacterial CAT gene, it drives CAT expression in an elicitor-inducible manner on electroporation of the CHS-CAT Nopaline synthase (NOS) 3′ construct into protoplasts of soybean, tobacco, or alfalfa (Dron et al., 1988; Choudhary et al., 1990).

The functional architecture of the *CHS15* promoter was dissected by a novel homologous plant *in vitro* transcription initiation system, in which whole-cell and nuclear extracts from suspension-cultured soybean cells direct accurate and efficient transcription from an immobilized promoter template (Arias et al., 1993). 5′ deletion from −130 to −72 abolished *CHS15* transcript by the soybean whole-cell extract. Preincubation of the soybean whole-cell extract with *CHS15* sequences from −4 to +105 had little effect on subsequent transcription of the immobilized CHS 15 promoter, indicating that this region of the promoter does not contain *cis* elements that bind *trans* factors in the soybean extract essential for *CHS15* transcription. In contrast, depletion of the extract by preincubation with *CHS15* sequences from either positions −132 to −80 or from positions −80 to −40 as *trans* competitors essentially abolished activity, indicating that these specific regions contain *cis* elements that bind *trans* factors in the soybean whole-cell extract that are essential for transcription from the *CHS15* promoter *in vitro* (Arias et al., 1993). Assay of the effects of depletion of the soybean whole-cell extract by preincubation with small regions of the *CHS15* promoter or defined *cis* elements showed that *trans* factors that bind to G-box (CACGTG, −74 to −69) and H-box (CCTACC, −61 to −56 and −121 to −126) *cis* elements, respectively, make major contributions to the transcription of the *CHS15* promoter *in vitro*. Both *cis* element/*trans* factor

interactions in combination are required for maximal activity (Arias et al., 1993). Combination of H-box (CCTACC (N)7-CT) and G-box (CACGTG) *cis* elements has been shown to be necessary for feed-forward stimulation of a CHS promoter by the phenylpropanoid-pathway intermediate *p*-coumaric acid (Loake et al., 1992).

CHI from bean catalyzes the stereospecific isomerization of both 2′,4,4′-trihydroxy chalcone (isoliquiritigenin) and 2′,4,4′,6′-tetrahydroxy chalcone to yield the corresponding (−) flavanones (Dixon et al., 1983b). A single isomerase enzyme may be involved in the biosynthesis of both kievitone and phaseollin in bean (Dixon, 1986). CHI appears to be encoded by a single gene (Dixon et al., 1986). CHI activity is usually high in unelicited bean cell cultures and its activity increased because of elicitor treatment (Robbins et al., 1985). In the induction of CHI in *Colletotrichum lindemuthianum*-infected or elicitor-treated cells, de novo synthesis of this enzyme has been shown (Cramer et al., 1985a,b). A significant proportion of elicitor-induced enzyme activity may be due to the activation of the preexisting enzyme (Robbins and Dixon, 1984). Elicitor may induce the formation of both active and inactive CHI molecules (Robbins and Dixon, 1984). Isoflavone synthetase (isoflavone synthase, IFS), isoflavone 2′-hydroxylase, and prenyl transferase are difficult to be purified. However, their increased activities lead to induction of phytoalexin synthesis (Dixon and Harrison, 1990; Tiemann et al., 1991).

The induction of phenylpropanoid phytoalexin synthesis appears to be associated with coordinated induction of relevant biosynthetic enzymes (Daniel et al., 1988; Dalkin et al., 1990a). PAL, CHS, and CHI are coordinately regulated in bean cell cultures treated with the elicitor (Cramer et al., 1985a; Dixon, 1986). *CHI* mRNA is coordinately induced with *PAL* and *CHS* mRNAs in elicitor-treated bean cell cultures (Mehdy and Lamb, 1987). *CHS* mRNA activity was higher than *PAL* mRNA at later times (Robbins and Dixon, 1984; Dixon et al., 1986).

The appearance of increased activities of phenylpropanoid biosynthetic enzymes and levels of their transcripts in plant suspension cultures is both rapid and transient, implying tight control of both initiation and cessation of transcription (Dixon and Harrison, 1990). Cinnamic acid, the immediate product of the PAL reaction, may be involved in the down-regulation of the phenylpropanoid pathway (Edwards et al., 1990). Exogenous additions of cinnamic acid prevented the induction of PAL enzyme activity in bean cell suspension cultures (Bolwell et al., 1986). Exogenously added cinnamic acid affected the rate of PAL synthesis by inhibiting appearance of PAL transcripts (Bolwell et al., 1988). Cinnamic acid may increase the rate of removal of active PAL enzyme by inducing synthesis of a proteinaceous inhibitor of the enzyme (Bolwell et al., 1986).

When bean cells were treated with *C. lindemuthianum* elicitor, all three PAL (*PAL1*, *PAL2*, and *PAL3*) genes were induced. Concentrations of cinnamic acid above 10^{-4} M inhibited appearance of all three *PAL* transcripts (Mavandad et al., 1990). L-α-Aminooxy-3-phenyl propionic acid (AOPP) is a potent and specific inhibitor of PAL activity *in vivo*. Blocking cinnamic acid production in elicitor-treated cells with AOPP resulted in increased levels of each of the three individual PAL transcripts. It suggests that the cinnamic acid may act as a component in a regulatory feedback system operating at the level of phenylpropanoid gene transcription (Mavandad et al., 1990).

CHS is also inhibited by application of relatively high concentrations (10^{-4}–10^{-3} M) of *trans*-cinnamic acid at which concentrations PAL is also inhibited (Bolwell et al., 1988; Mavandad et al., 1990). CHS appears to be regulated by cinnamic acid at the transcriptional level and not posttranslationally as in the case of PAL (Bolwell et al., 1988). The inhibition of PAL activity by the AOPP results in a delayed superinduction of transcripts encoding CHS (Bolwell et al., 1988).

Cinnamic acid at low concentrations stimulated the expression of bean CHS 15 promoter in the alfalfa protoplast system. At high concentrations, it inhibited the expression of the promoter (Loake et al., 1991). The stimulatory effects of low cinnamic acid concentrations may result from metabolism to *trans-p*-coumaric acid (Loake et al., 1991). The stimulation of

expression of the CHS 15 promoter by low concentrations of cinnamic acid and *trans-p*-coumaric acid appears to occur through specific *cis*-acting sequences because this stimulation was totally abolished by deletion of the promoter to position −130. The effects of this deletion appear to be due to the removal of a binding site for a *trans*-acting factor because electroporation of the sequence from −183 to −130 in *trans* with the plasmid pCHC1 also results in inhibition of stimulation by cinnamic acid and *p*-coumaric acid, presumably by competition for this factor (Loake et al., 1991).

Thus, cinnamic acid synthesized because of the activation of PAL may induce the activation of the enzyme leading to synthesis of *p*-coumaric acid that in turn activates CHS. The coordinated induction of PAL and CHS induces CHI mRNA synthesis (Mehdy and Lamb, 1987). This kind of coordinated expression leads to the synthesis of phytoalexins. If there is any difference in timing of this coordination there may be accumulation of cinnamic acid or *p*-coumaric acid, which in turn may inhibit PAL, CHS, and CHI (Hahlbrock and Scheel, 1989).

The different phytoalexins of bean are also induced in sequence. Kievitone accumulated rapidly to a maximum level of approximately 40 nmol/g fresh weight at between 8 and 12 h after treatment with the elicitor isolated from *C. lindemuthianum*, whereas phaseollin was present in very low levels up to 24 h postelicitation, and it accumulated to levels in excess of 500 nmol/g fresh weight at around 48 h postelicitation (Robbins et al., 1985).

7.3.2 Glyceollins

Four isomeric isoflavonoid pterocarpan phytoalexins, glyceollins I–IV, have been characterized in soybean and their biosynthetic pathways have been studied in detail. Due to *Phytophthora sojae* infection or elicitor treatment these phytoalexins accumulate following increases in all the enzymes of general phenylpropanoid metabolism as well as enzymes specifically involved in later steps of glyceollin biosynthesis (Figure 7.2).

Infection with *P. sojae* resulted in increased PAL mRNA activity and de novo synthesis of PAL in soybean (Borner and Grisebach, 1982; Lawton et al., 1983b). The importance of PAL activity in glyceollin biosynthesis has been demonstrated. PAL activity was inhibited in soybean roots by treatment with AOPP or R-(1-amino 2-phenyl-ethyl) phosphonic acid (APEP). This treatment completely abolished glyceollin induction and changed the phenotype of the incompatible to compatible (Moesta and Grisebach, 1982).

The increase in glyceollin biosynthesis was accompanied by increased 4CL in cell suspension cultures and cotyledons of soybean due to fungal elicitor treatment (Ebel et al., 1984). Increase in mRNA encoding 4CL was observed in soybean hypocotyls infected with *P. sojae,* and it was followed by the accumulation of glyceollin (Schmelzer et al., 1984). Several isoenzymes of 4CL have been reported in soybean. The activity of isoenzyme 2 alone increased because of elicitor treatment and this isoenzyme has been shown to be involved in the phytoalexin biosynthesis (Hille et al., 1982).

CHS activity increased in soybean because of fungal infection. CHS has been purified from soybean (Welle et al., 1991). CHS genes from soybean have been cloned and characterized (Akada et al., 1991). Among the three CHS genes, only one gene (*Chs1*) was found to be transcribed after elicitor treatment (Wingender et al., 1989). The 5′ region of *Chs1* of soybean was subjected to deletion analysis with the help of chimeric CHS-neomycin phosphotransferase (NPT II)/GUS gene constructs. This analysis delimited the sequences necessary for elicitor inducibility to −175 and −134 of the *Chs1* promoter (Wingender et al., 1990).

Glyceollin synthesis was accompanied by increased transcription and activity of PAL and CHS, followed by CHI (Bonhoff et al., 1986a; Esnault et al., 1987). CHI catalyzes the stereospecific isomerization of a chalcone to give a (2S)-flavanone, the immediate precursor of both flavones and isoflavones (Ebel, 1986). The reductase involved in the biosynthesis of

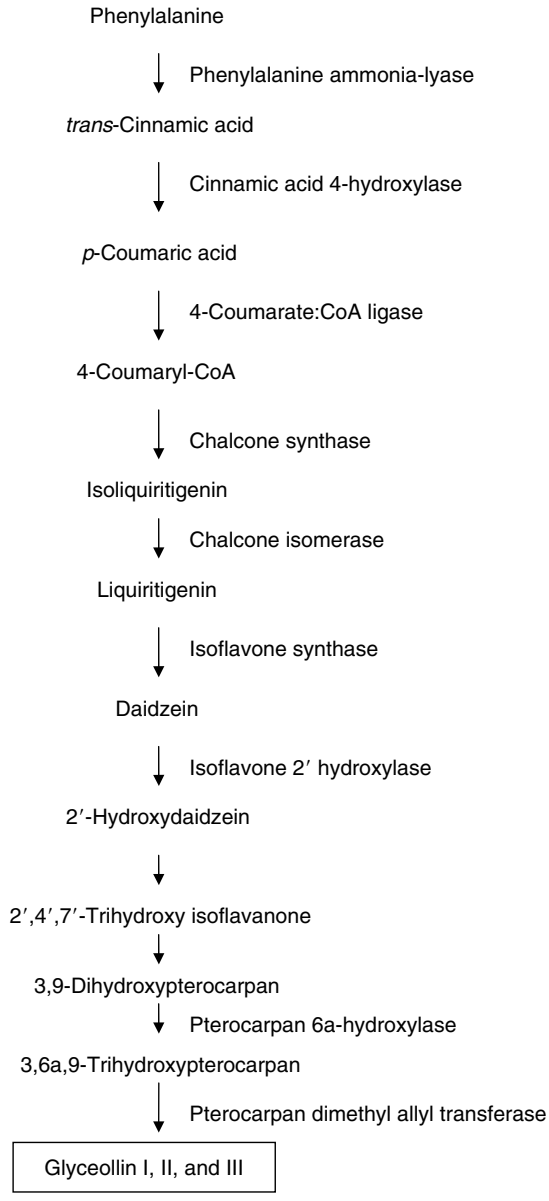

FIGURE 7.2 Biosynthesis of glyceollins in soybean.

6′-deoxychalcone, the flavanones naringenin and its 5-deoxy derivative are formed in soybean (Welle and Grisebach, 1989). An isoflavone synthetase has been shown to be involved in the biosynthetic pathway (Hagmann and Grisebach, 1984). The isoflavone genistein is formed from naringenin through a two-step 2,3-aryl migration (Kochs and Grisebach, 1986). A 2,3-aryl migrates with a flavonoid intermediate (Dewick, 1982). An NADPH- and dioxygen-dependent rearrangement of (2S)-naringenin (5,7,4′-trihydroxy-flavanone) to genistein (5,7,4′-trihydroxyflavonone) has been demonstrated (Hagmann and Grisebach, 1984).

The isoflavones daidzein and genistein are present constitutively in large quantities as conjugates in all seedling organs of soybean cultivars. The conjugates have been identified as the 7-*O*-glucosyl-isoflavones. The conjugates are hydrolyzed to free daidzein and high levels

of glyceollin subsequently accumulate. The glyceollin biosynthesis may not be solely dependent on the induction of enzymes of early phenylpropanoid and flavonoid metabolism, but may depend partly on the rapid release of the isoflavone aglycones from their conjugates and in the later steps of glyceollin biosynthesis (Graham et al., 1990). Addition of daidzein to infection sites on hypocotyls results in a large increase in daidzein accumulation but not in the levels of glyceollins (Morris et al., 1991). It indicates that metabolism of daidzein to the glyceollins is a rate-limiting step, and that following infection, the supply of substrates through the phenylpropanoid pathway is more than adequate for glyceollin biosynthesis, with accumulation of daidzein and its glucosides occurring as a consequence after infection (Morris et al., 1991).

Induction of several of the later enzymes in glyceollin biosynthesis has been reported in infected soybean roots (Bonhoff et al., 1986a,b). A pterocarpan 6a-hydroxylase has been shown to convert 3,9-dihydroxypterocarpan sterospecifically to a 3,6a,9-trihydroxypterocarpan (glycinol) in the presence of NADPH and dioxygen in soybean (Hagmann et al., 1984). A dimethyl-allylpyrophosphate:3,6a,9-trihydroxypterocarpan dimethylallyltransferase catalyzes the formation of the 4- as well as 2-dimethylallyl-trihydroxypterocarpan (Welle and Grisebach, 1988). The products of the dimethylallyl transfer may be the intermediates in the biosynthesis of the various glyceollin isomers in soybean (Ebel, 1986).

7.3.3 Medicarpin

The pterocarpan medicarpin is the major phytoalexin of alfalfa (Dalkin et al., 1990a). The biosynthetic pathway of medicarpin is presented in Figure 7.3. When alfalfa-cultured cells were treated with elicitor from *C. lindemuthianum*, the phytoalexin medicarpin and related compounds accumulated. The phytoalexin accumulation resulted from rapid and extensive increases in the extractable activities of PAL, 4 CL, CHS, CHI, and isoflavone-*O*-methyltransferase (Dalkin et al., 1990a). PAL activity increased approximately fourfold, reaching maximum values from 10 to 14 h after elicitor treatment (Kessmann et al., 1990a). Gowri et al. (1991) showed that PAL activity was induced 50-fold in alfalfa cell suspension cultures within 12 h of exposure to elicitor. PAL transcripts accumulated rapidly and massively within 2 h after elicitation (Gowri et al., 1991). Three isoforms of PAL have been purified from alfalfa cell suspension cultures treated with the fungal elicitor (Jorrin and Dixon, 1990). However, it has been suggested that there may be more than four PAL genes in alfalfa (Gowri et al., 1991). CA4H activity also increased fourfold (Kessmann et al., 1990a).

The presence of at least six elicitor-inducible CHS isopolypeptides has been reported in alfalfa cell suspension cultures (Dalkin et al., 1990a). Five distinct CHS cDNAs, namely, CHS 1, 2, 4, 8, and 9 have been isolated from an alfalfa cell suspension culture after exposure to an elicitor from *C. lindemuthianum* (Dalkin et al., 1990a). Comparison of the deduced amino acid sequences from all five alfalfa CHS clones showed extensive sequence conservation, although many of the amino acid differences between the individual CHS cDNA were not conservative changes. The calculated isoelectric points of the five CHS proteins ranged from 5.7 to 6.1 (Junghans et al., 1993). Exposure of an alfalfa cell suspension culture to a fungal elicitor resulted in a rapid and transient increase in CHS transcripts. Maximum transcript levels were attained around 4 or 5 h after elicitation and a large increase had occurred as early as 1 h after elicitation (Junghans et al., 1993). CHS activity rose approximately 10-fold to a maximum value 19 h postelicitation (Kessmann et al., 1990a). The transcripts corresponding to the five alfalfa CHS clones (CHS 1, 2, 4, 8, and 9) were not expressed in healthy leaves and were induced in leaves in response to *Phoma medicaginis* infection (Junghans et al., 1993). When alfalfa leaves were inoculated with *P. medicaginis*, CHS transcripts were induced rapidly within 2 h and the elevated transcript level was maintained up to 72 h after

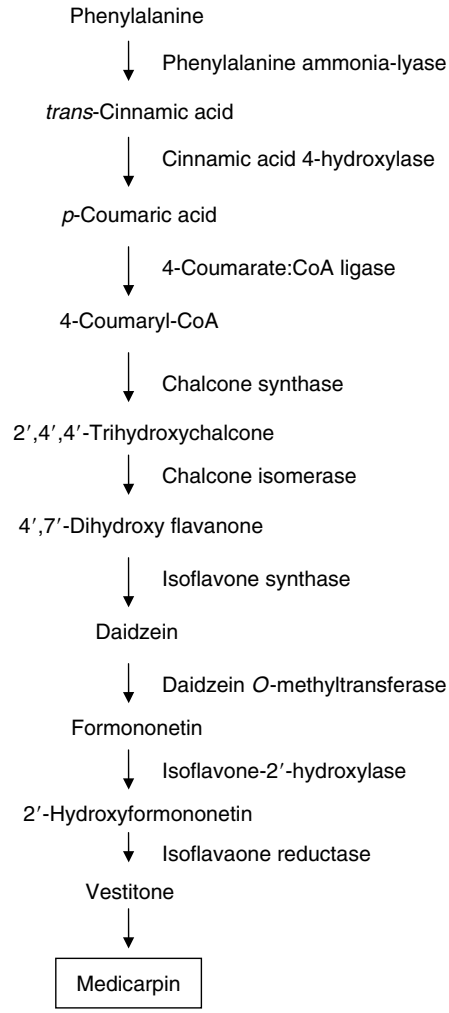

FIGURE 7.3 Biosynthesis of medicarpin in alfalfa.

inoculation (Junghans et al., 1993). CHI catalyzes the isomerization of the chalcone to give a (2S)-flavanone (Hagmann and Grisebach, 1984). CHI shows approximately fourfold increase within 10–14 h in alfalfa suspension-cultured cells because of elicitor treatment (Kessmann et al., 1990a).

The membrane-associated cytochrome P_{450} isoflavone synthase (IFS) activity was strongly induced reaching maximum activities at 11 h after elicitor treatment (Dalkin et al., 1990a; Kessmann et al., 1990a). Isoflavone 4′-*O*-methyltransferase, IFMT (daidzein-*O*-methyltransferase, DOMT) increased rapidly and remained elevated up to 48 h postelicitation (Dalkin et al., 1990a).

The alfalfa cells contained both daidzein and genistein-*O*-methyltransferase, and activity against genistein was induced in a manner similar to the DOMT activity. The alfalfa DOMT and genistein-*O*-methyltransferase activities may be functions of the same enzyme (Dalkin et al., 1990a). Isoflavone 2′-hydroxylase activity was strongly induced and the maximum activity was observed 14 h after elicitor treatment (Kessmann et al., 1990a). Isoflavone reductase (IFR) and pterocarpan synthase were also induced because of the fungal elicitor treatment (Dalkin et al., 1990a).

Treatment of alfalfa cell suspension cultures with elicitor from *C. lindemuthianum* results in the rapid and transient induction of 11 enzyme activities involved in the biosynthesis of the isoflavonoid phytoalexin medicarpin (Dalkin et al., 1990a; Kessmann et al., 1990a). PAL and CHS mRNAs were synthesized with a time lag of less than 1 h. 4 CL mRNA appeared at 1 h time lag. CHS was the major newly synthesized enzyme (Dalkin et al., 1990b). Most of the induced enzymes started declining very soon. By 12 h, PAL transcript levels started declining. CHS transcripts declined rapidly after 3 h postelicitation and IFR transcripts declined 8 h after elicitation (Gowri et al., 1991). However, caffeic acid-*O*-methyltransferase (COMT) transcripts remained at elevated levels up to 48 h after elicitation. IFR and COMT enzyme activities remained at high levels up to 48 h after elicitation (Gowri et al., 1991). These observations indicate that transcriptional activation, mRNA turnover, and enzyme stability may all be important for regulation of phytoalexin biosynthetic enzyme production in response to elicitation (Gowri et al., 1991).

The isoflavanoid conjugates medicarpin-3-*O*-glucoside-6″-*O*-malonate (MGM), afrormosin-7-glucoside (AG), and afrormosin-7-*O*-glucoside-6″-*O*-malonate (AGM) have been isolated from cell suspension cultures of alfalfa. They were the major constitutive secondary metabolites in the cells. Due to *C. lindemuthianum* elicitor treatment rapid accumulation of medicarpin with maximum levels at 14 h has been observed. Following the maximum of medicarpin accumulation, peak levels of MGM were observed at 24 h. The MGM accumulated during the period in which free medicarpin was declining (12–48 h), suggesting that the phytoalexin was actively glucosylated. However, the decline in medicarpin content during this period (800–320 nmol/g fresh weight) was not accompanied by a directly corresponding increase in MGM (40–100 nmol/g), suggesting that the disappearance of medicarpin can only be partially explained by the formation of the malonyl glucoside (Kessmann et al., 1990b). The concentration of AG and AGM were not affected by treatment of cells with fungal elicitor (Kessmann et al., 1990b).

Treatment of alfalfa cells with elicitor together with 100 μm AOPP reduced the incorporation of (^{14}C) phenylalanine into isoflavonoids to control levels for the first 4 h of treatment. To determine whether elicited alfalfa cells could potentially mobilize MGM into free medicarpin, changes in the specific activity of (^{14}C) medicarpin were compared in cells treated with elicitor alone or elicitor plus AOPP. During the first 4 h after addition of AOPP, during which period the inhibitor greatly decreased incorporation from (^{14}C) phenylalanine into medicarpin, similar quantities of medicarpin appeared in cells treated with AOPP and in cells treated with elicitor alone (Kessmann et al., 1990b). As the specific activity of ^{14}C in this medicarpin was much lower than that in the medicarpin accumulating at this period in the absence of AOPP, it was concluded that the medicarpin can originate from a preformed precursor such as MGM in elicited cells in which the flux into the isoflavonoid pathway is blocked (Kessmann et al., 1990b).

The isoflavonoid conjugates may act as a source of isoflavonoid metabolites that may be mobilized under stress conditions if the availability of carbon sources for phenylpropanoid biosynthesis becomes rate limiting, for example, in the late stages of an infection that depleted phenylalanine pools (Kessmann et al., 1990b). A pathway for the biosynthesis of isoflavonoid conjugates in alfalfa has been suggested as given in Figure 7.4.

In unelicited alfalfa cells, the major products are afrormosin conjugates. It suggests that under such conditions the first enzyme specific for medicarpin biosynthesis, formononetin 2′-hydroxylase, is rate limiting, and that flow occurs instead through the 6-hydroxylase, the constitutive pathway (Kessmann et al., 1990b). Isoflavone 2′-hydroxylase activity is undetectable in unelicited alfalfa cells but is induced approximately 5- to 10-fold on treatment with elicitor (Kessmann et al., 1990a). It activates the synthesis of medicarpin through inducible pathway.

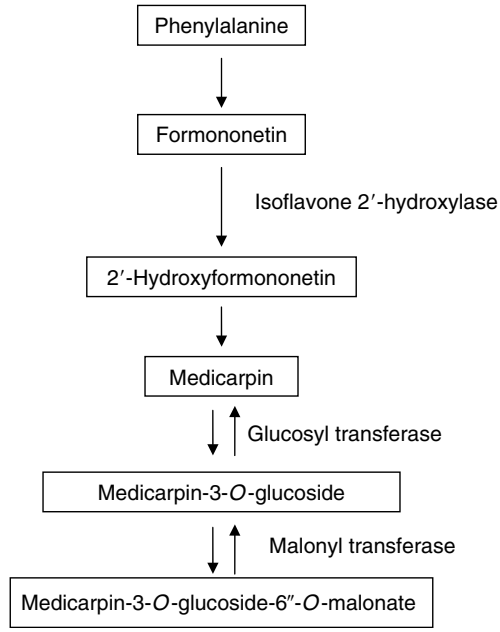

FIGURE 7.4 Biosynthesis of medicarpin through isoflavonoid conjugates in alfalfa.

In chickpea also medicarpin is the major phytoalexin. The enzymes catalyzing isoflavonoid conjugation have been shown to be constitutively expressed glucosyl and malonyl transferases with high activity that exhibit strict specificity for their respective aglycones/conjugates (Barz et al., 1989). The conjugated isoflavonoids that accumulate are derivatives of precursors of medicarpin rather than the phytoalexin itself (Barz et al., 1989). An isoflavone-*O*-methyltransferase has been purified from chickpea cell cultures and it is active specifically toward daidzein and genistein (Wengenmayer et al., 1974).

7.3.4 Pisatin

Pisatin, the phytoalexin of pea, is synthesized almost in the same pathway of phaseollin biosynthesis in bean. Increases in the activity of a number of biosynthetic enzymes and concomitant accumulation of the phytoalexin pisatin have been reported in pea (Loschke et al., 1981; Sweigard et al., 1986).

Fungal infection results in de novo synthesis of PAL and the increased synthesis results from increased mRNA activity in pea (Hahlbrock et al., 1981). PAL from pea has been cloned (Kawamata et al., 1992). CHS is encoded by a multigene family in pea (Harker et al., 1990). Three members of the CHS multigene family, *CHS1*, *CHS2*, and *CHS3*, have been detected in pea. *CHS1* and *CHS3* are expressed in both petal and root tissue, whereas *CHS2* expression was detected only in root tissue. The expression of all three CHS genes is induced by an abiotic elicitor (Harker et al., 1990).

Pea possesses an inducible methyltransferase, which catalyzes the terminal step in the biosynthesis of pisatin (Sweigard et al., 1986). When pea seedlings were infected with *Nectria haematococca* or *Aphanomyces euteiches*, the methyltransferase activity increased severalfold. This increase was followed by accumulation of pisatin (Sweigard et al., 1986).

The methyltransferase activity in pea is dependent on 6a-hydroxymaackiain (Sweigard et al., 1986). 6a-Hydroxymaackiain is incorporated more efficiently than maackiain and much

more efficiently than pterocarpan (Banks and Dewick, 1982; Sweigard et al., 1986). The high degree of substrate discrimination suggests that the 6a-hydroxymaackiain-dependent reaction is catalyzed by a specific enzyme for pisatin biosynthesis, rather than by a fortuitous activity of a nonspecific methyltransferase. Furthermore, the enzyme is induced by treatments, which elicit pisatin biosynthesis (Sweigard et al., 1986).

7.4 BIOSYNTHESIS OF FLAVANONE PHYTOALEXINS

Sakuranetin is an important flavanone phytoalexin detected in rice leaves (Kodama et al., 1988). It is synthesized through phenylpropanoid pathway (Dixon, 2001). PAL, cinnamate-4-hydroxylase (C4H), 4CL, CHS, chalcone reductase (CHR), and CHI are the key enzymes in biosynthesis of sakuranetin in rice (Figure 7.5; Dixon, 2001). Naringenin-7-*O*-methyltransferase is the terminal enzyme in biosynthesis of sakuranetin. Methylation of the hydroxyl group at position 7 of naringenin yields sakuranetin, with *S*-adenosyl-L-methionine as the methyl donor (Rakwal et al., 1996; Tamogami et al., 1997).

7.5 BIOSYNTHESIS OF COUMARIN PHYTOALEXINS

Coumarin phytoalexins have been detected in parsley. These include the linear furanocoumarins, marmesin, and psoralen, their coumarin precursor, umbelliferone, and the methoxylated psoralen derivatives, xanthotoxin, bergapten, and isopimpinellin (Scheel et al., 1986). They accumulate in parsley leaves inoculated with *Phytophthora sojae* (Scheel et al., 1986; Jahnen and Hahlbrock, 1988). 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase) is the first enzyme of the shikimate pathway, which is common to the synthesis of the three aromatic amino acids: phenylalanine, tryptophan, and tyrosine. These amino acids

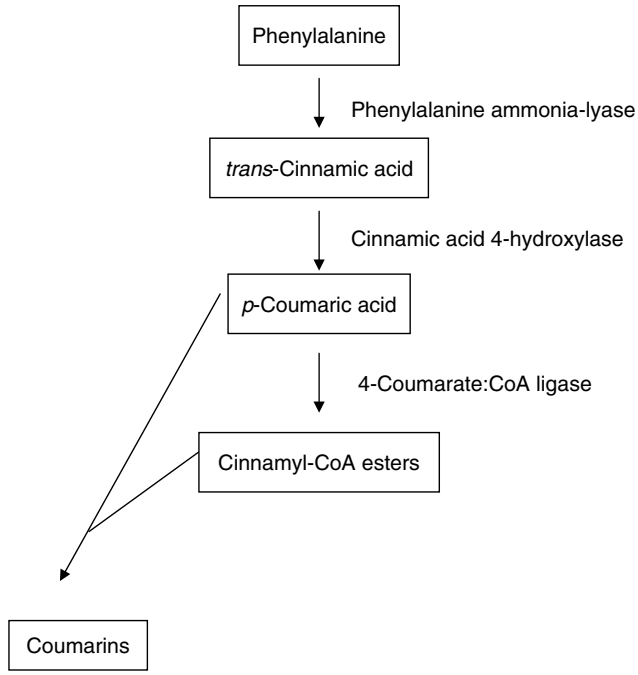

FIGURE 7.5 Biosynthesis of coumarins in parsley. (Adapted from Collinge, D.B. and Slusarenko, A.J., *Plant Mol. Biol.*, 9, 389, 1987.)

serve dual purposes as substrates in protein synthesis and the synthesis of secondary products such as phytoalexins (Henstrand et al., 1992). Parsley cell cultures treated with an elicitor show a twofold increase of DAHP synthase (McCue and Conn, 1989).

Elicitor from *P. sojae* causes a rapid accumulation of DAHP synthase mRNA in parsley cells grown in suspension cultures (Henstrand et al., 1992). The elicitor-induced accumulation of DAHP synthase mRNA reached maximal levels within 2 h and remained elevated for atleast 24 h after elicitor treatment. Actinomycin D, an inhibitor of transcription, caused a significant decrease in DAHP synthase mRNA accumulation (Henstrand et al., 1992). PAL mRNA also accumulated after elicitor treatment. The expression pattern showed a similar increased rate of accumulation between 45 and 60 min after treatment with elicitor (Henstrand et al., 1992). It is possible that DAHP synthase transcription is responding to a depletion of phenylalanine pools caused by increased PAL enzyme activity (Henstrand et al., 1992). PAL, CA4H, and 4 CL appear to be the enzymes involved in biosynthesis of furanocoumarins (Figure 7.6; Collinge and Slusarenko, 1987).

Increases in mRNA activities for PAL and 4 CL were followed by accumulation of furanocoumarins in parsley cells treated with fungal elicitor (Kuhn et al., 1984). 4 CL mRNA synthesis was at its maximum at 1.5 h in parsley cells treated with the elicitor (Chappell and Hahlbrock, 1984). Increases in mRNAs of these enzymes were shown to be due to de novo transcription (Chappell and Hahlbrock, 1984). Different PAL and 4 CL genes have been found to express in parsley because of fungal elicitor treatment (Douglas et al., 1987).

PAL is encoded by a family of four genes and out of the four genes, three appear to be activated by fungal elicitors (Lois et al., 1989a). The parsley *PAL1* has an open reading frame encoding a 716 amino acid polypeptide subunit, with a single intron of approximately 800 bp flanked with AG/GT consensus sequences (Lois et al., 1989a). *Cis*-acting elements have been identified and DNA sequences within these elements are conserved in the promoters of atleast one PAL gene (*PAL1*) (Lois et al., 1989b).

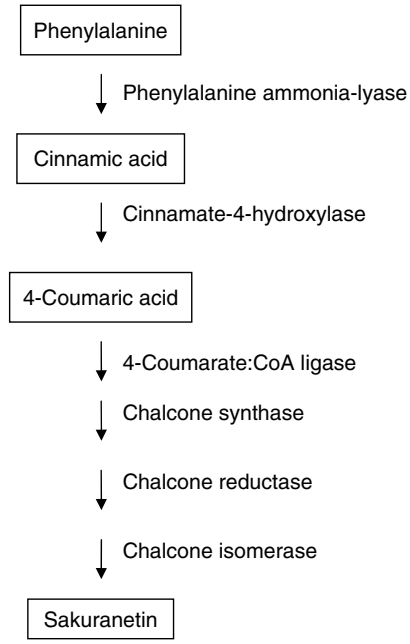

FIGURE 7.6 Biosynthesis of Sakuranetin in rice. (Adapted from Dixon, R.A., *Nature*, 411, 843, 2001.)

4 CL is a monomeric enzyme and it occurs in two isoforms in parsley, each of which is encoded by a single-copy gene (Douglas et al., 1987; Lozoya et al., 1988). The two genes are similar in exon–intron structure and nucleotide sequence (Douglas et al., 1987). The exceptions are a small (54 bp) and a larger (600 bp) insertion in the second intron of one 4 CL gene relative to the other. Both the genes were activated in elicitor-treated cells (Douglas et al., 1987). The two genes encode 4 CL isoenzymes that differ in three amino acid residues (Lozoya et al., 1988). 4CL mRNA is rapidly and transiently accumulated in small, confined areas around fungal penetration sites in infected parsley leaves (Hahlbrock and Scheel, 1989).

The biosynthetic steps of furanocoumarin biosynthesis in parsley appear to include the intermediate formation of glucosides or glucose esters. These may be cleaved by cell wall- or plasma membrane-associated glucosidases as part of the excretion mechanism (Hahlbrock and Scheel, 1989). Elicitor treatment rapidly induces UDP-glucose:cinnamate, UDP-glucose:4-coumarate, and UDP-glucose:ferulate *O*-glucosyl transferases (Hahlbrock and Scheel, 1989). Dimethylallyl diphosphate:umbelliferone dimethylallyl transferase catalyzes the prenylation of umbelliferone to give demethylsuberosin (Tietjen and Matern, 1983), which is converted to (+) marmesin by marmesin synthase (Hahlbrock and Scheel, 1989). Marmesin is a substrate for psoralen synthase (Wendorff and Matern, 1986). Psoralen is the precursor for all linear furanocoumarins (Hauffe et al., 1986). Psoralen synthase catalyzes the oxidative removal of the hydroxypropyl residue from (+) marmesin to yield psoralen (Hauffe et al., 1986). The two methyl transferases *S*-adenosylmethionine: xanthotoxol-*O*-methyltransferase (XMT) and *S*-adenosylmethionine:bergaptol-*O*-methyltransferase (BMT) catalyze the formation of xanthotoxin, and the methylation of bergaptol and 5-hydroxyxanthotoxin to bergapten and isopimpinellin, respectively (Tietjen et al., 1983). The elicitor induces dimethylallyl diphosphate:umbelliferone dimethylallyl transferase, marmesin synthase, psoralen synthase, XMT, and BMT in cultured parsley cells (Hahlbrock and Scheel, 1989). The BMT mRNA activation and enzyme accumulation occurred slowly in infected parsley tissue (Scheel et al., 1986). Thus, coumarin phytoalexins in parsley may be synthesized through phenylpropanoid pathway and through cleavage of glucosides or glucose esters.

7.6 BIOSYNTHESIS OF STILBENE PHYTOALEXINS

Stilbene phytoalexins have been detected in peanut (*Arachis hypogaea*) (Fliegmann et al., 1992), grapevine (*Vitis vinifera*) (Gehlert et al., 1990), and pine (*Pinus sylvestris*) (Sandermann et al., 1989). The precursor molecules for the formation of stilbenes are malonyl-CoA and coumaryl-CoA (Hain et al., 1993). PAL, CA4H, 4CL, and stilbene synthase are involved in the formation of the stilbene skeleton in the plants (Figure 7.7; Fliegmann et al., 1992; Dixon, 2001). The stilbene phytoalexins accumulate following the increase in these enzyme activities (Fritzemeier and Kindl, 1981). Stilbene synthase is the key enzyme in synthesis of stilbene phytoalexins. It is almost inactive in nonelicited cells (Fritzemeier and Kindl, 1981) and is induced severalfold (sometimes more than 100 times) because of fungal infection (Gehlert et al., 1990) or because of elicitor treatment (Steffens et al., 1989).

7.7 BIOSYNTHESIS OF TERPENOID PHYTOALEXINS

Several terpenoid (sesquiterpenoid, diterpenoid, and triterpenoid) phytoalexins have been detected in potato (Komaraiah et al., 2003), tobacco (Chappell et al., 1991; Klarzynski et al., 2000), castor bean (Dudley et al., 1986), sweet potato (Fujita and Asahi, 1985), rice (Kodama et al., 1988; Ren and West, 1992), and cocoa (Dixon, 2001). Three sesquiterpenoid phytoalexins, namely rishitin, lubimin, and solavetivone, are synthesized because of fungal

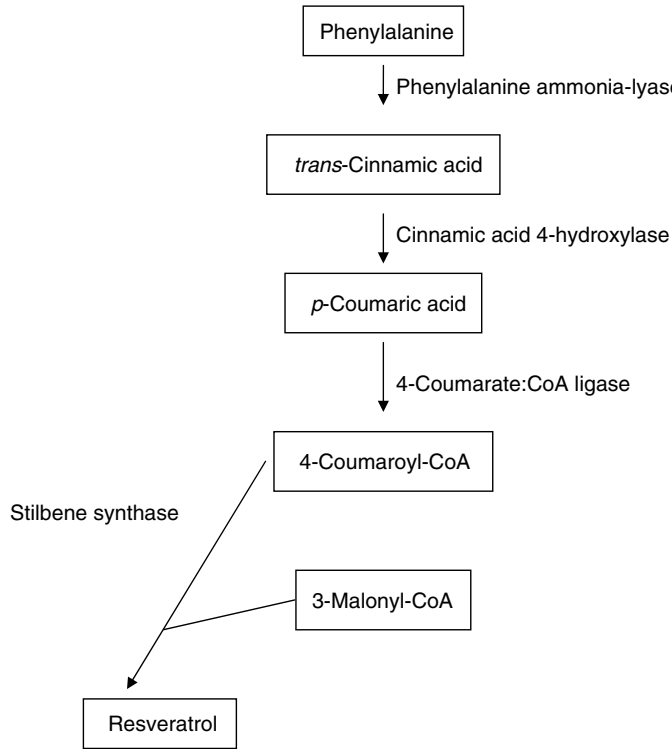

FIGURE 7.7 Biosynthesis of resveratrol in peanut. (Adapted from Fritzemeier, K.H. and Kindl, H., *Planta*, 151, 48, 1981.)

infection in potato (Coolbear and Threlfall, 1985). Their biosynthetic pathway is given in Figure 7.8 (Coolbear and Threlfall, 1985). The enzymes involved may be isopentenyl pyrophosphate isomerase, farnesyl pyrophosphate (FPP) synthase, FPP cyclase, 3-hydroxy-3 methylglutaryl coenzyme A reductase, and various oxidative enzymes (Coolbear and Threlfall, 1985; Stermer et al., 1991). Monooxygenases are involved in the later stages of the biosynthesis of sesquiterpenoid phytoalexins in potato (Brindle et al., 1985). Radiolabeled isopentenyl pyrophosphate was found to be incorporated into the sesquiterpenoid phytoalexin lubimin in potato tissues treated with elicitor from *Phytophthora infestans* (Coolbear and Threlfall, 1985).

The terpenoid phytoalexin ipomeamarone and other structurally related compounds have been detected in sweet potato infected with pathogens. When *Ceratocystis fimbriata* infects sweet potato, the activities of enzymes responsible for the conversion of mevalonate to isopentylpyrophosphate increase, and this increase is correlated with the production of furanoterpenoid phytoalexin ipomeamarone and structurally related compounds (Brindle and Threlfall, 1983; Fujita and Asahi, 1985). The fungal infection induces 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which catalyzes the NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl-CoA to mevalonate in sweet potato (Suzuki et al., 1975). HMGR activity in diseased tissue was largely associated with microsomes. The enzyme activity preceded the terpene formation and reached a maximum in 2 days (Suzuki et al., 1975). The phytoalexin accumulation has also been correlated with activity increases in pyrophosphomevalonate decarboxylase and dehydroipomeamarone reductase (Inoue et al., 1984). Pyrophosphomevalonate decarboxylase was greatest in the 12–24 h period after inoculation with *C. fimbriata*. The synthesis of the enzyme protein was required for the induction of terpene production in sweet potato root tissue (Oba et al., 1976).

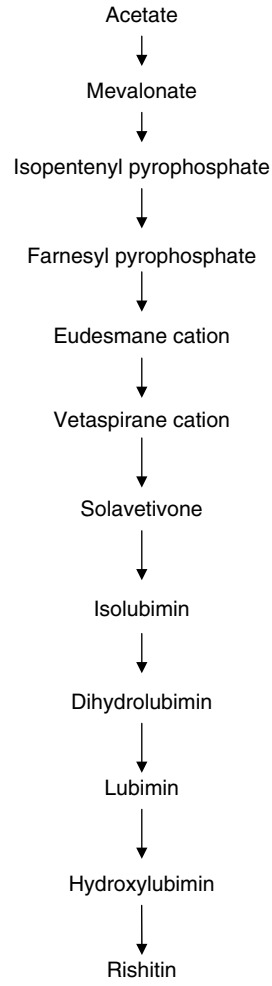

FIGURE 7.8 Biosynthesis of sesquiterpenoid phytoalexins in potato. (Adapted from Coolbear, T. and Threlfall, D.R., *Phytochemistry*, 24, 1963, 1985.)

Several sesquiterpene phytoalexins, such as rishitin, phytuberin, phytuberol, and capsidiol, have been detected in tobacco (Table 7.3). The elicitor-induced synthesis of sesquiterpenoids in tobacco cell suspension cultures was correlated with the induction of two enzymes of sesquiterpenoid biosynthetic pathway, HMGR and sesquiterpene cyclase (Vogeli and Chappell, 1988). The induction of sesquiterpenoid accumulation was correlated with a suppression of sterol biosynthesis, an isoprenoid branch pathway that competes with sesquiterpene biosynthesis between these two pathways. The suppression of sterol biosynthesis was correlated with a suppression of squalene synthetase, the first committed enzyme for this biosynthetic pathway (Vogeli and Chappell, 1988). There was coordinated inhibition of squalene synthetase and induction of enzymes of sesquiterpenoid phytoalexin biosynthesis in tobacco (Threlfall and Whitehead, 1990).

The elicitor from *Phytophthora parasitica* cell wall induced both accumulation of capsidiol and browning, whereas pectolyase from *Aspergillus japonicus* induced only browning in tobacco cell suspension cultures. The elicitor from *P. parasitica* induced more HMGR than the level found in control cell cultures. Incubation of the cell cultures with pectolyase did not induce HMGR enzyme activity significantly, but both the fungal wall elicitor and pectolyase

TABLE 7.5
Enzymes Involved in Biosynthesis of Capsidiol in Tobacco

Treatment	Induction of Capsidiol	Phenylalanine Ammonia-Lyase (nmol/mg Protein/h)	Sesquiterpene Cyclase (nmol/mg Protein/h)	Squalene Synthetase (nmol/mg Protein/h)	3-Hydroxy-3-Methylglutaryl-CoA Reductase (nmol/mg Protein/h)
None	–	75	0.0	6.7	2.7
Fungal elicitor	+	550	6.8	1.2	46.0
Pectolyase	–	760	2.8	1.9	3.7

Source: Adapted from Chappell, J., Von Lanken, C., and Vogeli, U., *Plant Physiol.*, 97, 693, 1991.

induced PAL and sesquiterpene cyclase and suppressed squalene synthetase activities in tobacco cell suspension cultures (Table 7.5; Chappell et al., 1991).

The studies have demonstrated the importance of inducible HMGR activity for elicitor-stimulated sesquiterpenoid biosynthesis. Tobacco cell suspension cultures incubated with pectolyase did not exhibit a transient induction of HMGR activity, nor did they accumulate sesquiterpenoids. This was in spite of the fact that all the necessary biosynthetic machinery beyond HMGR was apparently induced, as well as other responses such as culture browning and induction of PAL (Chappell et al., 1991). Mevinolin, a competitive inhibitor of HMGR, inhibited capsidiol accumulation in elicitor-treated tobacco cell suspension cultures (Chappell and Nable, 1987). It clearly demonstrates that HMGR is the key enzyme in production of the phytoalexins. Casbene, a macrocyclic diterpene hydrocarbon phytoalexin, is produced in castor bean inoculated with *Rhizopus stolonifer* or treated with its enzyme (Bruce and West, 1982). The enzymes catalyzing the last four steps of casbene biosynthesis are isopentenyl pyrophosphate isomerase, geranyl transferase (FPP synthetase), farnesyl transferase (geranyl-geranyl pyrophosphate synthetase), and casbene synthetase (Dudley et al., 1986; Collinge and Slusarenko, 1987; Lois and West, 1990).

Farnesyl transferase has been purified from extracts of castor bean seedlings that were elicited by exposure for 10 h to *R. stolonifer* spores. The molecular weight of the enzyme was about 72,000 Da (Dudley et al., 1986). FPP in combination with isopentenyl pyrophosphate was the most effective substrate for the production of geranylgeranyl pyrophosphate. Dimethylallyl pyrophosphate was not used by the enzyme. One peak of farnesyl transferase activity and two peaks of geranyl transferase activity (FPP synthetase) from extracts of elicited castor bean seedlings were resolved by ion exchange chromatography. It suggests that the pathway for geranylgeranyl pyrophosphate synthesis in elicited castor bean seedlings involves the successive actions of two enzymes—a geranyl transferase that uses dimethylallyl-pyrophosphate and isopentenyl pyrophosphate as substrates and a farnesyl transferase that uses the FPP produced in the first step and isopentenyl pyrophosphate substrates (Dudley et al., 1986).

Casbene synthetase has been purified from castor bean seedlings infected with *R. stolonifer* (Moesta and West, 1985). The molecular weight of casbene synthetase is 59,000 Da (Moesta and West, 1985). No casbene synthetase mRNA has been detected in uninfected seedlings. Casbene synthetase activity preceded the formation of cyclic diterpene casbene in castor bean seedlings infected with the pathogen (West et al., 1985). mRNA for casbene synthetase increased in castor bean seedlings treated with the elicitor of the pathogen within 6 h of treatment (Moesta and West, 1985). These studies suggest that casbene synthetase is the key enzyme in synthesis of the phytoalexin casbene.

Momilactones A and B, Oryzalexin A–D, sakurnetin, and several other antifungal agents have been reported to accumulate in rice leaves infected with blast pathogen, *Magnaporthe*

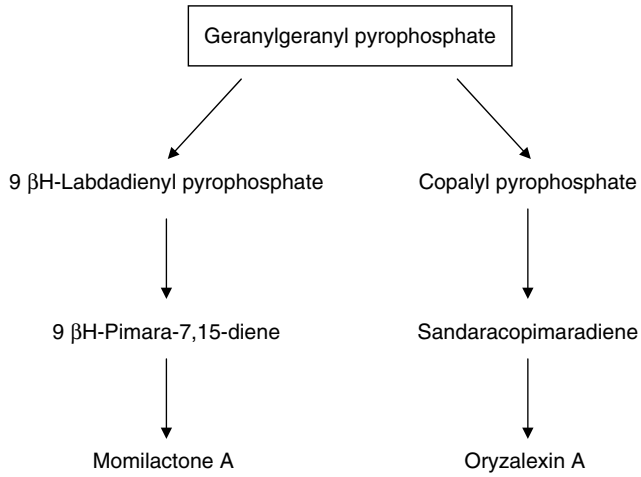

FIGURE 7.9 Biosynthesis of diterpene phytoalexins in rice. (Adapted from Ren, Y.Y. and West, C.A., *Plant Physiol.*, 99, 1169, 1992.)

grisea (Otomo et al., 2004; Jung et al., 2005). The momilactones are oxygenated derivatives of 9 βH-pimara-7,15-diene-19,6-β-olide and oryzalexins are oxygenated derivatives of a stereorically different pimaradiene, ent-sandaracopimara-8,(14),15-diene (Ren and West, 1992). Momilactones are formed by oxygenation of 9 βH-pimara-7,15-diene, which is generated by a two-step cyclization of geranylgeranyl pyrophosphate through the bicyclic intermediate, 9,10-syncopalyl pyrophosphate. The oryzalexins are formed by oxygenation of ent-sandaracopimaradiene, which originates in a two-step cyclization with ent-copalyl pyrophosphate as the bicyclic intermediate (Figure 7.9; Ren and West, 1992). The precursors of the rice phytoalexins have been identified as ent-sandara-copimara-9(14),15-diene and 9 βH-pimara-7,15-diene (Wickham and West, 1992).

The diterpene hydrocarbon synthase (cyclase), with geranylgeranyl pyrophosphate as the substrate, may induce synthesis of diterpene phytoalexins in rice (Ren and West, 1992). The cell wall preparation from the rice pathogen, *Fusarium moniliforme*, as well as chitin induced the cyclase enzyme activity. However, the activity was first detectable only after about 24 h, and the maximum activity was seen at about 40 h. The existence of a long period of time lag suggests the possibility that the induction of diterpene hydrocarbon synthase activity might be a secondary event dependent on some primary action of the elicitor treatment. A significant increase in chitinase activity was evident 12 h after addition of chitin to the rice culture. The production of chitinase may be an early event and phytoalexin biosynthesis coming on later. The chitinase produced by the plant may release water-soluble chitin fragments from the fungal cell wall and the soluble chitin may elicit phytoalexin synthesis by activating cyclase activity (Ren and West, 1992).

7.8 BIOSYNTHESIS OF INDOLE-BASED SULFUR-CONTAINING PHYTOALEXINS

Camalexin in *Arabidopsis thaliana*, Brasslexin in *Brassica* spp., and DIMBOA in *Zea mays* are the important indole-based sulfur-containing phytoalexins. The camalexin is synthesized through shikimic acid pathway. Indole-3-glycerol phosphate is the intermediate compound and indole is synthesized by the action of indole-3-glycerol phosphate lyase. Camalexin is synthesized from indole (Figure 7.10; Frey et al., 1997; Dixon, 2001). Benzoxazinone

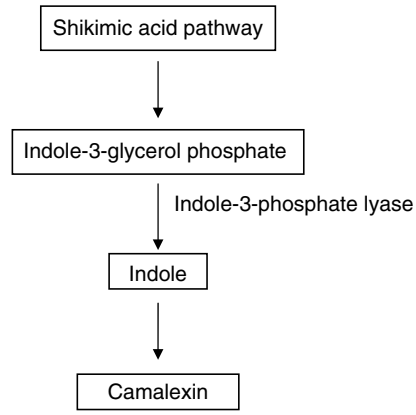

FIGURE 7.10 Biosynthesis of camalexin in *Arabidopsis*. (Adapted from Dixon, R.A., *Nature*, 411, 843, 2001.)

(DIMBOA) is synthesized from indole by the action of four consecutive cytochrome P450 enzymes (Dixon, 2001).

7.9 BIOSYNTHESIS OF ALKALOID PHYTOALEXINS

Benzophenanthridine alkaloid phytoalexin has been detected in *Papaver bracteatum* and benzylisoquinoline alkaloid phytoalexin is detected in *Berberis* spp. These are synthesized through shikimic acid pathway. Tyrosine is converted into (S)-reticuline by the action of cytochrome P450 80B1 and (S)-reticuline is converted as (S)-scoulerine by the action of berberine bridge enzymes. Benzophenanthridine alkaloids in *P. bracteatum* and benzylisoquinoline alkaloids in *Berberis* spp. are synthesized from (S)-scoulerine (Figure 7.11; Dixon, 2001).

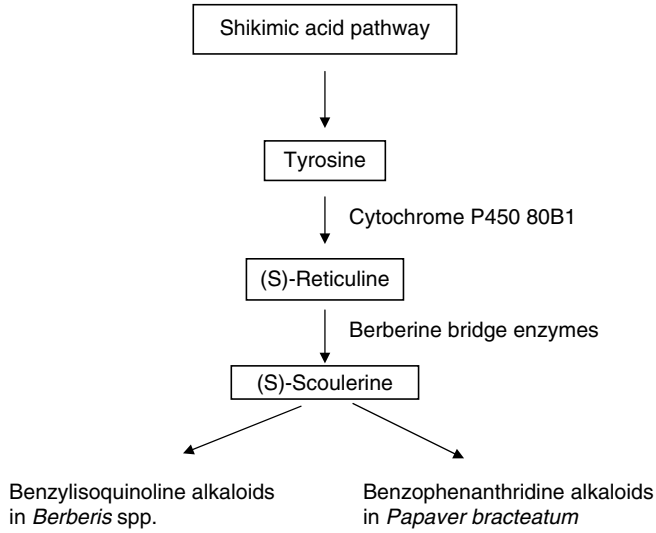

FIGURE 7.11 Biosynthesis of alkaloid phytoalexins. (Adapted from Dixon, R.A., *Nature*, 411, 843, 2001.)

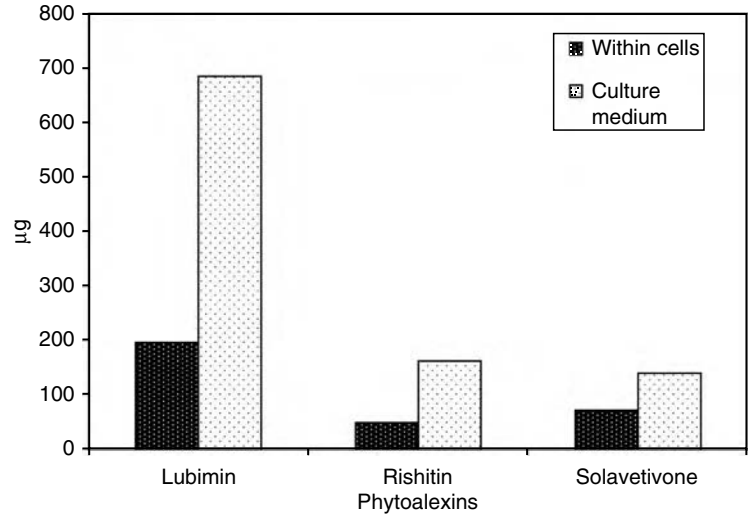

FIGURE 7.12 Secretion of phytoalexins from potato cultured cells infected by *Phytophthora infestans*. (Adapted from Brindle, P.A., Kuhn, P.J., and Threlfall, D.R., *Phytochemistry*, 22, 2719, 1983.)

7.10 SITE OF SYNTHESIS OF PHYTOALEXINS

Phytoalexin may be synthesized by living cells that are undergoing attack by the pathogen. These phytoalexins may be released toward the infection sites. The site of synthesis and accumulation of phytoalexins has been studied in sorghum–*Colletotrichum* interactions. In sorghum, three phytoalexins of the deoxyanthocyanidin class have been reported to accumulate in response to fungal infection (Hipskind et al., 1990). The important phytoalexins are luteolinidin and apigeninidin (Snyder et al., 1991). These phytoalexins are pigmented and they first accumulate in the cell that is mostly undergoing attack by *Colletotrichum graminicola* (Snyder and Nicholson, 1990). Subcellular vesicle-like inclusions appear in the host cell on which the appressorium had formed, migrate to the site of appressorium attachment, coalesce, and ultimately burst releasing their contents into the host cell itself (Snyder and Nicholson, 1990). The inclusions are directed to the fungal penetration sites (Nielsen et al., 2004). Nuclear migration, cytoplasmic streaming, and intracellular pH provided an environment for inclusion trafficking and release of the phytoalexins to the penetration sites (Nielsen et al., 2004).

The synthesized phytoalexins may be secreted from the cells. It was demonstrated using potato cell cultures (Brindle et al., 1983). Lubimin, rishitin, and solavetivone are the important phytoalexins of potato. These phytoalexins accumulated in potato cell suspension cultures inoculated with either an incompatible or a compatible race of *Phytophthora infestans* (Figure 7.12; Brindle et al., 1983). The large proportion of each phytoalexin in the culture medium suggests that the phytoalexins diffused into the medium from the cells in which they were synthesized. It is possible that the phytoalexins are synthesized in healthy living cells and then move out and accumulate in the adjoining necrotic tissue (Brindle et al., 1983).

7.11 PHYTOALEXINS ARE FUNGITOXIC

Phytoalexins are recognized only based on their antimicrobial activity (VanEtten et al., 1994). Most of them have been reported to be highly fungitoxic (Schutt and Netzly, 1991; Hrazdina et al., 1997; Lo et al., 1999). Malusfuran, the phytoalexin produced in apple

(*Malus domestica*), inhibited spore germination and growth of *Venturia inaequalis* at millimolar concentrations (Hrazdina et al., 1997). The rice phytoalexin sakuranetin is highly inhibitory to spore germination of *Magnaporthe grisea* (Kodama et al., 1992). The phytoalexin resveratrol inhibited hyphal growth of the alfalfa fungal pathogen *Phoma medicaginis* (Hipskind and Palva, 2000). Pisatin, the pea phytoalexin, inhibited mycelial growth of several fungi, including both pathogens and nonpathogens of pea (Delserone et al., 1999). The sorghum anthocyanidin phytoalexins inhibited growth of several fungi (Schutt and Netzly, 1991). Medicarpin, the phytoalexin of alfalfa, inhibited mycelial growth of *Phoma medicaginis*, *Nectria haematococca*, and *Phytophthora megasperma* f. sp. *medicaginis* (Blount et al., 1992). In *Cassia obtusifolia*, a flavonoid phytoalexin that was identified as 2-(*p*-hydroxyphenoxy)-5,7-dihydroxychromone was induced because of infection with *Alternaria cassiae* (Sharon and Gressel, 1991). It inhibited growth of *A. cassiae* at 0.3 mM concentration (Sharon et al., 1992). Maackiain, the phytoalexin of leguminous plants, inhibits fungal germ tube elongation (Lucy et al., 1988). The ethanol extract of wyerone acid significantly reduced *Botrytis fabae* spore germination with increased concentrations, but the phytoalexin had no effect on *in vitro B. fabae* germ tube growth (Nawar and Kuti, 2003).

Pathogens produce toxins that are responsible for disease symptom development. Phytoalexins may suppress toxin production by the pathogens. *Fusarium sporotrichoides* is a pathogen of parsnip (*Pastinaca sativa*). It produces trichothecenes, which are potent phytotoxins (Manka et al., 1985). The toxins could be isolated from diseased plant tissues (Miller et al., 1985). Xanthotoxin and angelicin are the furanocoumarin phytoalexins in parsnip (Desjardins et al., 1989b). Trichothecene toxin production by *F. sporotrichoides* was completely inhibited by furanocoumarins at concentrations well below those reported to accumulate in infected parsnips (Desjardins et al., 1988). In *F. sporotrichoides*-infected parsnip root tissues xanthotoxin and angelicin accumulated to high levels, whereas trichothecene toxin was present only at low levels. Trichodiene, the precursor of the toxin not detectable in liquid culture, accumulated. By contrast, in microwave-cooked parsnip root infected with the fungus very low levels of furanocoumarins and trichodiene were detected, and the level of trichothecene toxin was comparable with that observed in liquid cultures (Desjardins et al., 1989b). The results suggest that the furanocoumarins may inhibit biosynthesis of trichothecene from trichodiene in the parsnip roots infected with *F. sporotrichoides*.

7.12 HOW DO PATHOGENS OVERCOME THE ANTIFUNGAL PHYTOALEXINS?

7.12.1 Pathogens May Detoxify Phytoalexins

The pathogens have been reported to detoxify phytoalexins of the host. Pea pathogens are very tolerant to the pea phytoalexin pisatin and are known to detoxify pisatin (VanEtten et al., 1989). The phytoalexin tolerance and detoxification processes have been studied in detail using *Nectria haematococca* mating population MP VI (anamorph *Fusarium solani* f. sp. *pisi*), a pathogen of pea. *N. haematococca* demethylates pisatin to a nontoxic product, 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (VanEtten et al., 1982). The demethylation reaction is catalyzed by pisatin demethylase (pda), a cytochrome P450 (Matthews and VanEtten, 1983). The *PDA* gene has been cloned, and transformation of a *PDA* gene into a Pda^- isolate increases its pisatin tolerance (Ciuffetti and VanEtten, 1996). Disruption of a PDA gene in Pda^+ isolates reduces their pisatin tolerance (Wasmann and VanEtten, 1996). These results suggest that pisatin tolerance in *N. haematococca* is due to detoxification of pisatin by the pda.

Virulent isolates of *N. haematococca* are more sensitive to pisatin, have demethylating ability, and induce less pisatin in infected tissue compared with less-virulent isolates (Table 7.6; Tegtmeier and Van Etten, 1982).

TABLE 7.6
Relationship between Virulence and Sensitivity to Pisatin in the Pea Pathogen *Nectria haematococca*

N. haematococca Isolate	Lesion Length (cm)	Inhibition by Pisatin (%)	Demethylating Ability	Pisatin (μg/cm^3 Lesion Tissue)
Highly virulent	11.5	16	+	96
Less virulent	2.4	55	−	505

Source: Adapted from Tegtmeier, K.J. and VanEtten, H.D., *Phytopathology*, 72, 608, 1982.

Note: +, presence; −, absence.

Addition of *PDA* to a Pda$^-$ isolate increases its virulence on pea, and disruption of the *PDA* gene in a PDA$^+$ isolate reduces its virulence on pea (Schäfer et al., 1989). These results suggest that PDA may be a virulence factor. PDA is a cytochrome P450 enzyme. Seven similar cytochrome P450s that encode pda have been identified in *N. haematococca* (Miao et al., 1991; Maloney and VanEtten, 1994; Reimmann and VanEtten, 1994). Three different phenotypes have been identified in *N. haematococca* based on the lag period for induction of pda and the resulting amount of activity induced. They are Pda^{SH} (short lag, high activity), Pda^{SM} (short lag, moderate activity), and Pda^{LL} (long lag, low activity). Pda^{SH} and Pda^{SM} isolates are the most virulent ones (Kistler and VanEtten, 1984a,b; Mackintosh et al., 1989). It is suggested that a readily inducible enzyme detoxification system, Pda^{SH} or Pda^{SM} genes, confers tolerance to pisatin and allows a tolerant isolate to be a more-virulent isolate than an isolate that more slowly detoxifies pisatin (Pda^{LL}) or lacks pda (Delserone et al., 1999). Delserone et al. (1999) showed that the ability to demethylate pisatin was common in fungi, regardless of whether the particular isolate was pathogenic on pea or not. However, when the rate of pisatin demethylation was compared with virulence, all but one of the moderate to highly virulent isolates rapidly demethylated pisatin. These observations suggest that a specialized enzyme system for quickly detoxifying pisatin might be present in most pea pathogens.

The highly virulent isolates of pathogens of pea, *Ascochyta pisi* and *Rhizoctonia solani,* demethylate pisatin more rapidly than the less-virulent ones (VanEtten et al., 1989). *Phoma pinodella* and *Mycosphaerella pinodes*, the other pea pathogens, also demethylate pisatin (Delserone and VanEtten, 1987). A nonpathogen of pea, *Cochliobolus heterostrophus* (maize pathogen), was transformed with the *PDA* gene from the pea pathogen, *N. haematococca,* and the transformed maize pathogen became pathogenic on pea producing larger lesions (Schäfer et al., 1988). Overexpression of *PDA* gene from *N. haematococca* caused increased virulence of several independently generated *C. heterostrophus* transformants to pea (Oeser and Yoder, 1994). These studies have established that degradation of pisatin in pea is needed for pathogenesis by fungal pathogens. Maackiain and medicarpin are the phytoalexins produced in many leguminous plants. *Ascochyta rabiei* cleaves maackiain and medicarpin by three different ways. A reductive cleavage of ring C producing isoflavans, hydroxylation of ring A at position 1a resulting in production of 1a-hydroxydienones, and 9-*O*-methylation are the three different initial reactions in the metabolism of maackiain and medicarpin (Kraft et al., 1987). The enzyme responsible for the first reaction, reductive cleavage of the pterocarpan to isoflavan, has been purified. It is a soluble NADPH-dependent reductase with a strong preference for medicarpin and maackiain as substrates (Hohl and Barz, 1987). The enzyme preparations also catalyzed the 1a-hydroxylation and 9-*O*-demethylation (Hohl and

Barz, 1987). The 1a-hydroxylation by enzyme preparations obtained from *A. rabiei* is O_2 dependent (Hohl and Barz, 1987).

N. haematococca degrades maackiain and medicarpin by three alternative reactions. The products correspond to oxygen attack at positions 6a, 1a, and 11a (Denny and VanEtten, 1982), and all these reactions may be oxygenase catalyzed (Denny and VanEtten, 1982). The degraded products are less toxic than medicarpin and maackiain. The most virulent isolates of *N. haematococca* were relatively tolerant of maackiain and medicarpin, and almost all of these isolates could metabolize maackiain and medicarpin by atleast one route (Denny and VanEtten, 1981; Lucy et al., 1988). Three genes controlling maackiain metabolism have been reported in *N. haematococca* (Miao and VanEtten, 1987). *Mak1* and *Mak2* confer 1a-hydroxylation, and *Mak3* confers 6a-hydroxylation (Miao et al., 1986). The enzymes encoded by *Mak2* and *Mak3* are specific for maackiain, whereas *Mak1* may be identical to *Pda6* detected in *N. haematococca* (Miao et al., 1986). Maackiain and pisatin detoxification are required for pathogenicity of *N. haematococca* on chickpea and pea, respectively, and each reaction can be controlled independently (VanEtten et al., 1989).

Phaseollin, phaseollidin, phaseollinisoflavan, and kievitone are the important phytoalexins in French bean. *Fusarium solani* f. sp. *phaseoli* metabolizes all the four phytoalexins of bean (Smith et al., 1980; Zhang and Smith, 1983). The resulting metabolites are less toxic than their parent phytoalexins (Smith et al., 1980; VanEtten et al., 1982). Phaseollin is detoxified by 1a-hydroxylation by an intracellular oxygenase (Kistler and VanEtten, 1981). Isotope incorporation studies have provided direct evidence for participation of the monooxygenase in metabolism of phaseollin by *F. solani* f. sp. *phaseoli* (Kistler and VanEtten, 1981). Both phaseollin and its metabolite, 1a-hydroxyphaseollone, were detected in bean hypocotyls infected with *F. solani* f. sp. *phaseoli* (VanEtten et al., 1989). Phaseollidin is also detoxified by hydration of its isopentenyl side chain (Smith et al., 1980).

Phaseollinisoflavan is metabolized to a product that is less fungitoxic (Zhang and Smith, 1983; Wictor-Orlandi and Smith, 1985). When phaseollinisoflavan was added to the liquid cultures of *F. solani* f. sp. *phaseoli*, its concentration decreased for 21 h and thereafter no phaseollinisoflavan could be recovered. Conversely, a new compound, designated M-1, was detected in the culture medium after 9 h and increased steadily. The metabolite M-1 was less toxic to the fungus compared with the phytoalexin. Both M-1 and phaseollinisoflavan could be detected in bean hypocotyls inoculated with the pathogen indicating that they would have been metabolized in the plant cells (Wictor-Orlandi and Smith, 1985).

Kievitone hydratase is produced constitutively by *F. solani* f. sp. *phaseoli*. The enzyme induces hydration of isopentenyl side chain in kievitone resulting in the formation of kievitone hydrate (Smith et al., 1982). Kievitone and its metabolite, kievitone hydrate, have been detected in bean infected with the pathogen (Kuhn and Smith, 1978). The highly virulent isolates of *F. solani* f. sp. *phaseoli* produced kievitone hydratase (Smith et al., 1984). Two mutants of the pathogen produced less kievitone hydratase and showed less tolerance to kievitone. They were also less pathogenic on bean (Smith et al., 1984). These mutants were also less tolerant to phaseollin and phaseollinisoflavan (Choi et al., 1987). The potato pathogen, *Gibberella pulicaris*, degrades both lubimin and rishitin, the phytoalexins of potato (Desjardins and Gardner, 1989; Desjardins et al., 1989a). Seven metabolites of lubimin have been reported (Desjardins et al., 1989a). The lubimin may be metabolized to 15-dihydrolubimin or to 2-dehydrolubimin. Both are converted to isolubimin and then to cyclodehydroisolubimin (Desjardins et al., 1989a). Both 15-dihydrolubimin and isolubimin are toxic to *G. pulicaris*, whereas cyclodehydroisolubimin and two later tricyclic metabolites are not toxic to the fungus (Desjardins et al., 1989a). All isolates of *G. pulicaris* that were highly virulent on potato degraded both lubimin and rishitin rapidly and were relatively tolerant to them. The isolates that were less tolerant metabolized the phytoalexins more

TABLE 7.7
Detoxification of Pterostilbene by the Culture Filtrate of *Botrytis cinerea*

Treatment	Inhibition of *B. cinerea* Spore Germination (%)	Dead Conidia (%)
Pterostilbene	100	100
Pterostilbene + protein extract of culture filtrate	0	0

Source: Adapted from Pezet, R., Pont, V., and Hoang-Van, K., *Physiol. Mol. Plant Pathol.*, 39, 441, 1991.

slowly and they were low in virulence (Desjardins et al., 1989a). There may be atleast two genes in *G. pulicaris* that confer high tolerance to and rapid metabolism of rishitin (Desjardins and Gardner, 1989).

Botrytis cinerea is known to detoxify phytoalexins from a number of plants (Mansfield, 1980). The pathogenicity of isolates of *B. cinerea* toward French bean was found to be more closely correlated with their ability to metabolize phaseollin than with their ability to tolerate the phytoalexin (Van Den Heuvel, 1976). *B. cinerea* degraded resveratrol and pterostilbene, the stilbene phytoalexins of grapevine (Pezet et al., 1991). Pterostilbene solution lost its lethal property completely after the treatment with the protein extract of culture filtrate (Table 7.7; Pezet et al., 1991). Inhibitors of laccase, such as sodium diethyldithiocarbamate and sodium azide, inhibited the activity of the protein extract. This inhibition was also observed using thiourea, an OH^- scavenger and inhibitor of hydroxylation, indicating that hydroxylation is an important step in hydroxystilbene oxidation (Pezet et al., 1991). The results suggest that *B. cinerea* produces a hydroxystilbene-degrading enzyme, and the enzyme, stilbene oxidase, is a laccase. Stilbene oxidase oxidizes both pterostilbene and resveratrol to produce nontoxic products (Pezet et al., 1991). Oxidation of resveratrol facilitates pathogenesis of *B. cinerea* in grapevine (Hoos and Blaich, 1990). *B. cinerea* produces large amount of laccase [polyphenol oxidase (PPO)] secreted from the cytoplasm of its mycelial cells through the walls and mucilage sheath (Hodson et al., 1987).

Both the furanocoumarin phytoalexins xanthotoxin and angelicin were fungistatic, but not fungicidal to *Fusarium sporotrichoides*. Cultures of the fungus kept for more than 2 weeks at furanocoumarin concentrations completely inhibitory to growth had the same growth rate as control cultures when transferred to agar media without furanocoumarins. It suggests that the fungus is able to completely metabolize the phytoalexins (Desjardins et al., 1989a). All these studies suggest that virulent pathogens may degrade the phytoalexins to nontoxic products and the enzymes involved in the degradation may be pathogenicity factors.

7.12.2 Induction of Phytoalexins May Be Delayed in Susceptible Interactions

A delay in induction of phytoalexins is common in various susceptible interactions, and this delay would have helped the pathogen to escape from the toxic environment created by the accumulation of phytoalexins in the infection site. The early infection process of *Colletotrichum sublineolum* on mesocotyls of sorghum seedlings was similar in both compatible and incompatible interactions. About 6 h after inoculation, the conidia had germinated and formed appressoria. Infection vesicles were formed within epidermal cells at about 36 h after inoculation. During this period, a considerable amount of red phytoalexin pigments had accumulated in the resistant cultivar. The primary hyphae had emerged from the

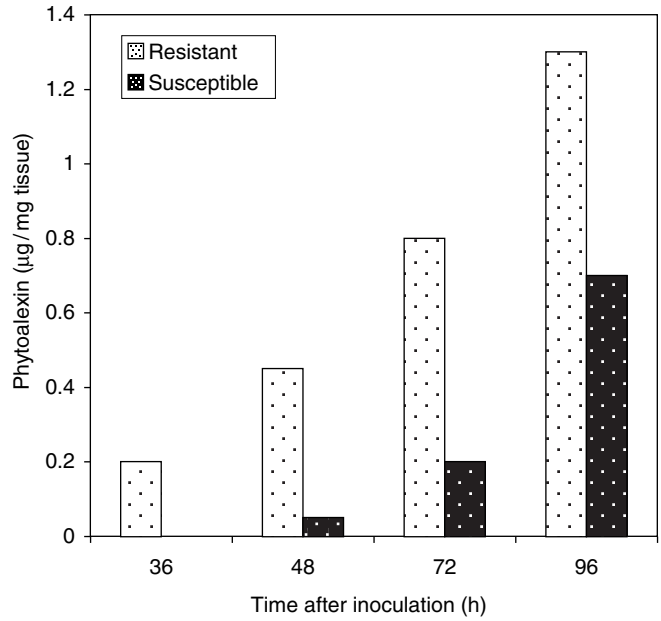

FIGURE 7.13 Accumulation of total 3-deoxyanthocyanidin phytoalexins in susceptible and resistant cultivars after inoculation with *Colletotrichum sublineolum*. (Adapted from Lo, S.C., Hipskind, J.D., and Nicholson, R.L., *Mol. Plant Microbe Interact*., 12, 479, 1999.)

infection vesicles by 48 h after inoculation. However, the emerged fungal structures were highly distorted in the resistant cultivar (Lo et al., 1999). In contrast, phytoalexins began to accumulate only at 48 h after inoculation in the susceptible interaction (Figure 7.13; Lo et al., 1999), and during this time, primary hyphae had emerged from infection vesicles. The morphology and proliferation of the primary hyphae did not appear to be affected in the susceptible interactions. In the susceptible cultivars, notable amount of phytoalexins accumulated only 72 h after inoculation (Figure 7.13), but by that time the pathogen had successfully penetrated the host tissue and proliferated intracellularly (Lo et al., 1999).

Glyceollins I, II, and III accumulate slower in susceptible soybean cultivar inoculated with *Phytophthora sojae* compared with that in the resistant variety (Bhattacharyya and Ward, 1986). Glyceollin accumulates in resistant soybean roots at 2 h after inoculation with the pathogen but not until 12 h in susceptible roots (Hahn et al., 1985). The delay in synthesis of phytoalexins in the infected susceptible host may be due to delayed stimulation of activity of phytoalexin biosynthetic enzymes. In the susceptible soybean tissues infected with *P. sojae*, the activity of phytoalexin biosynthetic enzymes remained the same as, or was only slightly higher than, in the uninoculated controls during the experimental period of 2–8 h after inoculation. In contrast, in the incompatible interaction, there was an early stimulation of the various enzyme activities starting at 2–4 h after inoculation with accumulation of phytoalexins (Hahn et al., 1985). In soybean roots inoculated with *P. sojae*, an increase in the activity of dihydroxypterocarpan 6a-hydroxylase, an enzyme that catalyzes the production of the immediate precursor of glyceollin, was observed 6 h earlier in resistant than in susceptible roots (Bonhoff et al., 1986a,b).

When soybean hypocotyls were inoculated with *P. sojae*, mRNAs for both PAL and CHS were rapidly produced even within 3 h following inoculation in a resistant variety, but in the susceptible variety only slight enhancement of mRNAs was observed at that time (Esnault et al., 1987). It suggests that the production of mRNAs for enzymes leading to

phenylpropanoid biosynthesis in soybean cotyledons is delayed in response to infection with compatible pathogen, but it is an early response in an incompatible interaction. Delayed production of CHS mRNA in a susceptible *Colletotrichum lindemuthianum*–bean interaction has been reported (Bell et al., 1984). CHS mRNA was produced at a slower rate in the susceptible interaction in soybean also (Schmelzer et al., 1984).

The delayed release of precursors of phytoalexin may also delay the synthesis of phytoalexins. The precursors of glyceollin, daidzein conjugates are present at higher levels in a soybean cultivar susceptible to *P. sojae* compared with that in a resistant variety. In incompatible reactions, there was a marked and immediate reduction in levels of the conjugates. These levels fell to less than half their original levels by 24 h and to nearly nondetectable levels within just 48 h. Concurrent with this, there was an accumulation of free daidzein and glyceollin. In contrast, the compatible reaction was characterized by a delayed disappearance of the daidzein conjugates, a nearly stoichiometric accumulation of daidzein, and little to no accumulation of glyceollin. In the compatible reactions, only low levels of glyceollin were synthesized despite the accumulation of very large amounts of free daidzein. It suggests that by the time the isoflavones are released, the majority of cells in the susceptible infected tissues are no longer capable of glyceollin biosynthesis. The delayed release of daidzein from its conjugates may be important in pathogenesis of the pathogen (Graham et al., 1990).

7.12.3 Pathogen May Suppress Accumulation of Phytoalexins in Susceptible Hosts

Pathogens may suppress accumulation of phytoalexins in the susceptible interactions. When rice leaves were inoculated with incompatible race of *Magnaporthe grisea*, the phytoalexin production was detected at 36–48 h after inoculation at which time the insertion of the infection hyphae had started. At this time, phytoalexin was not detected in rice leaves inoculated with compatible race of the pathogen. In general, the appearance of the phytoalexin was 12–24 h faster in incompatible interaction than in compatible interaction (Iwakuma et al., 1990). This race specificity disappeared when heat-killed spores were applied and the phytoalexin appeared at 48 h in both incompatible and compatible interactions. Hyphal wall components (elicitors) from both compatible and incompatible races of *M. grisea* induced similar amount of phytoalexins. The results suggest that the compatible pathogen may suppress the accumulation of phytoalexins, whereas its cell wall component may be able to induce phytoalexins faster. The race specificity in phytoalexin elicitation is observed when living spores are applied and not in the treatments with heat-killed spores or hyphal cell wall components. A suppressor or suppressing system in living fungal cells may exist (Iwakuma et al., 1990).

The induction of phytoalexin in barley leaves by an incompatible race of *Blumeria graminis* f. sp. *hordei* was suppressed by preliminary inoculation with a compatible race (Oku et al., 1980). Pycnospore germination fluid of *Mycosphaerella pinodes*, the pathogen of pea, contains both elicitors and suppressors for the accumulation of pisatin in pea (Shiraishi et al., 1978; Oku et al., 1987). The elicitors induce PAL gene in pea, whereas the suppressors suppress it (Hiramatsu et al., 1986). Similar elicitors and suppressors have been isolated from *P. sojae*, the soybean pathogen (Ziegler and Pontzen, 1982).

The suppressor isolated from the pea pathogen, *M. pinodes*, delayed the accumulation of PAL and CHS mRNAs by 3 h in pea. There was a 6 h delay in the increase of PAL enzyme activity, and a 6–9 h suppression of pisatin accumulation due to suppressor treatment (Yamada et al., 1989). The suppressor isolated from *M. pinodes* suppressed ATPase activity in pea plasma membrane. The ATPase activity in pea plasma membrane was unaffected by the addition of elicitor (Yoshioka et al., 1990). The inhibition of ATPase activity in pea plasma membrane *in vitro* seems to be closely correlated with the suppression of production of

pisatin *in vivo* (Yoshioka et al., 1990). The effect of the suppressor on host defense reactions seems to result from its inhibition of the ATPase in the host plasma membrane.

7.12.4 Amount of Accumulation of Phytoalexins May Be Less in Susceptible Interactions Compared with Resistant Interactions

In some susceptible interactions, only less amount of phytoalexins may accumulate compared with that in resistant interactions. In resistant oat plants, the phytoalexin avenalumin I accumulates at higher amounts compared with that in susceptible plants, and the infection hyphae of *Puccinia coronata* f. sp. *avenae* were significantly shorter in resistant oat plants (Mayama et al., 1995). When a cultivar of tobacco resistant to *Phytophthora nicotianae* var. *nicotianae* was inoculated with the pathogen, the sesquiterpenoid phytoalexins accumulated to higher level when compared with that in a susceptible variety (Nemestothy and Guest, 1990). Infection of broad bean (*Vicia faba* L.) leaves with *Botrytis fabae* (Sard.) resulted in wyerone acid phytoalexin accumulation in resistant and susceptible broad bean cultivars. Rapid wyerone acid accumulation was observed in leaves of resistant cultivars that reached levels greater than twofold of the susceptible cultivars (Nawar and Kuti, 2003).

The susceptible soybean leaves inoculated with *P. sojae* accumulate less amount of glyceollin than the resistant soybean leaves (Ebel and Grisebach, 1988). Two phytoalexins [1,3-dion-5-octyl-cyclopentaene(tsibulin 1d) and 1,3-dion-5-hexyl-cyclopentaene (tsibulin 2d)] accumulated to a very high level when onion bulbs were inoculated with a nonpathogen. When a pathogen of onion, *Botrytis allii*, was inoculated, the accumulation of the phytoalexins was almost negligible (Dmitriev et al., 1990). When susceptible and resistant chickpea cultivars were inoculated with *Ascochyta rabiei*, increase in medicarpin was negligible in the susceptible cultivar and the second phytoalexin, maackiain, could not be detected at all. In contrast, the resistant cultivar showed accumulation of large amounts of both the phytoalexins (Weigand et al., 1986). All these studies indicate that reduced accumulation of phytoalexins may confer susceptibility in plants.

7.12.5 Highly Toxic Phytoalexins May Not Accumulate in Susceptible Interactions

Plants accumulate multiple phytoalexin compounds. For example, luteolinidin, 5-methoxyluteolinidin, apigeninidin, and a caffeic acid ester of 5-*O*-arabinosyl-apigeninidin have been detected as phytoalexins in sorghum (Lo et al., 1999). All the four phytoalexins accumulated in resistant cultivar after inoculation with *Colletotrichum sublineolum*, whereas only apigeninidin and caffeic acid ester of 5-*O*-arabinosyl apigeninidin accumulated in the susceptible interaction. *In vitro* bioassays showed that luteolinidin and 5-methoxyluteolinidin exhibited higher toxicity than the other two phytoalexin components to the pathogen (Lo et al., 1999). It suggests that only less-toxic phytoalexins accumulate in susceptible interactions.

7.12.6 Some Phytoalexins May Not Be Produced in Susceptible Interactions

Although several phytoalexins are induced in both resistant and susceptible interactions, some of them were found to be induced only in resistant interactions. For example, the phytoalexin luteolinidin was induced only in resistant interactions in sorghum inoculated with *Colletotrichum sublineolum* (Lo et al., 1999). Cell suspension cultures of the scab-resistant apple cultivar produced the benzofuran phytoalexin, 2,4-methoxy-3-hydroxy-9-*O*-β-D-glucosyloxydibenzofuran (malusfuran), when stress was applied. Suspension cultures of the scab-susceptible apple cultivar, when similarly challenged, showed no detectable response (Hrazdina et al., 1997).

7.12.7 Some Phytoalexins May Not Have Any Role in Defense Mechanisms of Plants

In some host–pathogen interactions, phytoalexins may not have any role in disease resistance. Accumulation of phytoalexins may be only a metabolic process activated by stress. Phytoalexins may accumulate in both susceptible and resistant interactions due to infection. In pea, the pathogen, *Fusarium solani* f. sp. *pisi*, induced more pisatin than the nonpathogen, *F. solani* f. sp. *phaseoli* (Kendra and Hadwiger, 1987). Inoculation with *F. solani* f. sp. *pisi* induced PAL severalfold in pea and it was much higher than that induced by the nonpathogen *F. solani* f. sp. *phaseoli* (Loschke et al., 1981). The deoxyanthocyanidin phytoalexins accumulate rapidly in both sorghum mesocotyls and leaves following attempted fungal infection by pathogens as well as by nonpathogens (Nicholson et al., 1988).

7.13 CHEMICAL STRUCTURAL CLASSES OF PHYTOANTICIPINS

Phytoanticipins are low-molecular weight, antifungal compounds that are present in plants before challenge by fungal pathogens or are produced after infection solely from preexisting constituents (VanEtten et al., 1995). Numerous antifungal phytoanticipins have been detected in plants. They belong to several chemical classes including phenolic acids (Luthria and Mukhopadhyay, 2006), di- and trihydroxy phenolics (Vidhyasekaran, 1988), flavanones (Rakwal et al., 2000; Danelutte et al., 2003; Jung et al., 2005), flavonoids (Guetsky et al., 2005), isoflavones (Morkunas et al., 2005; Wegulo et al., 2005), isoflavonoids (Graham and Graham, 1991), isoflavans (Meragelman et al., 2005), isoflavanones (Monache et al., 1996), glucosides of isoflavonoids (Graham, 1991), pterocarpans (Meragelman et al., 2005), furanocoumarins (Lombaert et al., 2001), anthocyanidins (Hammerschmidt and Nicholson, 1977; Vidhyasekaran, 1988), chromene (Morandim et al., 2005), bibenzyl (Dixon, 2001), xanthone (Li et al., 2005), benzoxazinone (Frey et al., 1997), terpenoid saponins (Mert-Türk 2006; Thomas et al., 2006), steroid saponins (Mert-Türk, 2006), steroidal glycoalkaloid saponin (Morrissey and Osbourn, 1999), dienes (Wang et al., 2004b; Guetsky et al., 2005), glucosinolates (Brader et al., 2006), and cyanogenic glucosides (Wang and VanEtten, 1992).

7.14 PHENOLICS AS PHYTOANTICIPINS

Several phenolics and phenylpropanoids that may act as phytoanticipins have been detected in plants (Vidhyasekaran, 1988). Chlorogenic acid and caffeic acid are the common phenolic acids detected in eggplant (*Solanum melongena*) (Luthria and Mukhopadhyay, 2006). Ferulic, *p*-coumaric, and syringic acids are commonly found in wheat cultivars (Southerton and Deverall, 1990). The flavonoid epicatechin plays an important role as phytoanticipin in avocado fruits (Guetsky et al., 2005). Several isoflavonoids such as genistein, wighteone, and luteone have been detected in lupine (*Lupin luteus*) (Morkunas et al., 2005). Concentrations of these isoflavonoids increased in lupine after infection with *Fusarium oxysporum* (Morkunas et al., 2005). Daidzein, genistein, and glycitein are the isoflavones detected in soybean (Wegulo et al., 2005). Concentrations of these isoflavones increase after infection by *Sclerotinia sclerotiorum* (Wegulo et al., 2005). The antimicrobial isoflavanones desmodianones A, B, and C have been isolated from *Desmodium canum* (Monache et al., 1996). In the root extract of *Hildegardia barteri*, the antifungal pterocarpans 2-hydroxymaackiain and farrerol have been isolated (Meragelman et al., 2005).

Anthocyanins are glycosides of anthocyanidins and the final products of flavonoid metabolism. They are commonly found in corn leaves (Hammerschmidt and Nicholson, 1977). Furanocoumarins occur in many plants, especially in species of the Umbelliferae,

Leguminosae, Rutaceae, and Moraceae (Pathak et al., 1962). Furanocoumarins are heterocyclic compounds derived from coumarin by the addition of a furan ring at the 6,7 positions (linear furanocoumarins) or 7,8 positions (angular furanocoumarins). The furanocoumarins angelicin, bergapten, psoralen, trimethylpsoralen, xanthotoxin, isopimpinellin, and sphondin have been identified in celery plants (Ataga et al., 1993). Xanthotoxin and bergapten are the furanocoumarins detected in parsnip and parsley (Lombaert et al., 2001).

7.15 TOXICITY OF PHENOLICS TO PATHOGENS

Several phenolics are highly toxic to fungal pathogens. Both the toxicity of different phenolics and the sensitivity of pathogens to the phenolics vary. Differential sensitivity to monophenols and di- and trihydroxy phenols has been reported among *Phytophthora* spp. (Casares et al., 1986). Salicylic acid, *p*-coumaric acid, vanillic acid, and pyrogallol were highly inhibitory to *Phytophthora cambivora* and not to *Phytophthora cinnamomi*. Cinnamic acid inhibited both the fungi effectively. In general, *P. cinnamomi* was less sensitive to the phenolics than *P. cambivora* (Casares et al., 1986).

Effect of different phenolics in inhibiting growth of *P. cinnamomi* varied (Cahill and McComb, 1992). Cinnamic acid, *p*-coumaric acid, vanillic acid, ferulic acid, *p*-hydroxybenzoic acid, caffeic acid, and coumarin completely inhibited growth of the fungus at 10 mM, whereas chlorogenic acid, protocatechuic acid, epicatechin, and phloroglucinol did not inhibit the growth appreciably (Cahill and McComb, 1992).

The isoflavones even at low concentrations inhibit the fungi *in vitro* (Weidenborner et al., 1990). The isoflavone biochanin A and its dehydro derivatives (isoflavanones) show high antifungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* at a concentration of about 0.8 mM. Genistein isoflavan and other isoflavans with two hydroxyl groups and one methoxy group are also fungitoxic (Weidenborner et al., 1990).

The toxicity of phenolics has been demonstrated by artificially increasing the synthesis of phenolics in some plants. Treatment of susceptible tomato plants with quinic acid or phenylalanine increased their phenolic content, and this increase in phenolic content resulted in resistance to *F. oxysporum* f. sp. *lycopersici* (Carrasco et al., 1978). When tomato plants were fed with catechol, a marked accumulation of total phenols was observed and it resulted in suppression of disease symptom expression after infection by *F. oxysporum* f. sp. *lycopersici* (Retig and Chet, 1974). Inoculation of freshly harvested avocado fruits with a mutant strain of *Colletotrichum magna* inhibited subsequent decay development by the pathogen *Colletotrichum gloeosporioides*. The mutant strain induced higher levels of epicatechin. It suggests that enhanced resistance of avocado fruits to *C. gloeosporioides* may be due to the induction of the phenolic epicatechin (Prusky et al., 1994). These studies suggest that phenolics may be important phytoanticipins involved in fungal pathogenesis.

7.16 HOW DOES PATHOGEN OVERCOME THE ANTIFUNGAL PHENOLICS?

7.16.1 Pathogen May Degrade Phenolics to Nontoxic Products

Successful pathogens have been shown to be potent degraders of plant defense compounds, particularly phenolics. *Fusarium* spp. degrade isoflavones of chickpea (Willeke and Barz, 1982a,b). Biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), its 7-*O*-glucoside, and the biochanin A-7-*O*-glucoside-6″-*O*-malonate are the main phenolic constituents of chickpea (Koster et al., 1983). *Fusarium javanicum* (Willeke and Barz, 1982a) and *Fusarium solani* (Willeke et al., 1983) degrade biochanin A. The catabolic route first involves dihydrobiochanin A (5,7-dihydroxy-4′-methoxyisoflavone) and then leads through 3-(*p*-methoxyphenyl)-4,6-diketo-5,6-dihydro-4H-pyran to phenylacetic acids derived from the side chain phenyl ring

and carbon atoms 2 and 3 of the heterocycle. *O*-Demethylation of biochanin A has also been reported to occur (Willeke and Barz, 1982a; Willeke et al., 1983).

Ascochyta rabiei degraded biochanin A within 1–3 h after incubation in the nutrient medium; transient formation of two isoflavones pratensein and genistein was seen (Kraft and Barz, 1985). 3′-Hydroxylation and *O*-demethylation reactions led to the isoflavones pratensein, genistein, and orobol, which were rapidly further degraded. Dihydrogenistein and *p*-hydroxyphenylacetic were also identified as catabolites (Kraft and Barz, 1985). Biochanin A-7-*O*-glucoside was degraded leading to aglycone and pratensein. Biochanin A-7-*O*-glucoside-6″-*O*-malonate was very slowly degraded without subsequent accumulation of catabolites (Kraft and Barz, 1985). Removal of malonic acid from biochanin A-7-*O*-glucoside-6″-malonate by action of an esterase has been shown to occur with *Fusarium javanicum* (Schlieper et al., 1984).

The secondary spread of fungi that cause foliar disease results when sporulation occurs on the leaf surface and conidia are dispersed to previously uninfected tissue. Some fungi produce conidia in a water-soluble mucilage, and in this case, conidium dispersal depends on both water and wind since liquid water is typically involved in the movement of propagules away from the site of sporulation onto new, healthy tissues. Sporulation occurs within a zone of necrotic tissue that may contain high levels of phenols produced during lesion restriction. Toxic phenols may be exuded from the lesions and they may exist as normal components of the phylloplane. Analysis of the material that leached into water droplets on necrotic lesions showed the presence of significant levels of phenols within 1 h (Nicholson et al., 1989), whereas phenols could not be detected in leachate from green leaf tissue. The leachate from necrotic lesions contained *p*-coumaric and ferulic acids. These phenolic acids inhibited conidial germination *in vitro* (Nicholson et al., 1989). The phenols released into the water that carries conidia to new infection sites may inhibit conidium germination and germ tube elongation, resulting in suppression of disease spread (Nicholson et al., 1989). Conidia of *Colletotrichum graminicola*, the causal agent of corn anthracnose, are embedded within a mucilaginous matrix. The mucilage contains a proline-rich protein fraction capable of selectively binding to condensed tannins and other polyphenolic compounds (Nicholson et al., 1986). The phenols could inhibit conidium germination only when the mucilage was removed from conidia but not when mucilage was added back to the washed conidia (Nicholson et al., 1986). The mucilage contained a nonspecific esterase as well as a β-glucosidase (Ramadoss et al., 1985). The enzymes found in the mucilage may cleave the phenolic esters and glycosides, freeing the aglycones and making them more available for binding to the extracellular proline-rich proteins of the mucilage (Nicholson et al., 1986). As a consequence, their toxicity would be reduced. Thus, the proline-rich proteins of the spore mucilage may protect conidia from toxic phenols that accumulate in the water necessary for conidium dispersal and secondary spread of the fungus (Nicholson et al., 1989).

The flavonoid flavan-3-ol-epicatechin (epicatechin) has been shown to be the antifungal factor that contributes to the resistance of unripe avocado fruits to *Colletotrichum gloeosporioides* (Guetsky et al., 2005). Epicatechin inhibits lipoxygenase and the oxidative activity of lipoxygenase degrades the antifungal diene compounds found in the unripe fruits (Karni et al., 1989). Decline in epicatechin levels leads to increase in lipoxygenase activity, along with metabolism of the diene (Ardi et al., 1998). The avocado pathogen secretes laccase (*p*-diphenol-dioxygen oxidoreductase) in culture and in infected fruit tissues (Guetsky et al., 2005). Laccase degrades epicatechin and epicatechin levels decreased when *C. gloeosporioides* was grown in the presence of epicatechin in culture. Extracts of laccase enzyme obtained from decayed avocado fruit tissue and culture media fully metabolized the epicatechin substrate within 4 and 20 h, respectively (Guetsky et al., 2005). Inhibitors of fungal laccase, such as EDTA and thioglycolic acid, reduced *C. gloeosporioides* symptom development when applied to ripening susceptible fruits. Isolates of *C. gloeosporioides* with reduced laccase activity

and no capability to metabolize epicatechin showed reduced pathogenicity on ripening fruits (Guetsky et al., 2005). These results suggest that during pathogenesis the phenolics are degraded by fungal laccase and the fungal enzyme may be a pathogenicity factor.

7.16.2 Pathogen May Suppress Increased Synthesis of Phenolics in Plants

The pathogens may suppress increased synthesis of phenolics in susceptible tissues. Uninfected roots of *Eucalyptus calophylla* resistant to *Phytophthora cinnamomi* contained more than three times the amount of phenolic substances as those of *Eucalyptus marginata* (susceptible to the pathogen). A rapid increase in the concentration of total phenolics in roots of *E. calophylla* followed inoculation with the pathogen. The level increased up to 97% above control levels within 72 h after inoculation. In roots of *E. marginata*, there were only small increases in the concentration of total phenolics (Cahill and McComb, 1992). A correlation between accumulation of phenolic compounds and resistance in alfalfa stems inoculated with *Colletotrichum trifolii* has been reported (Baker et al., 1989). In resistant interactions, phenolics accumulated at high levels, whereas in susceptible interactions phenolic synthesis did not seem to be accelerated.

7.16.3 Pathogen May Suppress Phenol Biosynthetic Enzymes

PAL activity increased rapidly to a very high level in roots of *Eucalyptus calophylla*, a species resistant to *Phytophthora cinnamomi* after infection with the pathogen. However, in a susceptible species, *E. marginata*, the activity of PAL increased only slightly after inoculation with the fungus in the first 24 h. Subsequently, a rapid decline in the PAL activity was observed, and at 96 h after inoculation PAL activity declined even up to 77% of control levels (Cahill and McComb, 1992). The importance of suppression of PAL in reduction in phenolic synthesis was shown by using an inhibitor of PAL, aminooxyacetic acid (AOA). The increase in phenolic synthesis was suppressed when the resistant *E. calophylla* was treated with AOA (Cahill and McComb, 1992). AOA treatment made the resistant *E. calophylla* into a susceptible one (Cahill and McComb, 1992) and it suggests that suppression of phenolic synthesis leads to susceptibility.

7.16.4 Pathogen May Suppress Phenolic Metabolism by Its Suppressor Molecule

Pathogens are known to produce suppressors, which may suppress synthesis of phenolics. The culture broth of *Ascochyta rabiei*, the chickpea pathogen, showed inhibitory activity on isoflavone accumulation in chickpea cotyledons. The inhibitory activity was found in a glycoprotein fraction from the culture broth and this glycoprotein was called suppressor. The inhibitory activity of the suppressor was observed in the concomitant decrease in the accumulation of all phenolic constituents of chickpea (Table 7.8; Kessmann and Barz, 1986).

7.16.5 Pathogen May Suppress Phenolic Metabolism by Producing Toxins

Toxins produced by pathogens may also suppress phenolic biosynthesis in plants. When rice leaves were treated with toxin produced by *Helminthosporium oryzae* (Vidhyasekaran et al., 1986), phenolic content decreased and PAL activity was suppressed (Table 7.9; Vidhyasekaran et al., 1992). When *H. oryzae* was inoculated, phenolic content of rice leaves decreased at 24 h after inoculation. A decrease in PAL activity was observed on the third day. When an avirulent isolate that is not able to produce the toxin was inoculated on the rice leaves, there was no decrease in phenolic content or reduction in PAL activity. The results suggest that the toxin produced by the pathogen may suppress the phenolic metabolism of plants, resulting in severe incidence of the disease (Vidhyasekaran et al., 1992).

TABLE 7.8
Suppression of Phenolic Accumulation in Chickpea Cotyledons by the Suppressor of *Ascochyta rabiei*

	Isoflavonoids (nmol/g Fresh Weight)			
Treatment	Formononetin	Biochanin A	Formononetin 7-*O*-Glucoside-6″-Malonate	Biochanin 7-*O*-Glucoside-6″-Malonate
Control	37	104	140	57
Suppressor	30	78	45	27

Source: Adapted from Kessmann, H. and Barz, W., *J. Phytopathol.*, 117, 321, 1986.

7.16.6 Pathogen May Suppress Oxidation of Phenolics by Inhibiting Polyphenol Oxidase

Oxidized phenolics are more toxic than phenolics, and PPO is involved in oxidation of phenolics (Retig, 1974). Some pathogens may suppress oxidation of phenolics by inhibiting PPO. *Alternaria alternata* induces chlorotic symptoms in mung bean. Tentoxin is a cyclic tetrapeptide produced by the fungus and the toxin induces chlorosis (Duke et al., 1982). Mung bean plants showed high levels of PPO. PPO was absent from plastids of tentoxin-treated mung beans. Tentoxin reduced PPO activity by 90% in ivyleaf morning glory, a sensitive species. Tentoxin had no effect on PPO from soybean, a species that is insensitive to tentoxin (Vaughn and Duke, 1981). Tentoxin also inhibited PPO activity in *V. faba* (Vaughn and Duke, 1984).

7.16.7 Phenolics Are Fungitoxic but They May Not Accumulate to Fungitoxic Level during Pathogenesis in Some Plant–Pathogen Interactions

Phenolics may accumulate during infection but even the increased concentrations may not be sufficient to inhibit some pathogens. In an incompatible interaction there were large accumulations of the malonyl and neutral glucosides of daidzein and genistein at 24–36 h after

TABLE 7.9
Suppression of Phenolic Content and Phenylalanine Ammonia-Lyase Activity in Rice Leaves due to Treatment with *Helmintosporium oryzae* Toxin

Treatment	Phenolics (μg/g Fresh Weight)	Phenylalanine Ammonia-Lyase (nmol Cinnamic Acid Produced/g Fresh Weight)
Untreated control	1924	918
Toxin	865	597

Source: Adapted from Vidhyasekaran, P., Borromeo, E.S., and Mew, T.W., *Physiol. Mol. Plant Pathol.*, 41, 307, 1992.

inoculation with *Phytophthora sojae* in soybean leaves (Morris et al., 1991). However, no growth inhibition of *P. sojae* could be demonstrated on a medium that contained up to 200 μg mL^{-1} daidzein or formononetin or genistein (Morris et al., 1991). Constitutive levels of isoflavonoids and their glucosides both in leaves and hypocotyls of soybean were found to be too low to contribute to resistance based on their fungitoxicity (Graham, 1991; Morris et al., 1991).

7.17 SAPONINS AS PHYTOANTICIPINS

Saponins are high-molecular weight glycosides combining a sugar element and a steroid aglycone or triterpene molecule. Saponins can be divided into three major groups: triterpenoid saponins, steroid saponins, and steroidal alkaloid saponins (Mert-Türk, 2006). Avenacins are the major triterpenoid phytoanticipins detected in oat (Mert-Türk et al., 2005; Thomas et al., 2006), whereas α-tomatine is the major steroidal glycoalkaloid saponin detected in tomato (Morrissey and Osbourn, 1999).

Saponins have been implicated in disease resistance in some plants (Osbourn, 1996a,b). The saponin avenacin occurs in oat roots. Avenacin is a mixture of four major compounds (avenacins A-1, A-2, B-1, and B-2), which are all glycoconjugates containing β-linked glucosyl residues (Crombie et al., 1986a). Avenacin A-1 is the most abundant of the four in oat roots (Crombie and Crombie, 1986). Avenacin A-1 causes permeabilization in a sterol-dependent manner and it also affects membrane fluidity in fungal pathogens (Armah et al., 1999).

Avenacin A-1 has been shown to be involved in disease resistance. A diploid oat species, *Avena longiglumis*, has been shown to lack avenacin A-1, and this species was found to be highly susceptible to several fungal pathogens of oat (Mert-Türk, 2006). *A. longiglumis*, which lacked detectable levels of avenacin, was susceptible to infection even by the nonpathogen of oat *Gaeumannomyces graminis* var. *tritici* (Osbourn et al., 1994). Mutants of a avenacin-producing oat species, *Avena strigosa*, were generated and the saponin-deficient mutants were highly susceptible to various oat pathogens, unlike the wild species (Papadopoulou et al., 1999). The resistance of oats to nonpathogens has been attributed to the presence of avenacin. *Gaeumannomyces graminis* var. *avenae* is a pathogen of oats, whereas *G. graminis* var. *tritici* infects wheat and not oats. Avenacin inhibits the growth of *G. graminis* var. *tritici* in culture, whereas *G. graminis* var. *avenae* isolates are less sensitive to avenacin (Crombie et al., 1986b). Growth of all the *G. graminis* var. *tritici* isolates was completely inhibited at 5 μg avenacin A-1/mL of agar, whereas *G. graminis* var. *avenae* isolates were still able to grow in the presence of higher levels of avenacin A-1 (20–40 μg mL^{-1}) (Osbourn et al., 1991). These *G. graminis* var. *avenae* isolates deglucosylate avenacin A-1 to less-toxic derivatives by producing avenacinase with β-glucosidase activity. *G. graminis* var. *tritici* isolates produce less amount of avenacinase and hence they are unable to degrade avenacin. It is suggested that it is unable to infect oats because of its inability to degrade avenacin (Crombie et al., 1986b).

Although *G. graminis* var. *avenae* grew well in the presence of avenacin A-1, there was no relationship between avenacinase-producing ability of the isolates and their virulence (Osbourn et al., 1991). Although the isolate Gg 178 was the most pathogenic isolate on oats, it produced only less avenacinase. The isolate Hornby 61 is the least virulent; but it produced high amount of avenacinase. The results indicate that the ability to produce avenacinase may be an absolute requirement during early infection of oat roots (since var. *tritici* isolates lacking ability to produce avenacinase could not infect oat roots), but once within the root cortex, the subsequent extent of infection may depend on other factors. Avenacin is restricted to the outer cell layers of oat roots only (Osbourn et al., 1991).

Avenacinase activity was shown to reside in a single polypeptide species of 100 kDa. This protein was able to generate both the mono- and bis-deglucosylated forms of avenacin A-1 (Osbourn et al., 1991). Specific mutations have been made by transformation-mediated gene disruption procedures that eliminate the ability of *G. graminis* var. *avenae* to detoxify avenacin (VanEtten et al., 1995). The mutants lost their pathogenicity on oats (VanEtten et al., 1995). Both *G. graminis* var. *avenae* and *G. graminis* var. *tritici* can infect creeping bentgrass (*Agrostis stolonifera*). Avenacin could not be detected in any creeping bentgrass cultivars and hence avenacinase activity is not required for creeping bentgrass infection by *G. graminis* (Thomas et al., 2006). Other oat-infecting fungi, *Fusarium avenaceum* and *Phialophora* sp., degrade avenacin (Crombie et al., 1986b). These results suggest that avenacin degradation is important in pathogenesis of *G. graminis* and other pathogens of oats.

α-Tomatine is a steroidal glycoalkaloid found in tomato. It belongs to the group of saponins. It is highly toxic to fungi. The mechanism of action of α-tomatine may be its ability to complex with sterols in fungal membranes, resulting in loss of membrane integrity (Steel and Drysdale, 1988). It is reported that α-tomatine is present in tomato leaves in the concentration around 1 mM, which is sufficient to inhibit the growth of many pathogens of tomato (Mert-Türk, 2006). Hence this molecule could protect the tomato leaves from fungal pathogens. However, tomato pathogens such as *Alternaria solani*, *Septoria lycopersici*, *Botrytis cinerea*, *Verticillium albo-atrum*, *Fusarium oxysporum* f. sp. *lycopersici*, and *Phytophthora infestans* could produce a glucosidase, called α-tomatinase that detoxifies α-tomatine (Morrissey and Osbourn, 1999). The importance of α-tomatinase in fungal pathogenesis was demonstrated by producing transgenic fungal pathogens. The biotrophic tomato pathogen, *Cladosporium fulvum*, is sensitive to α-tomatine, and is unable to detoxify this saponin. The cDNA encoding tomatinase from the necrotroph *Septoria lycopersici* was transferred to *C. fulvum* (Melton et al., 1998). The tomatinase producing *C. fulvum* transformants showed increased sporulation on cotyledons of susceptible tomato lines. They also caused more extensive infection of seedlings of tomato resistant lines (Melton et al., 1998). α-Tomatinases hydrolyze sugar molecules from the tetrasaccharide group attached to the C-3 carbon of α-tomatinase. The deglycosylated products are less inhibitory to fungal growth. It suggests that α-tomatine degradation may be an important strategy for successful infection of tomato plants. Even nonpathogenic *Fusarium oxysporum* isolates, which are capable of producing tomatinase, have been shown to colonize roots and stems of tomato without producing disease symptoms (Ito et al., 2005). It suggests that tomatinase production is essential to colonize tomato tissues. It is also reported that tomatinase may suppress the plant defense mechanisms (Martin-Hernandez et al., 2000).

Fungal pathogens of tomato are more resistant to α-tomatine *in vitro* than are fungi that do not infect tomato (Steel and Drysdale, 1988; Suleman et al., 1996). Minimum molar concentration of tomatine that completely inhibits mycelial growth of pathogens of tomato such as *S. lycopersici*, *A. solani*, *P. infestans*, *F. oxysporum* f. sp. *lycopersici*, and *V. albo-atrum* ranges from 0.01 to 0.85, whereas concentration of α-tomatine required to inhibit mycelial growth of nonpathogens of tomato such as *Cercospora beticola*, *Alternaria tenuis*, *Septoria linicola*, *F. oxysporum* f. sp. *conglutinans*, and *Exerohilum turcicum* ranged from 0.00013 to 0.0020 M (Arneson and Durbin, 1968).

The ability of fungal pathogens to colonize tomato tissue has been shown to be due to their intrinsic resistance to α-tomatine at the membrane level (Steel and Drysdale, 1988). Sterol-deficient mutants of a tomato-attacking isolate of *Fusarium solani* showed increased resistance to α-tomatine and were able to infect green tomato fruits, which contained over 20 times than the red fruits. The wild-type *F. solani* isolate was pathogenic only to ripe fruits. The results suggest that the membrane composition of the pathogen is important in determining the ability of fungi to infect tomato (Defago et al., 1983).

7.18 GLUCOSINOLATES AS PHYTOANTICIPINS

7.18.1 Biosynthesis of Glucosinolates

Glucosinolates are sulfur-containing glycosides and they are commonly found in the family Cruciferae (Porter et al., 1991). They comprise a common glycoside moiety and a variable side chain (Mithen, 1992). Three major classes of glucosinolates have been recognized in *Brassica*. The first one possesses side chains that may have aliphatic alkenyl or hydroxyalkenyl groups. The second one has side chains that contain an indolyl group and the third one contains aralkyl side chains (Mithen, 1992).

The major glucosinolates found in *Brassica* species are sinigrin, gluconapin, glucobrassicanapin, progoitrin, hapoleiferin, glucoiberverin, glucoraphanin, hydroxyglucobrassicin, glucobrassicin, methoxyglucobrassicin, neoglucobrassicin, gluconasturtium, and glucotropaeolum (Mithen, 1992; Mithen and Magrath, 1992). The content of individual glucosinolates varies widely between different *Brassica* plants. *Brassica oleracea* and *Brassica cretica* subsp. *cretica* contain more of gluconapin, whereas *Brassica rupestris* does not contain gluconapin and contains only glucoiberin. *B. oleracea* var. *capitate* contains mostly glucobrassicin and sinigrin and methoxyglucobrassicin are also present in appreciable amounts in it. *Brassica depranensis* contains glucoiberverin and glucoiberin, whereas *Brassica macrocarpa*, *Brassica insularis*, and *B. cretica* subsp. *laconica* contain more of sinigrin (Mithen et al., 1987b). *Brassica rapa* contains gluconapin and glucobrassicanapin (Mithen et al., 1987b).

Glucosinolates are derived from amino acids along a biosynthetic pathway that consists of two parts. Initially, there is a chain elongation phase that is followed by the biosynthesis of the specific part of the glusinolate molecule (Underhill et al., 1973). The side chains are derived from amino acids like methionine, tryptophan, and phenylalanine (Underhill et al., 1973). The indolyl glucosinolates are derived from tryptophan (Mithen and Magrath, 1992). The glycone moiety is developed through a complex series of general nitrogenous and sulfur-containing intermediates (Underhill et al., 1973).

Glucosinolates are hydrolyzed by an endogenous enzyme called myrosinase (thioglucoside glucohydrolase). The hydrolysis products include nitriles, isothiocyanates, oxazolidinethiones, and thiocyanates (Mithen, 1992). The enzyme at low pH (3 to 4) hydrolyzes glucobrassicin to 3-indoleacetonitrile (IAN), glucose, sulfate, hydrogen sulfide, and sulfur. Isothiocyanates are derived from the hydrolysis of alkenyl glucosinolates such as sinigrin and gluconapin. Thiocyanate ion is derived from indole glucosinolate hydrolysis, whereas vinyloxazolidine-2-thione is derived from the hydrolysis of the hydroxyalkenyl glucosinolate progoitrin (Mithen et al., 1987a). Indolyl glucosinolates may produce nitrile derivatives also. Under acidic conditions, the predominant hydrolytic products from alkenyl and hydroxyalkenyl glucosinolates are nitriles rather than isothiocyanates (Mithen, 1992). Aldoxime is an intermediate in the pathway in biosynthesis of aliphatic glucosinolates as well as in the biosynthesis of cyanogenic glycosides (Underhill et al., 1973).

Myrosinase, the enzyme that hydrolyzes glucosinolates, occurs in multiple forms. They are all glycoproteins with 2–4 subunits with a molecular weight between 125 and 153 kDa (Mithen, 1992). Myrosinase may be associated within a group of histochemically distinct cells known as myrosin cells (Thagstad et al., 1991). The myrosinase is associated with the tonoplast-like membrane that surrounds the myrosin grains within the myrosin cells. Iverson et al. (1979) have demonstrated myrosinase activity within tissues that lack myrosin cells. Hence myrosinase and myrosin grains may occur within many cells. Myrosinase may be associated with membrane, whereas glucosinolates may be located within the vacuole (Luthy and Matile, 1984). Ascorbic acid promotes the activity of myrosinase (Mithen, 1992).

7.18.2 Toxicity of Glucosinolates to Fungal Pathogens

Glucosinolates have been shown to be involved in disease resistance (Brader et al., 2006). Glucosinolates are not toxic to fungal pathogens, but the hydrolysis products of glucosinolates are fungitoxic (Doughty et al., 1991). 1-Methoxyglucobrassicin and sinigrin did not inhibit growth of the oilseed rape pathogen *Leptosphaeria maculans in vitro* (Mithen et al., 1986). However, all glucosinolates (sinigrin, gluconapin, glucobrassicin, and 1-methoxyglucobrassicin) reduced fungal growth when added to cultures containing myrosinase. Progoitrin was not fungitoxic even in the presence of myrosinase. Sinigrin in the presence of myrosinase had the most pronounced effect. Volatile products from sinigrin and gluconapin were strongly inhibitory to *L. maculans* and no growth occurred on petri plates exposed to these products (Mithen et al., 1986). The results suggest that hydrolysis products of glucosinolates are fungitoxic rather than glucosinolates themselves and toxicity of these hydrolysis products varies among glucosinolates.

Mithen et al. (1986) showed that hydrolysis products of glucobrassicin are highly fungitoxic. Further antifungal activity of the hydrolysis products was attributable to the products possessing an indole nucleus and not to the thiocyanate ion. Of the indole compounds, indolyl-3-carbinol had the greatest activity (Mithen et al., 1986).

The volatile isothiocyanate products such as allyl-(or 2-propenyl-) and 3-butenyl isothiocyanates, which are formed from the glucosinolates, sinigrin and gluconapin, respectively, have toxic activity against many fungi (Drobnica et al., 1967; Hartill, 1978). Isothiocyanates are toxic toward *Peronospora parasitica* (Greenhalgh and Mitchell, 1976) and *Mycosphaerella brassicae* (Hartill and Sutton, 1980). The germination of resting spores of *Plasmodiophora brassicae* was severely inhibited by allyl and β-phenethyl-isothiocyanate concentrations as low as 5 μg/mL. Davis (1964) showed that β-phenethyl isothiocyanate inhibited growth of *Fusarium oxysporum* at a concentration of 30 μg/mL.

Glucosinolates may serve as source for synthesis of phytoalexins in oilseed rape leaves (Conn et al., 1988). The phytoalexin cyclobrassinin accumulates in oilseed rape leaves because of infection with *Alternaria brassicae*. This compound is derived from tryptophan and indolyl glucosinolates are also derived from tryptophan. Hence it has been suggested that the phytoalexin is derived from indolyl glucosinolates (Hanley and Parsley, 1990). The phytoalexin is inhibitory to pathogens.

7.18.3 How Does the Pathogen Overcome Toxicity of Glucosinolates?

7.18.3.1 Concentration of Glucosinolates May Be Less in Susceptible Tissues

The plants susceptible to pathogens have been shown to contain less glucosinolates. Genotypes in both *Brassica oleracea* and *B. rapa* susceptible to *L. maculans* show lesser glucosinolate content than the resistant genotypes (Table 7.10; Mithen et al., 1987a).

High levels of resistance to *Peronospora parasitica* have been found in wild members of the *B. oleracea* group (Greenhalgh and Mitchell, 1976). Wild populations contain higher total glucosinolate content than cultivars (Mithen et al., 1987b). The cabbage variety susceptible to *Peronospora parasitica* contained lesser concentrations of allyl isothiocyanate than the susceptible cultivars (Greenhalgh and Mitchell, 1976).

7.18.3.2 Glucosinolates May Not Be Involved in Disease Resistance Unless the Tissue Is Damaged

Glucosinolates, unless hydrolyzed, are not toxic to pathogens. Hydrolysis requires high activation of myrosinases in plants. Similar activation may not occur unless the leaf tissue is highly damaged like by insect injury (Mithen and Magrath, 1992). Volatile derivatives of

TABLE 7.10
Glucosinolate Content of Genotypes of *Brassica oleracea* and *B. rapa*, Susceptible or Resistant to *Leptosphaeria maculans*

Brassica Species	Genotypes	Total Glucosinolates (μmol/g Fresh Weight)
B. oleracea	Susceptible	1.16
	Resistant	9.42
B. rapa	Susceptible	0.92
	Resistant	7.94

Source: Adapted from Mithen, R.F., Lewis, B.G., Heaney, R.K., and Fenwick, G.R., *Trans. Brit. Mycol. Soc.*, 88, 525, 1987a.

the glucosinolate sinigrin are produced on mechanical maceration of *Brassica* leaf tissue. Walker and Stahmann (1955) concluded that glucosinolates may not be important in the resistance of crucifers to *Plasmodiophora brassicae* since hydrolysis of a glucoside occurs only when host cells are severely damaged, but there was intimate contact of the plasmodium with the cytoplasm of the invaded cell, indicating less or no damage of host cells. Although it is known that fungi can also produce myrosinase, it has not yet been established that enough myrosinase is produced in infected leaves to release sufficient fungitoxic hydrolytic products (Mithen, 1992).

In many host–pathogen interactions, no relationship between glucosinolates and disease resistance has been observed. The rape cultivar Bienvenu contains high glucosinolate content, whereas the rape cultivar Cobra contains very low glucosinolate content. The cultivar Cobra had a reduced activity of myrosinase also (Porter et al., 1990), but both the cultivars were equally susceptible to *Alternaria brassicae* (Doughty et al., 1991). Inoculation with *A. brassicae* increased the concentration of glucosinolates in rape leaves by 4- to 10-fold, with the greatest increases in aromatic and indolyl glucosinolates. Both the cultivars Cobra and Bienvenu responded similarly to infection (Doughty et al., 1991).

Synthetic lines of *Brassica napus* were derived by combining the genomes of *Brassica atlantica* and *B. oleracea* var. *alboglabra*, which were, respectively, resistant and susceptible to *Leptosphaeria maculans* with a susceptible line of *B. rapa* (Mithen and Magrath, 1992). Resistance was expressed in the synthetic lines containing the genome of *B. atlantica*. The leaves of the synthetic *Brassica napus* line 235, derived from *B. atlantica*, were highly resistant to *L. maculans*. The synthetic *B. napus* line 235 contained predominantly glucobrassicanapin and gluconapin, with smaller amounts of sinigrin and low levels of indolyl glucosinolates. The synthetic line 233, which is susceptible to *L. maculans*, contained smaller amounts of alkenyl glucosinolates than line 235 but higher levels of the indolyl glucosinolate glucobrassicin. The F_1 hybrid between these lines contained intermediate levels of both alkenyl and indolyl glucosinolates (Mithen and Magrath, 1992). Due to infection, glucosinolate content was very much reduced in both susceptible and resistant lines. Those from the resistant synthetic *B. napus* line 235 contained predominantly alkenyl glucosinolates, whereas those from the susceptible *B. napus* line 233 had predominantly indolyl glucosinolates with only trace levels of alkenyl glucosinolates. Within the F_2 population of a cross between the synthetic lines, there was a segregation of both disease resistance and glucosinolates, but there was no correlation between the levels of alkenyl or indolyl glucosinolates and disease resistance (Mithen and Magrath, 1992). Thus it appears that resistance genes are not related to the glucosinolate profile.

7.19 CYANOGENIC GLUCOSIDES

Some of the plants contain hydrocyanic acid (Vidhyasekaran, 1988). Young sorghum plants contain abundant hydrocyanic acid (Vidhyasekaran et al., 1971). Sudan grass (*Sorghum vulgare* var. *sudanensis*), flax, and birdsfoot trefoil are the other important cyanogenic plants. Hydrocyanic acid is liberated from nitrile glucosides and it is released only when the cells are injured (Vidhyasekaran et al., 1971). Cyanide is toxic to most organisms because it inhibits respiration in mitochondria (Fry and Evans, 1977). It is toxic to fungi (Vidhyasekaran, 1988). The growth of *Fusarium oxysporum* f. sp. *lini*, the flax wilt fungus, was strongly inhibited *in vitro* at 135 μg/mL hydrocyanic acid (Trione, 1960). Hydrocyanic acid was toxic to germination of uredospores of *Puccinia purpurea*, the sorghum pathogen, even at 15 μg/mL (Vidhyasekaran et al., 1971).

It appears that pathogens of the cyanogenic plants are able to tolerate hydrocyanogenic acid. *Stemphylium loti* is a pathogen of birdsfoot trefoil (*Lotus corniculata*), whereas *S. botryosum*, *S. sarcinaeforme*, and *S. consortiale* are nonpathogens of trefoil. *S. loti* is more tolerant to hydrocyanic acid than the nonpathogens. An enzyme has been suggested to be the cause for tolerance of *S. loti* to hydrocyanic acid (Fry and Millar, 1969). Fungi that are capable of causing diseases in cyanogenic plants are known to produce cyanide hydratase (Wang et al., 1992; Cluness et al., 1993). Cyanide hydratase is a substrate-inducible fungal enzyme capable of converting cyanide to formamide (Fry and Evans, 1977). Cyanide hydratase of the sorghum pathogen, *Gloeocercospora sorghi*, has been characterized. It was cyanide inducible and functioned as an aggregated protein consisting of 45 kDa polypeptides (Wang et al., 1992). The corresponding gene has been cloned (Wang and VanEtten, 1992). Several studies have indicated that degradation of the fungitoxic hydrocyanic acid is important in pathogenesis of fungal pathogens of cyanogenic plants.

7.20 DIENES

Dienes are antifungal substances found in plants. Avocado fruits contain 1-acetoxy-2-hydroxy-4-*oxo*-heneicosa-12,15-diene, which has been suggested as the basis of resistance to fruit decay (Wang et al., 2004a,b). The concentration of the preformed diene is enhanced when the fruit is inoculated with nonpathogenic strains of *Colletotrichum gloeosporioides* or *C. magna* (Prusky et al., 1994). When avocado fruits were exposed to 30% carbon dioxide for 24 h, level of the antifungal compound increased to a greater extent. This increase in diene level resulted in delay in symptom development because of *C. gloeosporioides* infection (Prusky et al., 1991; Prusky and Keen, 1993). Accumulation of the diene was accompanied by the transcriptional activation of *avfad9* encoding a Δ^9 stearoyl-ACP desaturase and *avfadI2-3* encoding a Δ^{12} fatty acid desaturase (Madi et al., 2003; Wang et al., 2004a). The diene molecule is longer than its precursor, linoleic acid, and the elongation of the linoleic acid is an important step in biosynthesis of the diene (Wang et al., 2004a). Enhanced expression of *avfae1* encoding a long-chain fatty acid elongase is involved in biosynthesis of the diene (Wang et al., 2004a). Pathogens could not infect avocado fruits when diene content is more, but diene content decreases to subfungitoxic concentrations during fruit ripening, permitting the development of disease symptoms caused by *C. gloeosporioides* (Prusky et al., 1982).

7.21 CONCLUSION

Several antifungal secondary metabolites have been detected in plants. Some of these metabolites are synthesized de novo in response to infection (phytoalexins), whereas others are preformed infectional inhibitors (phytoanticipins). Transcriptional activation of enzymes involved in biosynthesis of phytoalexins has been observed within a few minutes of

recognition of pathogen invasion. The phytoalexins may be synthesized by living cells undergoing attack by pathogen and secreted from these cells toward the site of infection. The secreted phytoalexins are inhibitory to fungal spore germination and hyphal growth. They may also suppress toxin production by pathogens. However, potential pathogens may be able to detoxify the phytoalexins of their host. They produce specialized enzymes to degrade these phytoalexins. These enzymes appear to be pathogenicity factors and are shown to be essential for fungal pathogenesis.

Induction of phytoalexin synthesis is delayed in susceptible interactions when compared with that in resistant interactions. Probably, the phytoalexins would accumulate at a time when the pathogen had already invaded the host tissues. This delay may be due to delayed release of elicitors from the fungal cell. Even the amount of release of elicitors may be less in compatible interactions, and induction of phytoalexins is generally dependent on the dosage of elicitors. The amount of phytoalexins produced may not be sufficient enough to inhibit pathogen growth in susceptible interaction. Suppressors of phytoalexin synthesis have been detected in some virulent pathogens. Spore germination fluids of some pathogens contain both elicitors and suppressors, and their interaction may determine susceptibility or resistance.

Each host plant produces several phytoalexins, sometimes even structurally very much different. All these phytoalexins may not be equally fungitoxic and it has been shown that some less-toxic phytoalexins alone accumulate in some susceptible interactions. Some phytoalexins may not have any role in the fungal pathogenesis.

Several phytoanticipins have been detected in plants. Several phenolics act as phytoanticipins, and most of them are highly toxic to fungal pathogens. Potential pathogens degrade these phenolics into nontoxic or less-toxic ones. Specific phenolics-degrading enzymes have been identified in spore germination fluid. Pathogens may suppress increased synthesis of phenolics in susceptible tissues, probably by suppressing phenol biosynthetic enzymes. Suppressors may be produced by pathogens, and they may suppress the accumulation of phenolics in the infection zone. Toxins produced by pathogens may also suppress accumulation of these phenolics. Some phenolics may not accumulate to fungitoxic level at the infection site.

Some saponins have been shown to be involved in suppressing fungal disease development. Avenacins in oats and tomatine in tomato are the important saponins involved in suppressing fungal disease development. However, virulent pathogens produce enzymes avenacinase and tomatinase to degrade these saponins. These enzymes are important pathogenicity factors of fungal pathogens affecting oats and tomato.

Glucosinolates play an important role in suppressing fungal pathogenesis in cruciferous plants. The hydrolysis products of glucosinolates are highly fungitoxic. Unless the tissue is damaged, glucosinolates may not be hydrolyzed and become toxic. Some pathogens produce myrosinase to hydrolyze the glucosinolates. The exact role of these glucosinolates in fungal pathogenesis is not known. Dienes and cyanogenic glucosides are also inhibitory to fungal pathogens and their role in fungal pathogenesis has been discussed.

Several hundreds of secondary metabolites have been described in plants and most of them are highly toxic to fungi. However, virulent pathogens have evolved their own mechanism to detoxify them and cause disease. Knowledge on these detoxifying mechanisms may help to evolve novel methods of disease management.

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8 Toxins in Disease Symptom Development

8.1 INTRODUCTION

Necrotrophic fungal pathogens are known to produce host-specific/host-selective and host-nonspecific phytotoxins. *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat (Ptr ToxA toxin) (Martinez et al., 2004; Manning et al., 2004; Manning and Ciuffetti, 2005; Sarma et al., 2005), *Pyrenophora teres*, the net-blotch pathogen of barley (aspergillomarasmine) (Friis et al., 1991), *Mycosphaerella zeae-maydis*, the corn yellow leaf blight pathogen (PM-toxin) (Yun et al., 1998), *Cochliobolus victoriae*, the oat leaf blight pathogen (victorin) (Churchill et al., 1995), and *Cochliobolus carbonum*, the corn leaf blight pathogen (HC-toxin) (Pitkin et al., 2000) are known to produce host-specific toxins. *Helminthosporium maydis* T strain, the maize pathogen, produces HMT-toxin, a host-specific one (Turgeon et al., 1995). *Helminthosporium oryzae*, the rice brown spot pathogen, produces a host-specific toxin (Vidhyasekaran et al., 1986). *H. oryzae* produces several nonspecific toxins including ophiobolin A, 6-epiophibolin A, anhydroophiobolin A, 6-epianhydroophiobolin A, and ophiobolin 1 (Xiao et al., 1991). *Bipolaris zeicola*, the pathogen of maize and rice, produces BZR-toxin (Xiao et al., 1992). *Helminthosporium sacchari*, the sugarcane pathogen, produces a host-specific toxin (HS-toxin) (Livingston and Scheffer, 1984). *Exserohilum turcicum*, the corn leaf blight pathogen, produces a peptide toxin (Bashan et al., 1995).

Several *Alternaria alternata* pathotypes have been shown to produce host-specific toxins and these include Japanese pear pathotype, which causes black spots in pear (AK-toxin); apple pathotype, the apple *Alternaria* blotch pathogen (AM-toxin); tobacco pathotype, the tobacco brown spot pathogen (AT-toxin); tangerine pathotype, the leaf and fruit spot pathogen of tangerines (*Citrus reticulata*) (ACT-toxin); rough lemon (*Citrus limon*) pathotype, causing brown spot in lemon (ACR-toxin); strawberry pathotype, causing *Alternaria* black spot of strawberry (AF-toxin); and tomato pathotype, the pathogen causing *Alternaria* stem canker in tomato (AAL-toxin) (Kohmoto and Otani, 1991; Tanaka et al., 1999; Brandwagt et al., 2000; Johnson et al., 2000; Tanaka and Tsuge, 2000; Spassieva et al., 2002). *A. alternata* pathotype infecting knapweed (*Centaurea maculosa*) produces a host-specific toxin, maculosin, which causes chlorotic spots developing into black necrotic lesions on the leaves of knapweed (Park et al., 1994). *Alternaria panax*, the causal agent of *Alternaria* blight of American ginseng, produces a host-specific toxin, AP-toxin (Quayyum et al., 2003). Germinating spores of *Alternaria brassicicola*, the causal agent of black leaf spot in *Brassica* spp., produce a host-specific toxin, AB-toxin (Otani et al., 1998). *Alternaria brassicae*, another species pathogenic to *Brassica* species, produces a cyclic peptide host-specific toxin, destruxin B (Bains and Tewari, 1987).

Several other *Alternaria* spp. produce nonspecific toxins. *Alternaria macrospora*, the cotton pathogen, produces a nonspecific toxin (Krishnamohan and Vidhyasekaran, 1988).

Alternaria helianthi, the sunflower pathogen produces several phytotoxins, radicinin, radianthin, deoxyradicinol, and 3-epideoxyradicinol (Tal et al., 1985). *Alternaria chrysanthemi*, the pathogen of chrysanthemum (*Chrysanthemum morifolium*), produces radicinin (Tal et al., 1985). *Alternaria zinniae*, the pathogen of zinnia, produces a nonspecific toxin, zinniol (Robeson and Strobel, 1984). *Alternaria carthami*, the safflower (*Carthamus tinctorius*) pathogen, produces three toxins, brefeldin A, zinniol, and 7-dehydrobrefeldin A (Kneusel et al., 1994).

Magnaporthe grisea, the rice blast pathogen, produces several toxins, piricularin, picolinic acid, pyriculol, and tenuazonic acid (Umetsu et al., 1972; Zhang et al., 2004). The rice sheath blight pathogen *Rhizoctonia solani* produces a host-specific toxin (Vidhyasekaran et al., 1997; Paranidharan et al., 2005). *Rhynchosporium secalis*, the leaf scald pathogen of barley, produces three phytotoxic peptides (Wevelsiep et al., 1991). *Ascochyta rabiei*, the causal agent of blight of chickpea produces three toxins, solanapyrones A, B, and C (Bahti and Strange, 2004). Large number of *Cercospora* spp. produce a toxin, designated cercosporin (Melouk and Schuh, 1987; Jenns et al., 1989). *Cercospora beticola*, the causal agent of leaf spot disease of sugar beet (*Beta vulgaris*), produces cercosporin and a family of other toxins named beticolins. Beticolins form a family of 20 nonpeptidic compounds, named beticolin 0 to 19 (Goudet et al., 1998, 2000). *Ramularia collo-cygni*, the fungus causing *Ramularia* leaf spot disease on barley, produces rubellin B, C, and D (Heiser et al., 2004).

Besides the foliar pathogens, the pathogens causing wilt diseases also produce toxins. Various *Fusarium* spp. produce a nonspecific toxin, fusaric acid (FA) (Shahin and Spivey, 1986). *Fusarium amygdali*, the causal agent of peach and almond wilts, produces a toxin, designated fusicoccin (Marre, 1979). *Verticillium dahliae*, the cotton *Verticillium* wilt pathogen, produces an 18.5 kDa protein toxin (Palmer et al., 2005). *Ceratocystis ulmi*, the Dutch elm (*Ulmus americana*) disease pathogen, produces a toxin called cerato-ulmin (Pijut et al., 1990; Sorbo et al., 2000). Pathogens causing root and stem rots such as *Sclerotium rolfsii* (Bateman and Beer, 1965), *Sclerotinia sclerotiorum* (Godoy et al., 1990), and *Sclerotium cepivorum* (Stone and Armentrout, 1985) produce oxalic acid, which is considered as a toxin. *Fusarium culmorum* attacks wheat earheads and it produces trichothecene derivative toxins, deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol (Kang and Buchenauer, 1999).

Most of the purified toxins have been shown to reproduce the typical symptoms of the diseases caused by the toxin-producing pathogens. Vidhyasekaran et al. (1997) have shown that the toxin produced by *Rhizooctonia solani* reproduced all symptoms of the rice sheath blight disease. *H. oryzae* toxin reproduces brown spot disease symptoms in rice (Vidhyasekaran et al., 1986). The symptoms of Northern corn leaf blight are characterized by necrotic lesions and the toxin produced by the pathogen *Exserohilum turcicum* reproduced these symptoms (Bashan et al., 1995). *A. panax* causes *Alternaria* blight in leaves of American ginseng (*Panax quinquefolius*) and produces characteristic water-soaked necrotic spots. The AP-toxin produced by it reproduces the same symptoms (Quayyum et al., 2003). The toxin (cerato-ulmin) produced by *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*, the causal agents of Dutch elm disease, reproduced typical Dutch elm disease symptoms (Sorbo et al., 2000). Injections of 1.5 μg Pt60 toxin produced by *Phoma tracheiphila*, the causal agent of mal secco disease of citrus, into the mesophyll of sour orange or lemon leaves induced chlorosis and necrosis of tissues. These symptoms closely resembled those caused by foliar inoculation with the pathogen (Fogliano et al., 1998). Cercosporin produced by *Cercospora zeae-maydis*, the maize gray leaf spot pathogen, induces typical disease symptoms in maize (Shim and Dunkle, 2003). The protein toxin produced by *Verticillium dahliae*, the causal agent of *Verticillium* wilt disease in seedlings of cotton, evokes the typical symptoms of the disease (Palmer et al., 2005). In this chapter, the role of toxins in disease development is discussed.

8.2 IMPORTANCE OF TOXINS IN DISEASE DEVELOPMENT

Importance of toxins in fungal disease development has been demonstrated by different ways. Toxin-less mutants of the toxin-producing pathogens have been developed and these mutants failed to cause disease symptoms on susceptible cultivars. *Mycosphaerella zeae-maydis*, the corn yellow leaf blight pathogen, produces PM-toxin and mutants lacking the PM-toxin were developed (Yun et al., 1998). All toxin minus mutants lost the ability to cause disease on corn (Yun et al., 1998). Victorin Tox^{-} mutants of *Cochliobolus victoriae* were generated and these mutants lost their ability to cause disease (Churchill et al., 1995). Toxin minus mutants of *Alternaria alternata* apple pathotype failed to cause disease on apple (Johnson et al., 2000).

Importance of toxins has been studied using transformation techniques. When a toxin-deficient strain of *Pyrenophora tritici-repentis* was transformed with *ToxA* gene encoding Ptr ToxA toxin isolated from virulent *P. tritici-repentis* strain, it became a pathogenic toxin-producing strain (Ciuffetti et al., 1997). The gene encoding cerato-ulmin, *cu*, has been cloned from the Dutch elm pathogen *Ophiostoma novo-ulmi* and the gene was transferred to a nonpathogenic saprophyte *Ophiostoma quercus*. The saprophyte became pathogenic by insertion of the toxin gene. The transformant produced cerato-ulmin and caused typical disease symptoms (Sorbo et al., 2000). A gene *CZK3* encoding a MAP kinase kinase kinase (MAPKKK) is involved in cercosporin production by *Cercospora zeae-maydis*. The mutant disrupted in *CZK3* gene was unable to produce the toxin. Complementation of disruptants with the *CZK3* restored the ability to produce cercosporin and cause typical disease symptoms in maize (Shim and Dunkle, 2003).

Addition of toxins to avirulent strains of the pathogen or even to saprophytes may make them pathogenic and disease symptoms may develop. When highly virulent isolate of *H. oryzae*, the rice brown spot pathogen, was inoculated on rice leaves, the spores germinated, formed appressoria, and penetrated the epidermal layers at 12 h after inoculation (Vidhyasekaran et al., 1986). Extensive inter- and intracellular mycelial growth was observed in mesophyll tissue of leaves at 48 h after inoculation. When an avirulent isolate of *H. oryzae* was inoculated on rice leaves, the isolate did not grow beyond the infection peg. The virulent isolate induced typical brown spot symptoms with yellow halo at 48 h, whereas the nonpathogenic isolate did not produce any visible symptoms. When spore suspensions of the avirulent isolate were prepared in *H. oryzae* toxin and inoculated on rice leaves, extensive inter- and intracellular mycelial growth was observed at 36 h. The growth rate of the mycelium of the avirulent isolate in the presence of the toxin was similar to that of the virulent isolate (Table 8.1; Vidhyasekaran et al., 1986) and typical brown spot

TABLE 8.1
Effect of Virulent *Helminthosporium oryzae* Isolate on Development of Nonpathogenic *H. oryzae* Isolate within Rice Leaf Tissues

Treatment	Extent of Fungal Growth Inside Leaf Tissue
Virulent isolate	+++
Nonpathogenic isolate	−
Toxin + nonpathogenic isolate	+++

Source: Adapted from Vidhyasekaran, P., Borromeo, E.S., and Mew, T.W., *Phytopathology*, 76, 261, 1986.

Note: − No mycelial growth; +++ abundant inter- and intracellular mycelial growth.

symptoms also developed in avirulent isolate inoculated leaves in the presence of toxin (Vidhyasekaran et al., 1992).

In a similar study, it was observed that when a small amount of HV-toxin produced by *Cochliobolus victoriae* was added, the nonpathogenic mutant of *C. victoriae*, which did not produce toxin, developed throughout susceptible leaves just as rapidly as did the pathogenic *C. victoriae* (Yoder, 1972). Essentially, the same results were obtained in a study of colonization of corn leaves by avirulent isolate of *Cochliobolus carbonum* in the presence of HC-toxin (Comstock and Scheffer, 1973). When avirulent spores of *Alternaria alternata* or *Cochliobolus heterostrophus* were inoculated on leaves of Tms-cytoplasm maize along with a trace of HM-toxin produced by *C. maydis* T strain, the rate of formation of infection hyphae of the avirulent pathogens greatly increased and necrosis developed (Kohmoto et al., 1989).

H. oryzae, the brown spot pathogen of rice, produces another toxin, ophiobolin A. When spore germination fluids of *H. oryzae* containing the toxin were applied along with spores of *A. alternata*, a nonpathogen of rice, the nonpathogen caused a large number of disease lesions on rice leaves (Xiao et al., 1989). When spores of *A. alternata* were suspended in ophiobolin A solution, the spores germinated well and formed appressoria and infection pegs on the rice leaf sheath surface. Extensive inter- and intracellular hyphal growth was observed only in the sheath tissues that had been inoculated in the presence of ophiobolin A or spore germination fluids containing the toxin. The spores without ophiobolin A or spore germination fluids could not grow beyond the infection peg and did not grow inside the cell (Xiao et al., 1991). Ophiobolin A at 3 μg/mL induced susceptibility of rice leaf tissues to *A. alternata* (Xiao et al., 1991).

The toxin produced by *Magnaporthe grisea* induced susceptibility to infection by nonpathogenic *A. alternata* on rice leaves (Fujita et al., 1994). When spores of the nonpathogenic species *A. alternata* were combined with the spore germination fluids of the pathogen *Alternaria brassicicola* containing the AB-toxin, the spores of the nonpathogen could invade *Brassica* leaves, just as the pathogen did (Otani et al., 1998). When spores of *A. alternata*, a nonpathogen of rice plants, were inoculated together with BZR-toxin produced by *B. zeicola* (syn. *Helminthosporium carbonum*) race 3, the pathogen of rice and maize, the spores of the nonpathogen successfully penetrated rice tissues. Typical invasion and fungus development also occurred in maize tissues when BZR-toxin was added to spore drops of *Helminthosporium victoriae*, the oat pathogen, which alone could not penetrate into maize tissues (Xiao et al., 1992). All these studies indicate that the toxins produced by pathogens aid in the development of fungal growth and disease symptom development.

8.3 TOXINS SUPPRESS HOST-DEFENSE MECHANISMS

Defense mechanisms are activated in both susceptible and resistant interactions. For successful colonization of pathogens, the defense mechanisms have to be suppressed. Toxins appear to play an important role in suppressing defense mechanisms of the host plants. *Ceratocystis ulmi*, the Dutch elm pathogen produces a toxin, cerato-ulmin. The cytoplasm of the resistant variety responds to the fungal invasion by depositing phenolic material, but in the susceptible variety the toxin suppressed the phenolic deposition (Pijut et al., 1990). HV-toxin, produced by *H. victoriae*, reduces the accumulation of the phytoalexins avenalumins in oats (Mayama et al., 1986). When *H. oryzae* toxin was applied on rice leaves, phenolic content decreased and phenylalanine ammonia-lyase activity was suppressed (Vidhyasekaran et al., 1992).

Japanese pear tissues possess a potential resistance mechanism to *Alternaria alternata* Japanese pear pathotype (*Alternaria kikuchiana*) invasion. Dysfunction of plasma membranes by AK-toxin, produced by the fungus, results in abolishing the induction of a resistance

mechanism in susceptible pear tissues (Kohmoto et al., 1987). Papilla formation at the penetration sites of *A. alternata* functions as a resistance mechanism in Japanese pear. When pear leaves were inoculated with avirulent spores plus AK-toxin, the rate of papilla formation reduced and the avirulent spores could invade susceptible leaves (Otani et al., 1995).

Inoculation of leaves of susceptible maize genotypes with the weakly virulent race of *H. carbonum* race 2 induces resistance to a challenge inoculation with the highly virulent race 1 (Cantone and Dunkle, 1990a). The host-specific toxin (HC-toxin) produced only by race 1 isolates abolished the induced resistance when applied along with the challenge inoculum (Cantone and Dunkle, 1990a). The limited infection by *H. carbonum* race 2, a weakly virulent, nontoxin producing race, induces the release of an inhibitory compound from affected maize cells, which then diffuses into water droplets on the leaf surface. The virulent race 1 conidia when applied together with these inhibitory compounds are unable to cause susceptible-type lesions (Cantone and Dunkle, 1990b). The synthesis of the inhibitory compound is suppressed by HC-toxin (Cantone and Dunkle, 1991). Thus, the pathogens appear to suppress the defense mechanisms of the host by producing toxins.

8.4 TOXINS CAUSE CELL MEMBRANE DYSFUNCTION

8.4.1 Permeability Changes

Plasma membrane in plant cells governs all permeability processes from simple diffusion to active transport (Otani et al., 1977). Changes in membrane permeability are the first detectable events in the onset of diseases caused by different pathogens (Wheeler and Hanchey, 1968; Park et al., 1992). Changes in membrane permeability are mostly induced by toxins produced by pathogens. Electron microscopic observation showed that many invaginations in the plasma membranes of the susceptible strawberry cells occurred within 1–3 h after *Alternaria alternata* strawberry pathotype toxin treatment (Park et al., 1992).

The toxins induce electrolyte leakage from plant cells. Increase in electrolyte leakage is the earliest symptom observed in fungus-infected plants and electrolyte leakage may lead to necrosis (Kwon et al., 1998). HV-toxin produced by *Cochliobolus victoriae*, the oat blight pathogen, causes rapid loss of electrolytes (Bronson and Scheffer, 1977). When rice leaves were treated with *Rhizoctonia solani* toxin, the increase in electrolyte leakage was observed within 30 min after treatment (Vidhyasekaran et al., 1997).

Alternaria alternata apple pathotype toxin (AM-toxin) causes invagination of plasma membranes and an increase in electrolyte leakage. The increase in electrolyte loss was observed at 1 h after the toxin treatment (Shimomura et al., 1991). Susceptible tissues of sorghum exposed to toxin from *Periconia circinata* had increased loss of electrolytes within 20 min (Gardner et al., 1972). Fusaric acid (FA), the toxin produced by *Fusarium* spp., induced a sudden, large increase in electrolyte leakage (Marre et al., 1993).

The toxin (AK-toxin) produced by *Alternaria alternata* Japanese pear pathotype, the pathogen of black spot disease of Japanese pear, caused rapid increases in efflux of K^+ from susceptible leaves of Japanese pear. On the contrary, the toxin caused a decrease in effluxes of Na^+, Mg^{2+}, and Ca^{2+}. The toxin-induced loss of K^+ was much greater in the presence of Na^+, Mg^{2+}, or Ca^{2+} in the ambient solution. Stimulation of toxin-induced leakage was evident immediately after adding these cations to toxin-treated tissues. When these cations were removed, loss of K^+ reverted to the lower level. When toxin-induced K^+ efflux was stimulated by other cations, the tissues took up such cations. The results suggest that AK-toxin affects K^+ transport across plasma membranes of susceptible cells; the transport is closely associated with movement of other inorganic cations in the cells (Otani et al., 1977). The toxin also induced Na^+ and phosphate leakage (Park et al., 1987).

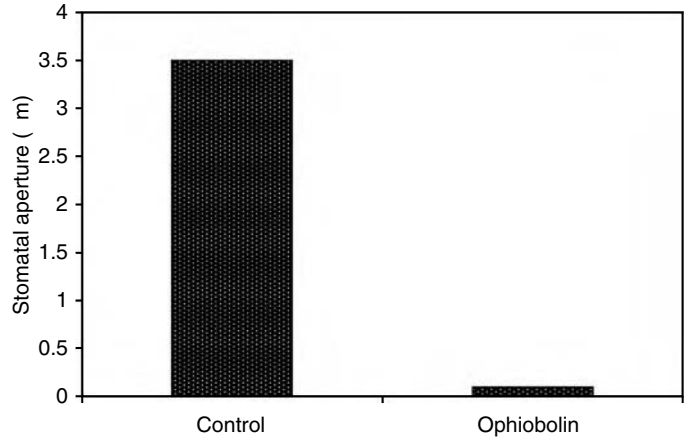

FIGURE 8.1 Effect of ophiobolin on stomatal opening in *Commelina communis*. (Adapted from Nejidat, A., *Plant Cell Physiol.*, 28, 455, 1987.)

Ophiobolin A is a nonspecific phytotoxin produced by *H. oryzae*, *H. maydis*, and *H. setariae*. The toxin increases ion leakage (Tipton et al., 1977) and membrane permeability for potassium ions (Cocucci et al., 1983). Stomatal movement is driven by potassium fluxes in and out of guard cells (Outlaw, 1983). Ophiobolin affects stomatal movement. Ophiobolin treatment affected stomata opening in *Commelina communis* and at 10 μM concentration stomata were tightly closed (Figure 8.1; Nejidat, 1987) and the tissue was damaged (Nejidat, 1987). The inhibitory effect of the toxin has been suggested to be due to its effect on membrane permeability and potassium leakage from guard cells (Leung et al., 1985).

Tipton et al. (1977) provided evidences to show that ophiobolin A produced by *H. maydis* does not cause general membrane disruption but inhibits specific membrane transport processes. The toxin stimulates net leakage of electrolytes and glucose from maize seedling roots. Treatment of the roots with ophibolin A inhibits uptake of 2-deoxyglucose by 85% but it does not show a similar effect on efflux of hexose (Tipton et al., 1977).

The fluidity of cell membranes of oat protoplasts was measured by electron-spin resonance with 5-doxyl stearic acid as a spin label (Briggs et al., 1984). No changes in fluidity of the protoplast membranes were detected following exposure to HV-toxin (Briggs et al., 1984). It suggests that the toxin-induced leakage of electrolytes may occur through the membrane matrix, possibly through a shuttle-type carrier.

8.4.2 Changes in Membrane-Bound ATPases

8.4.2.1 H^+-ATPase Is Stimulated

The plasma membrane plays a major role in the control of cell processes. Using ATP as the energy source, it pumps protons from the cytoplasm to the cell exterior, thus creating an electrochemical gradient across the plasma membrane that constitutes the driving force for nutrient uptake (Briskin, 1990). Cell wall loosening, a prerequisite for cell growth, is induced by extensive acidification of the apoplast (Rayle and Cleland, 1992). It results in alkalinization of the cytoplasm, which triggers cell division. Thus H^+-ATPase is involved in nutrient uptake as well as cell growth and division (Serrano, 1990).

Fusicoccin, the toxin produced by *Fusarium amygdali*, the pathogen of peach and almond, stimulates the activity of the plasmalemma-localized H^+-ATPase (Marre, 1979). Fusicoccin

leads to a twofold activation of ATP hydrolytic activity in the microsomal membrane fraction obtained from cell-suspension cultures of *Corydalis sempervirens* (Schulz et al., 1990). There was a stimulation of H^+ pumping by 50%–130% and stimulation of ATP hydrolysis by 20%–45% after incubation of microsomal membrane fraction from radish with fusicoccin (Rasi-Caldogno and Pugliarello, 1985).

Although fusicoccin stimulates H^+-ATPase, there does not seem to be direct interaction between the toxin and the H^+-ATPase activity and a fusicoccin-binding protein could be separated (Stout and Cleland, 1980). Furthermore, stimulation by fusicoccin of the ATPase from *Vicia faba* was lost after solubilization of the enzyme (Blum et al., 1988). The results suggest that fusicoccin may have sites of action in addition to the H^+-ATPase (De Michelis et al., 1989). A high-affinity fusicoccin receptor of 30–34 kDa, distinct from the 100 kDa H^+-ATPase, has been identified in plasma membrane fractions (De Boer et al., 1989; Meyer et al., 1989). Proteolytic removal of a 7–10 kDa fragment from the C-terminal end of the 100 kDa H^+-ATPase strongly activates ATP hydrolytic activity and H^+ pumping by the enzyme (Palmgren et al., 1991). It was suggested that a part of the C-terminal region may constitute an autoinhibitory domain of the H^+-ATPase and this domain may be the ultimate target for toxins that function as regulators of H^+ pumping across the plasma membrane (Palmgren et al., 1991).

When spinach leaves were infiltrated with 5 μM fusicoccin, plasma membranes from the leaves showed a twofold increase in ATP hydrolytic activity (Johansson et al., 1993) and a threefold increase in H^+ pumping (Johansson et al., 1993) compared with controls. Lysophosphatidylcholine stimulates the H^+-ATPase activity in isolated plasma membrane vesicles (Palmgren et al., 1988, 1990a,b; Palmgren and Sommarin, 1989). H^+-ATPase activated by lysophosphatidylcholine was not further activated by proteolytic removal of the C-terminal region, and it was suggested that activation by lysophosphatidylcholine involved a displacement of the C-terminal inhibitory domain (Palmgren et al., 1991). Activation by lysophosphatidylcholine was not additive to activation by fusicoccin in plasma membrane preparations from spinach leaves. It suggests that the latter process may also involve a displacement or removal of the C-terminal inhibitory domain (Johansson et al., 1993). Trypsin treatment of plasma membrane vesicles from fusicoccin-incubated leaves did not increase the ATPase or the H^+ pumping activity of these vesicles. Trypsin treatment resulted in the appearance of a 90 kDa band in addition to the native 100 kDa H^+-ATPase band in both the control and the fusicoccin-activated material (Johansson et al., 1993). The results suggest that activation of the H^+-ATPase by fusicoccin proceeds by a mechanism involving a displacement of the C-terminal inhibitory domain.

The fusicoccin-mediated increase in H^+-ATPase was rapid and largely completed within the first 4 min of incubation (Johansson et al., 1993). This relatively rapid activation suggests that de novo synthesis of H^+-ATPase did not contribute to the observed increase in activity. The intensity of the 100 kDa H^+-ATPase band is not increased but rather decreased in samples from fusicoccin-incubated leaves compared with controls (Johansson et al., 1993). No increase in H^+-ATPase polypeptide was observed after incubation with fusicoccin. These observations strengthen the conclusion that *in vivo* activation of the H^+-ATPase by fusicoccin proceeds by a mechanism involving a displacement of the C-terminal inhibitory domain (Johansson et al., 1993).

Fusicoccin opens stomata (Brown and Outlaw, 1982). Fusicoccin enhances K^+ influx (Blatt and Clint, 1989; Clint and Blatt, 1989). The K^+ influx may be due to activation of H^+-ATPase pump in plasma membrane (Assmann and Zeiger, 1987). Fusicoccin-promoted stomatal opening may be primarily due to the ability of fusicoccin to stimulate the plasma membrane H^+-ATPase of plant cells (Marre, 1979). In guard cells, activation of H^+ pump in plasma membrane creates an electrochemical gradient, which is presumed to drive the K^+ influx necessary for guard cell swelling and stomatal opening (Assmann and Zeiger, 1987).

K^+-selective ion channels present in the guard cell membrane may provide a pathway for K^+ movement across the cell membrane in response to an electrochemical driving force (Schroeder et al., 1988).

Fusicoccin may activate a carrier that mediates K^+ uptake against its electrochemical gradient and may inactivate ion channels that mediate K^+ efflux (Blatt and Clint, 1989; Clint and Blatt, 1989). In 1 mM KCl, low concentrations of fusicoccin combined with exposure to white light synergistically stimulated stomatal opening (Assmann and Schwartz, 1992). With increasing fusicoccin concentrations, stomatal opening occurred even in the darkness and the synergistic effect diminished (Assmann and Schwartz, 1992). The synergistic effect of light and 0.1 μM fusicoccin also diminished as KCl concentrations were increased (Assmann and Schwartz, 1992). Stomata were either opened by light on the intact leaf or opened in isolated peels by light and 0.1 μM fusicoccin could close to a significant extent upon imposition of darkness and then reopen upon exposure to white light, indicating that 0.1 μM fusicoccin did not prevent ion efflux. These results show that the stomata remained functional after treatment with this low fusicoccin concentration. In the absence of fusicoccin, stomata in 1 mM KCl closed in darkness and remained closed even in the light. The K^+ channel blocker, tetraethylammonium (TEA), was equally effective in inhibiting stomatal opening by either 0.1 μM fusicoccin + 1 mM KCl or 120 mM KCl (Assmann and Schwartz, 1992). Fusicoccin (1 μM) prevented stomatal closure and this effect could be completely eliminated by inclusion of 1 mM vanadate, a H^+-ATPase inhibitor, in the incubation solution, despite little direct effect of vanadate on stomatal closure. In whole-cell patch-clamp experiments with guard cell protoplasts of *Vicia fabia*, fusicoccin (1 or 10 μM) stimulates an increase in outward current that is essentially voltage independent between −100 and +60 mV, and occurs even when the membrane potential is held at voltage (−60 mV) at which K^+ channels are inactivated and therefore could not be attributed to altered flux through K^+ channels. The fusicoccin-stimulated current was essentially voltage independent over the range from −100 to +60 mV. This voltage independence contrasts sharply with the voltage dependence of K^+ and anion channels over the same voltage range. Hence it was concluded that the observed increase in outward current results from fusicoccin activation of H^+-ATPase at the guard cell plasma membrane (Assmann and Schwartz, 1992). Fusicoccin may activate H^+ pumps and it may not function merely by blocking K^+ efflux channels. The plasma membrane H^+-ATPase of guard cells may be a primary locus for the fusicoccin effect on stomatal apertures.

Fusicoccin has been shown to open stomata in bean, tobacco, sorghum, cucumber, lucerne, and pokeweed (*Phytolacca americana*) (Turner and Graniti, 1969). By this process, the toxin increases stomatal transpiration (Turner and Graniti, 1969; Lado et al., 1972). The uncontrolled water loss due to irreversible stomatal opening may result in a rapid reduction of available water in the intercellular spaces and in a subsequent loss of turgor of the mesophyll cells (Turner and Graniti, 1969). Large areas of the leaf blade may wilt and wither (Turner and Graniti, 1969; Graniti and Turner, 1970).

Rhynchosporium secalis, the leaf scald pathogen of barley grows subcuticularly, primarily above anticlinal epidermal walls during early stages of pathogenesis (Lehnackers and Knogge, 1990). Initial symptoms in barley cultivars comprise the swelling of epidermal cells and a subsequent loss of rigidity of anticlinal epidermal walls. This is accompanied by a separation of the plasmalemma from the cell wall (Lehnackers and Knogge, 1990). An increased opening of stomata is also observed (Branchard and Laffray, 1987). It leads to increases in transpiration and permeability of cells (Ayers and Jones, 1975). Cell necrosis occurs (Wevelsiep et al., 1991). A small family of necrosis-inducing peptides has been identified in culture filtrates of *R. secalis* (Wevelsiep et al., 1991). Two of these peptides, NIP1 and NIP3, correlated with the development of necrotic lesions. Both the peptides stimulated the activity of the plasmalemma-localized Mg^{2+}-dependent, K^+-stimulated H^+-ATPase (Wevelsiep et al., 1993). When ATPase from barley plasma membrane vesicles was partially purified by centrifugation in a

glycerol gradient, no stimulation of enzyme activity by peptides was detectable. Hence the peptides do not seem to interact directly with the ATPase. An NIP3-binding protein of ~65 kDa has been detected in barley (Wevelsiep et al., 1993).

8.4.2.2 H^+-ATPase Is Inhibited

In some host–pathogen interactions, ATPase is inhibited. AF-toxin produced by *Alternaria alternata* strawberry pathotype affected the electrogenic proton pump (H^+ pump) in the plasma membrane of susceptible strawberry cells (Namiki et al., 1986a), but no significant inhibition of the ATPase activity was observed by *in vitro* toxin treatment (Lee et al., 1990, 1992). Probably a binding site for the toxin may be needed as shown in case of action of *Rhynchosporium secalis* toxin (Wevelsiep et al., 1993). Similar observations have been reported in HS-toxin treated sugarcane cells (Kohmoto and Otani, 1991). The toxin produced by *Cercospora beticola* (CBT) induces leakage of betacyanin and amino acids from red beet root tissue (Schlosser, 1971). CBT inhibits H^+ extrusion and depolarizes transmembrane potential (Macri and Vianello, 1979), but does not affect ATP and pyruvate levels (Macri et al., 1980). The toxin inhibits ATP-dependent proton translocation in microsomal vesicles and it has a primary effect on ATPases associated with plasmalemma or tonoplast membranes (Macri et al., 1983).

CBT was inhibitory to the ATP-induced intravesicular proton accumulation and this effect increased with the time of incubation. CBT inhibited ATPase activity and this inhibition also increased with the time of preincubation. However, CBT was more inhibitory to proton transport than to ATP hydrolysis. The results suggest a direct effect of CBT on ATPase associated with the plasma membrane (Blein et al., 1988).

8.4.3 Inhibition of Calmodulin Activity

Calmodulin is a Ca^{2+}-binding protein in plasma membrane. Calmodulin is a mediator of calcium transport (Hepler and Wayne, 1985). Ophiobolin A, the toxin produced by *H. oryzae*, *H. maydis*, and *H. setariae*, reacts *in vitro* with bovine brain and spinach calmodulins so that they are unable to activate calmodulin-dependent cAMP phosphodiesterase (Leung et al., 1984). Ophiobolin A inhibited maize calmodulin-dependent cAMP phosphodiesterase activity (Figure 8.2; Leung et al., 1985). The inhibition of maize calmodulin activity by ophiobolin was strongly dependent of Ca^{2+} (Leung et al., 1985).

The extract of ophiobolin A-treated maize roots had less active calmodulin than that of the untreated roots. It suggests that ophiobolin A interacts with calmodulin in situ (Leung et al., 1985). Calmodulin appears to be a target molecule for the toxicity of ophiobolin A in root cells. Transport enzymes such as calmodulin-dependent Ca-ATPase may be affected and in turn may affect calcium transport in the membrane (Leung et al., 1985). Low concentrations of calcium inhibit stomatal opening (Fischer, 1972). Calcium chloride at concentration of 0.1 mM inhibited stomatal opening of *Commelina communis* (Nejidat, 1987). Addition of 0.1 μM ophiobolin significantly abolished the Ca^{2+} effect. Chloropromazine, a potent inhibitor of calmodulin, enhanced stomatal opening and significantly reversed the effect of 0.1 mM $CaCl_2$ (Nejidat, 1987). Calmodulin inhibitors stimulate stomatal opening (Donovan et al., 1985). The results suggest that ophiobolin may affect calmodulin activity (Nejidat, 1987). Ophiobolin appears to be a natural calmodulin inhibitor (Leung et al., 1984, 1985).

8.4.4 Alteration in Membrane Potential

The electrical potential difference (PD) across biological membranes is due to a diffusion potential resulting from unequal distribution of ions (Dainty, 1962). There is also evidence

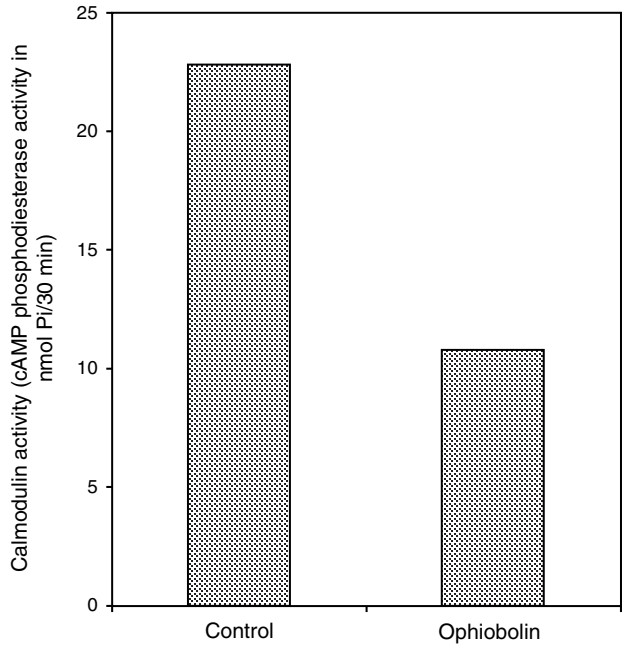

FIGURE 8.2 Effect of ophiobolin A in inhibition of calmodulin activity in maize. (Adapted from Leung, P.C., Taylor, W.R., Wang, J.H., and Tipton, C.L., *Plant Physiol.*, 77, 303, 1985.)

that energy requiring active transport processes (electrogenic pumps) contribute to PD in plant cells (Higginbotham, 1970). Due to fungal infection, depolarization of the membranes takes place. The toxins produced by *H. maydis* (Mertz and Arntzen, 1978), *H. victoriae* (Novacky and Hanchey, 1974), and *H. sacchari* (Schroter and Novacky, 1985) depolarized the membranes of their respective susceptible hosts such as maize, oat, and sugarcane. Beticolin, the toxin produced by *Cercospora beticola*, caused membrane depolarization in tobacco cells (Gapillout et al., 1996).

Treatment of susceptible oat roots with victorin, the toxin produced by *H. victoriae*, caused a measurable depolarization within 2–5 min and averaged ~30% depolarization within 10 min (Novacky and Hanchey, 1974). Membrane depolarization was detected at concentrations as low as 50 nm *H. sacchari* toxin. The onset of membrane depolarization was observed after a lag period of 4–10 min. The energy-dependent component of membrane potential was inhibited by the toxin (Schroter and Novacky, 1985). HS-toxin may cause a loss of H^+ gradient across the membrane (Schroter and Novacky, 1985).

FA is the toxin produced by *Fusarium* spp. FA has both direct and indirect effects on the plasma membrane. The direct effect is indicated by a rapid depolarization, possibly due to change in membrane permeability. The indirect effects are indicated by: (i) transient hyperpolarization, possibly reflecting a FA–mediated influx of protons followed by their electrogenic extrusion and (ii) a slow depolarization, possibly caused by reduced ATP levels inhibiting electrogenic extrusion of H^+ (D'Alton and Etherton, 1984).

FA induces reductions in ATP concentrations (D'Alton and Etherton, 1984), which are necessary for electrogenic extrusion of H^+ and hence maintenance of the membrane PD between the inside of a plant cell and the external solution. FA induced an early transient hyperpolarization followed by a depolarization of transmembrane electrical PD (E_m) in cells of *Egeria densa* leaves. Both effects were enhanced by higher concentration of FA. The effects of 1 mM FA were progressively reduced as the pH of the incubating medium increased as

the ratio of the uncharged to the charged form of the acid (pK FA = 5.59) was reduced (Marre et al., 1993).

The initial hyperpolarization may be due to an early stimulation of the proton pump by acidification of the cytosol consequent on the entry into the cell of the undissociated form of the acid. The depolarization observed after ~5 min of treatment with FA may be due to inhibition of oxidative phosphorylation (Marre et al., 1993). FA inhibited malate-dependent O_2 uptake. Succinate oxidation coupled to phosphorylation was also inhibited by FA (Marre et al., 1993). Inhibition of oxidative phosphorylation by FA has been reported in tomato roots (D'Alton and Etherton, 1984), maize roots (Arias, 1985), and sorghum seedlings (Dunkle and Wolpert, 1981). Inhibition of respiratory activity (Arias, 1985) and decrease in ATP levels (Koehler and Bentrup, 1983) due to FA treatment in different plant species have been reported.

8.4.5 Toxins Form Ion Channels in Plant Cell Membranes

Some toxins form ion channels in plant cell membranes. Addition of 10 μM concentrations of beticolins produced by *Cercospora beticola* induced discrete current transitions, similar to those occurring upon opening and closing of ion channels, indicating that the beticolins are able to form ion channels (Goudet et al., 2000). Beticolin channels show poor ionic selectivity. Cations like K^+, Na^+, or Li^+ are permeable than Cl^- but all those ions have a significant permeability through beticolin channels. Beticolins are able to chelate Mg^{2+} and to form $(B\text{-}Mg)_2$ dimeric complexes. $(B\text{-}Mg)_2$ dimers have been suggested to be the basic element of the pore. The pore will enable loss of electrolytes and amino acids (Goudet et al., 2000). The pore formation has been suggested to be responsible for membrane permeabilization, collapse of ionic and electrical gradients, and cell lysis (Goudet et al., 2000).

8.4.6 Modification of Membrane Phospholipids

The plasma membrane consists mainly of proteins and phospholipids. Changes in phospholipid content in cell organelles have been observed in several plants due to infection (Sednina et al., 1981). When the cells are treated with toxins produced by pathogen, phospholipases of the host are activated. AF-toxin, produced by *Alternaria alternata* strawberry pathotype, induced a considerable decrease in phospholipid content in microsomes of strawberry protoplasts (Figure 8.3; Lee et al., 1992).

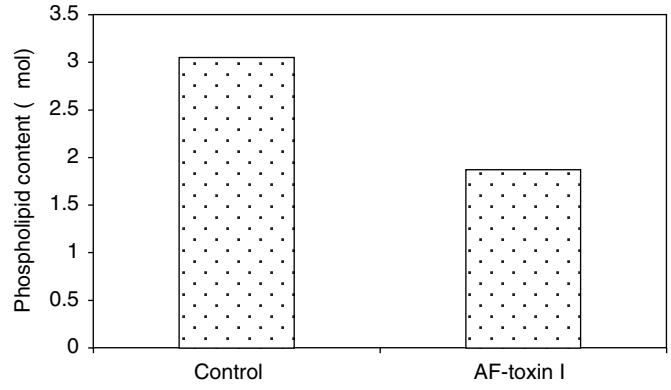

FIGURE 8.3 Decrease in phospholipids content in microsomes of strawberry protoplasts treated with *Alternaria alternata* strawberry pathotype AF-toxin I. (Adapted from Lee, S.S., Kawakita, K., Tsuge, T., and Doke, N., *Physiol. Mol. Plant Pathol.*, 41, 283, 1992.)

Phospholipase A_2 causes dysfunction of membranes by hydrolyzing phospholipids and releasing unsaturated fatty acids (Waite, 1985). When suspension-cultured cells of the susceptible cultivar were treated with AF-toxin I, an enhanced level of phospholipase A_2 activity in the microsomes was detected at relatively low concentrations of the toxin that are known to reduce cell viability (Lee et al., 1992). Furthermore, *in vitro* toxin treatment of microsomes prepared from susceptible cells significantly stimulated the phospholipase A_2 activity at much lower concentrations. Phospholipase A_2 activity was detected in strawberry microsomes in Ca^{2+}-dependent manner. A significant increase in the enzyme activity was observed when 5 mM or more $CaCl_2$ was added to the reaction mixture. It suggests that AF-toxin I affects cytosolic Ca^{2+} concentrations, which in turn affect activity of phospholipase A_2 (Lee et al., 1992).

The phospholipid-degrading enzymes, especially phospholipase A_2 and phospholipase C, are involved in signal transduction in plant cells in response to various stimuli (Scherer and Andre, 1989; Scherer et al., 1990). The toxins may serve as external stimuli and phospholipase A_2 activation in response to AF-toxin may be a consequence of signal transduction in strawberry cells (Lee et al., 1992). The removal of certain phospholipids from microsomes alters the activity of membrane-bound enzyme systems. ATPase activity is inhibited by the action of phospholipase (Faull and Gay, 1983) and it may lead to depolarization of active membrane potential (Lee et al., 1992). The removal of phospholipids from membranes causes damage to membrane structure, sometimes followed by cell death (Hoppe and Heitefuss, 1975).

8.4.7 Toxin-Induced Active Oxygen Species Induce Membrane Dysfunction

Some toxins have been shown to induce production of active oxygen species, which are involved in cell membrane dysfunction. *Cercospora* spp. cause severe diseases in many crops. A large number of *Cercospora* spp. produce a toxin named cercosporin. Cercosporin is structurally related to several photosensitizing compounds, that is, compounds that sensitize cells to visible light (Foote, 1976). Cercosporin causes peroxidation of plant lipids (Daub, 1982b), resulting in major changes in the structure and composition of plant cell membranes (Daub and Briggs, 1983). Cercosporin absorbs light to form a long-lived electronically excited state (triplet state), which can then react with molecular oxygen to produce compounds which are toxic to living cells. The triple sensitizer (^3S) may react with O_2 in several ways. It may react with a reducing substrate (R or RH) by the transfer of a hydrogen atom or electron. The resulting sensitizer radical may then react with O_2 to produce superoxide ions (O_2^-). Alternatively, the triplet sensitizer may react directly with O_2 by an energy transfer process yielding the electronically excited singlet state of O_2 ($^1O_2$) (Foote, 1976).

Cercosporin, when illuminated in the presence of O_2, reacted with cholesterol to form the 5α-hydroperoxide of cholesterol, which is only produced by reaction with singlet oxygen. Cercosporin, in the presence of light, O_2, and a reducing substrate, was able to reduce *p*-nitroblue tetrazolium chloride, a compound readily reduced by superoxide. Superoxide dismutase, a scavenger of superoxide, inhibited this reaction (Daub and Hangarter, 1983). The results suggest that cercosporin can induce the production of both $^1O_2$ and O_2^- (Daub and Payne, 1989).

Both $^1O_2$ and O_2^- are extremely toxic to cells, causing the oxidation of fatty acids, sugars, cellulosic materials, guanine, and several amino acids (cysteine, methionine, histidine, tryptophan, and tyrosine) which in turn results in damage to DNA, inactivation of enzymes, and destruction of cell membranes (Foote, 1976). Cercosporin induces ion leakage from potato, carrot, beet, and corn tissue when these tissues are irradiated with an incandescent bulbs and oxygen is involved in the reaction (Macri and Vianello, 1979).

Tobacco leaf discs treated with cercosporin showed a large increase in electrolyte leakage 1 to 2 min after irradiation with light. All tobacco protoplasts exposed to cercosporin in the light were damaged within 45 min. Tobacco cell culture mutants, which show resistance to the generation of O_2^-, show decreased sensitivity to cercosporin (Daub and Hangarter, 1983). Compounds capable of quenching $^1O_2$ prevent the killing of tobacco suspension-cultured cells by cercosporin (Daub, 1982a). Suspension-cultured tobacco cells were treated with bixin, which is known to quench $^1O_2$. Bixin prevented killing response by cercosporin (Daub, 1982a). These results suggest that cercosporin is involved in cell membrane dysfunction by producing active oxygen species.

Several other toxins also act by producing active oxygen species. *Fusarium solani* toxin, dihydrofusarubin, reduces molecular oxygen under oxidation, triggering the formation of active oxygen species such as superoxide and hydrogen peroxide on tobacco leaves and spinach chloroplasts (Albrecht et al., 1998). Rubellin D toxin produced by *Ramularia collo-cygni*, the pathogen causing *Ramularia* leaf spot disease of barley, triggered the formation of active oxygen species (Heiser et al., 2003). Rubellin D induces the fatty acid peroxidation of α-linolenic acid in barley leaves. Singlet oxygen was produced and it played an important role in fatty acid peroxidation. Ethane formation was observed along with fatty acid peroxidation and chlorophyll bleaching also occurred in toxin-treated leaves (Heiser et al., 2003).

8.4.8 Mitochondrial Membrane Dysfunction

Mitochondria control the respiratory activity of plant cells. They produce ATP by oxidative phosphorylation. Regulation of the molecular traffic through membranes is particularly important in mitochondrion, where electron transfer across the inner membrane creates a proton motive force, which drives phosphorylation producing cellular energy (Braun et al., 1989). Inhibition of ATP synthesis will result in suppression of defense mechanisms of the host and will facilitate extensive colonization of the pathogens (Berville et al., 1984; Holden and Sze, 1984). *H. maydis* race T produces a host-specific toxin, HMT-toxin. HMT-toxin affects mitochondrial activities (Holden and Sze, 1989) and induces rapid swelling of mitochondria of the susceptible Texas line of cytoplasmic male-sterile corn (Koeppe et al., 1978). It increases permeability of the inner mitochondrial membrane to calcium (Holden and Sze, 1984) and protons (Klein and Koeppe, 1985). It dissipates mitochondrial membrane potential (Berville et al., 1984) and induces leakage of small molecules and ions (NAD^+ and Ca^{2+}) from the matrix space (Holden and Sze, 1984). It induces uncoupling of oxidative phosphorylation (Holden and Sze, 1987). It stimulates NADH- or succinate-mediated respiration (Bednarski et al., 1977) and mitochondrial ATPase activity (Bednarski et al., 1977; Holden and Sze, 1987). HMT-toxin inhibits malate-driven respiration (Berville et al., 1984).

A unique mitochondrial gene from cms-T plants, designated *μrf13-T*, which encodes a 13 kDa protein URF13-T, associated with the cms trait in maize has been isolated (Dewey et al., 1987). A truncated version of the *μrf13-T* gene, *pJG13-T*, which produces a form of the 13 kDa protein missing amino acids 2 through 11 was also constructed and expressed in *Escherichia coli* by means of the plasmid vector pJG 200. Oxygen consumption was completely inhibited by the addition of HMT-toxin in cell producing the complete 13 kDa protein. In contrast, respiration was not altered by toxin in control cells transformed with the pATH 3 vector containing no insert, or in the cells producing the truncated version of the 13 kDa protein. HMT-toxin induced dramatic swelling in spheroplasts from *E. coli* cells that produced 13 kDa polypeptide. No effect was seen with spheroplasts containing the pATH 3 vector containing no insert or in the spheroplasts producing the truncated 13 kDa protein from plasmid pJG13-T (Dewey et al., 1988).

Incubation of cms-T maize mitochondria with dicyclohexylcarbodiimide (DCCD) prevented toxin-induced inhibition of malate-dependent oxidation, dissipation of the membrane potential, and leakage of accumulated calcium (Bouthyette et al., 1985). Two major DCCD-binding proteins ~6 and 14 kDa in size were observed when normal and cms-T mitochondria were incubated with ^{14}C-labeled DCCD. Immunoprecipitation of ^{14}C-labeled DCCD maize mitochondrial proteins with an antiserum directed against the μrf13-T protein product revealed that DCCD binds to the 13 kDa protein. Similar to its characteristics in cms-T mitochondria, the 13 kDa polypeptide produced in *E. coli* by pATH 13-T was also localized in the membrane and was shown to bind DCCD (Dewey et al., 1988). The results suggest that the amino-terminal region of the 13 kDa polypeptide is involved in conferring HMT-toxin susceptibility to cms-T mitochondria (Dewey et al., 1988). The amino-terminal portion of the protein is the region most likely associated with the membrane, the site where the toxin action is believed to occur.

The observed inhibition of whole-cell respiration by HMT-toxin could be explained by pore formation, which leads to a loss of intermediate species associated with glycolysis and the TCA cycle as well as any unbound intracellular electrolytes. Addition of HMT-toxin to *E. coli* cells expressing URF13 induces a rapid increase in the conductivity of the external medium. A massive and immediate efflux of radiolabeled ^{86}Rb accumulated in *E. coli* cells expressing URF13 after the addition of HMT-toxin (Braun et al., 1990). The rate at which ^{86}Rb is lost from cells after toxin addition (<1 min) is consistent with the formation of hydrophilic pores within the plasma membrane; added uncouplers which break down the proton motive gradient driving Rb uptake but do not permeabilize the membrane to Rb, also promote a cellular Rb, but do so with a half-life of tens of minutes. The amount of accumulated ^{86}Rb in control cells lacking URF13 is not affected by addition of HMT-toxin. These results provide evidence that effects of T-toxin in *E. coli* cells expressing *URF13* are analogous to those seen in cms-T mitochondria and result from the formation of hydrophilic pores within URF13-containing membranes (Levings and Siedow, 1992).

A chimeric gene composed of *T-μrf13* fused at its amino terminus to the mitochondrial targeting sequence of the ATPase subunit from *Neurospora crassa* has been constructed (Huang et al., 1990). When this construct was transformed into yeast, it produced a protein that was localized to the mitochondrial membrane fraction and was processed to a size similar to that of standard URF13. Growth of yeast cells expressing the chimeric URF13 construct was inhibited by added HMT-toxin (Huang et al., 1990). Yeast transformed with full-length T-μrf13 lacking the mitochondrial transit peptide was insensitive to HMT-toxin (Huang et al., 1990). The results suggest that *μrf13* needs to be localized in mitochondria to produce a toxin-sensitive phenotype. Glab et al. (1990) have also shown that *T-μrf13* gene confers HMT-toxin sensitivity to yeast.

Removal of carboxyl-terminal 32 amino acids producing a truncated URF13 of 83 amino acids instead of the 115 found in the standard URF13 had no effect on sensitivity to HMT-toxin (Braun et al., 1989). Deletion of the next amino acid (Leu 83) to give an 82 amino acid product, however resulted in a complete loss of toxin sensitivity (Braun et al., 1989). A toxin-insensitive mutant (T-4) was detected in cms-T maize cell tissue culture. In the T-4 mutant, the *T-μrf13* reading frame was shortened to 74 codons by a frameshift mutation. The URF13 protein in the mutant contained only 74 amino acids (Wise et al., 1987a,b).

Membrane-bound proteins had stretches of hydrophobic amino acid residues that may associate with the lipid bilayer. URF13 contains several hydrophobic domains. Site-directed mutations demonstrated that DCCD binds to URF13 at two separate residues, positions 12 and 39 (Braun et al., 1989). DCCD binding to aspartate at position 12 is expected because it is located between the two largest hydrophobic domains and the residues adjacent to the aspartate at position 12 are similar to those found in other DCCD-binding sites. DCCD binding to the aspartate at position 39 is unexpected because this residue is located in an area

predicted to be hydrophilic; DCCD stably binds only in nonaqueous environments. Taken together, the results suggest that amino acids 12 and 19 are membrane bound. In addition, aspartate at position 39 is required for functional HMT-toxin–URF13 interaction since substitutional mutations at this aspartate residue eliminate sensitivity to toxin. Although DCCD binds at two sites on URF13, only binding at position 39 confers protection against T-toxin (Braun et al., 1989). The binding of T-toxin to URF13 causes rapid permeabilization of the inner mitochondrial membrane, which results in leakage of NAD^+ and other ions from the matrix. A pore consisting of at least six transmembrane-helices is required for NAD^+ leakage (Levings et al., 1995). URF13 oligomers are involved in hydrophilic pore formation (Levings et al., 1995).

The results suggest that HMT-toxin inhibits cms-T maize mitochondrial function by their capacity to permeabilize the inner mitochondrial membrane after interaction with the *T-urf13* gene product, URF13, which is able to form membrane pores. This, in turn, promotes the loss of metabolic integrity that results in the large-scale fungal colonization and subsequent necrotic lesions that are symptomatic of leaf blight in cms-T maize (Levings and Siedow, 1992). *Phyllosticta maydis* also affects maize plants carrying cms-T cytoplasm. This fungus produces Pm toxin. Pm toxin is also capable of affecting cms-T mitochondria (Levings and Siedow, 1992).

FA produced by *Fusarium* spp. reduced the respiratory activity of isolated mitochondria of tomato (Paquin and Waygood, 1957). Mitochondria isolated from FA-treated tomato plants have reduced respiratory activity (Kuo and Scheffer, 1964). Mitochondria in susceptible host cells are affected by toxins produced by *Alternaria alternata* rough lemon pathotype (ACR-toxin), *A. alternata* f. sp. *lycopersici* (AAL-toxin) and *A. alternata* tobacco pathotype (AT-toxin). AT-toxin and AL-toxin induced ultrastructural damage to mitochondria. ACR-toxin increased the permeability of the inner mitochondrial membrane (Kohmoto and Otani, 1991). The toxin produced by *Ceratocystis ulmi*, the pathogen of Dutch elm disease, induced the swelling of mitochondria and disruption of the outer membrane (Pijut et al., 1990).

8.5 HOW DO PATHOGENS INDUCE MEMBRANE DYSFUNCTION ONLY IN SUSCEPTIBLE HOSTS?

8.5.1 Detoxification of Phytotoxins, Which Occurs in Resistant Hosts, Does Not Occur in Susceptible Hosts

Phytotoxins are produced by pathogens and they induce membrane dysfunction in host cells. Phytotoxins cause membrane dysfunction only in compatible, susceptible hosts. These toxins are detoxified in many incompatible resistant hosts. *Cochliobolus carbonum*, the maize leaf spot pathogen, produces a toxin named HC-toxin. The toxin is a cyclic tetrapeptide with a structure of cyclo(D-prolyl-L-alanyl-D-alanyl-L-2-Aeo) and the Aeo stands for amino-9,10-epoxy-8-oxodecanoic acid (Kawai et al., 1983). The epoxide and the 8-carboxyl groups in Aeo are required for biological activity of HC-toxin (Kim et al., 1987). When HC-toxin is fed to maize leaves through the transpiration stream, it is metabolized to a single, nontoxic derivative in which 8-carboxyl group of Aeo is reduced to the corresponding alcohol (Meeley and Walton, 1991). Cell-free extracts of maize seedlings metabolize HC-toxin to the same compound, 8-hydroxy-HC-toxin, as whole green leaves. HC-toxin reductase has been shown to be involved in the metabolization of the toxin and this enzyme has been purified and characterized (Meeley and Walton, 1991). This enzyme inactivates HC-toxin by pyridine nucleotide-dependent reaction of an essential carbonyl group. This enzyme activity was detectable only in extracts of maize that are resistant to *C. carbonum* race 1. The results indicate that detoxification of the toxin occurs in resistant varieties, whereas such detoxification does not

occur in susceptible varieties (Meeley et al., 1992). Conidia of *C. carbonum* secrete HC-toxin during appressorium formation on maize leaves (Weiergang et al., 2004). Extensive fungal growth occurred in the susceptible interaction by 24 h. The fungus failed to become established in the resistant host even after 36 h. The resistance was conditioned by *Hm1* in the resistant plants. The *Hm1* gene encoded the toxin reductase, and it caused inactivation of the toxin early in the interaction (Weiergang et al., 2004).

Cercospora oryzae produces a red toxin, cercosporin (Batchvarova et al., 1992). Louisiana red rice is resistant to the toxin and the rice cells grew in the presence of cercosporin concentrations that were completely toxic to Labelle, the most susceptible variety. When the rice cells were treated with the toxin, red rice cells contained about one-tenth as much cercosporin as cells of Labelle. It suggests that resistant cells have mechanism for excluding, exporting, or destroying the toxin. Treating cell suspensions of red rice with norflurazon abolished the cercosporin resistance in the cultivar (Batchvarova et al., 1992). Norflurazone is an inhibitor of carotenoid biosynthesis and it reduced the carotenoid content of cells derived from red rice (Batchvarova et al., 1992). The results suggest that carotenoids are involved in resistance to the toxin. Illuminated cercosporin produces active oxygen species (Daub and Payne, 1989). Carotenoids quench such species (Goodwin, 1980), particularly singlet oxygen (Daub and Hangarter, 1983). The resistant varieties may show increased levels of superoxide dismutase, catalase, and peroxidase which are all scavengers of oxygen species (Daub and Hangarter, 1983).

Alternaria alternata, a pathogen of spotted knapweed, produces a host-specific toxin, maculosin and when the toxin was applied on leaves of knapweed, it was converted into maculosin-β-*o*-glucoside which is not phytotoxic toward spotted knapweed (Park et al., 1994). Ferulic acid induced resistance to *Magnaporthe grisea* in rice seedlings and it showed ability to detoxify piricularin, the toxin produced by *M. grisea* (Tamari et al., 1966). Phenylalanine and tyrosine detoxified *Alternaria macrospora* toxin (Krishnamohan and Vidhyasekaran, 1988). Petasol, a toxin produced by *Drechslera gigantea*, is conjugated with amino acids to yield an inactive conversion product (Bunkers and Strobel, 1991).

Detoxification of the toxins induces resistance even in the susceptible varieties. Treating tomato roots with soil bacteria which are able to degrade and detoxify FA, as well as with toxin-degrading mutants of *Ralstonia solanacearum* and clones of *Escherichia coli* transformed with engineered plasmids from *Cladosporium werneckii*, protected plants from wilting caused by *Fusarium oxysporum* f. sp. *lycopersici* (Utsumi et al., 1989). Bacterial strains capable of degrading oxalic acid, the toxin produced by *Sclerotinia sclerotiorum*, have been identified and these bacterial strains prevented *S. sclerotiorum* infection in *Arabidopsis thaliana* (Dickman and Mitra, 1992). These results also suggest the possibility of existence of detoxification mechanism in resistant plants and similar mechanisms may not exist in susceptible ones.

8.5.2 Susceptible Tissues May Have Toxin Receptors Which May Be Absent in Resistant Tissues

Toxin uptake by susceptible tissue is considered to involve adsorption to a receptor that may be lacking in resistant tissues (Scheffer and Pringle, 1964). Many circumstantial evidences support the presence of toxin receptors in susceptible host tissues. In culture filtrates of pathogens, toxoids have also been detected along with toxins. Since toxoids are structurally related to the toxin, they may compete for the same toxin receptor sites. Pretreatment of susceptible tissues with toxoids prevented toxicity of the corresponding native toxin in case of interactions between HS-toxin and sugarcane (Livingston and Scheffer, 1984), HV-toxin and oats (Wolpert and Macko, 1989), and AF-toxin and strawberry (Namiki et al., 1986b).

However, toxin receptor sites have been identified only in few cases. Only oat genotypes carrying the dominant Vb allele are both susceptible to *Cochliobolus victoriae* and sensitive to the toxin produced by the fungus, victorin (Wolpert et al., 1986; Wolpert and Macko, 1989). When oat leaf slices were incubated with ^{125}I-Bolton-Hunter derivative of victorin C (BHC), a 100 kDa protein was labeled only in the susceptibles oat genotype. The genotype-specific toxin-induced labeling of the 100 kDa protein appeared to be covalent as it persisted after phenol extraction, precipitation, and PAGE. Reduced form of victorin C (RC) is not toxic but it behaves as a protectant, presumably by competitive displacement of victorin. When oat leaf slices were incubated with vicotrin C, the toxic effects of victorin C were decreased or eliminated, depending on the concentration of RC (Wolpert and Macko, 1989).

In vitro binding analysis revealed a requirement for an exogenous reducing agent (2-mercaptoethanol or dithiothereitol). When leaf tissue homogenates were prepared in the presence or absence of reducing agent and tested for covalent binding, labeling was observed only in the presence of a reducing agent. *In vitro* binding experiments demonstrated the presence of a 100 kDa victorin-binding protein (VBP) in both susceptible and resistant genotypes. Reduced form of victorin and methyl ester victorin C prevented ^{125}I-BHC-mediated labeling of the 100 kDa protein (Wolpert and Macko, 1989). The reduction of the aldehyde to a primary alcohol, as in RC, eliminates toxicity of victorin. RC may nevertheless associate with the active site, because treatment of oat leaf slices with RC prevents the toxic effect of victorin C. Many aldehydes are known to bind covalently to proteins. Hence the aldehyde may mediate a covalent association of victorin with its site of action and the covalent binding may be essential for toxicity (Wolpert et al., 1988; Wolpert and Macko, 1989).

The 100 kDa protein binds ^{125}I-BHC in a genotype-specific manner only *in vivo* but has no genotype specificity *in vitro*. Binding *in vitro* required a reducing agent that was not required for *in vivo* binding. It is possible that the difference between S and R genotypes is due to the presence of a reducing group either in the 100 kDa protein or in associated molecules. The 100 kDa protein may be the victorin receptor (Wolpert and Macko, 1989).

Polyclonal antibodies against victorin were raised in rabbits immunized with a victorin bovine serum albumin conjugate (Akimitsu et al., 1992). The antibodies were purified from serum by protein A column chromatography. *In vivo* and *in vitro* covalent binding of victorin to proteins in susceptible and resistant oat tissue was examined by Western blot bioassays using antivictorin antibody. *In vivo* binding of victorin to proteins of 100 and 45 kDa was observed in both susceptible and resistant cultivars of oats. Victorin also bound *in vitro* to proteins of 100, 65, and 45 kDa in both susceptible and resistant oats (Akimitsu et al., 1992). These results are in contrast to findings of Wolpert and Macko (1989). According to Akimitsu et al. (1992), there was also no evidence for host-specific binding of victorin *in vivo*. Binding *in vivo* occurred in both S and R oats, and not in just S oats. Hence, host specificity may be due to some other components in the victorin-transduction pathway after victorin binds to oat tissue tissue proteins (Akimitsu et al., 1992). However, Wolpert et al. (1994) argued that the genotype-specific binding could not be detected *in vivo* with the antivictorin antibody by Akimitsu et al. (1992), because the binding observed was as a result of *in vitro* binding that occurred as a consequence of homogenizing the tissue in a nondenaturing buffer. Wolpert and Macko (1991) developed antisera to the *in vitro*-labeled 100 kDa protein from the susceptible oat genotype and these preparations reacted with the *in vivo*-labeled 100 kDa protein from the susceptible genotype. The antibodies directed against the 100 kDa protein isolated from the susceptible genotype reacted with the 100 kDa protein from the resistant genotype. The results suggest that the proteins from the susceptible and resistant genotypes may differ by only a single amino acid residue, or may be structurally identical (Wolpert and Macko, 1991).

The 100 kDa VBP may be the site of action (Wolpert et al., 1994). A 3.4 kb cDNA clone was isolated that when subjected to a 400 bp 5′ deletion was capable of directing the synthesis of a protein in *Escherichia coli*, which reacted to an antibody specific for the 100 kDa protein.

Peptide mapping, by limited proteolysis, indicated that the protein directed by the cDNA is the 100 kDa protein (Wolpert et al., 1994).

The 100 kDa VBP could be detected in many plant species such as *Arabidopsis*, pea, tomato, wheat, sorghum, maize, and oats (Wolpert et al., 1994). It could be detected in various organisms tested such as *Chlorella*, bovine lever, and *E. coli* (Wolpert et al., 1994). Thus the 100 kDa protein is somewhat universal (Loschke et al., 1994).

DNA sequence analysis of the 3427 bp cDNA for the VBP revealed a 3096 bp open reading frame coding a protein containing 1032 amino acid residues (Wolpert et al., 1994). A comparison of the deduced amino acid sequence of the 100 kDa VBP cDNA and the pea cDNA indicated 83% amino acid identity. The pea cDNA encodes for the P protein component of the glycine decarboxylase multienzyme complex. The mature form of the 100 kDa VBP also displays 71% amino acid similarity to the P protein of *E. coli*, humans, and chicken (Wolpert et al., 1994). The glycine decarboxylase is found in animals, plants, and prokaryotes and is located in the mitochondria of eukaryotic organisms (Kume et al., 1991). Hence, Wolpert et al. (1994) concluded that the 100 kDa VBP is the P protein component of glycine decarboxylase based on the widespread species distribution of the 100 kDa VBP, suggesting a common and basic metabolic function of the 100 kDa VBP, and nucleotide sequence homology of the cDNA for the 100 kDa VBP with the P protein component of glycine decarboxylase. In plant tissues, glycine decarboxylase has a major role in the recovery of carbon and energy due to photorespiration, and mutations in the photorespiration are lethal to plants. The effect of victorin interaction with the P protein may be the inhibition of glycine decarboxylase activity, and this would cause cell death in green tissue (Wolpert et al., 1994). Another VBP, a 15 kDa protein, has also been isolated (Wolpert et al., 1995). This protein was also a component of the multienzyme complex, glycine decarboxylase (Wolpert et al., 1995).

A putative receptor protein for dothistromin, the toxin produced by *Dothistroma pini*, the pathogen of *Pinus radiata*, has been identified (Jones et al., 1995). Results of experiments with treatments that protect sorghum seedlings against the effects of peritoxin produced by *Periconia circinata* (e.g., proteinase, heat shock, inhibitors of protein synthesis, and biotinylation of membrane proteins) suggest that disease symptoms result from an interaction of peritoxin with a proteinaceous receptor (Dunkle and Macko, 1995).

The plasmalemma of susceptible sugarcane contains a protein of 48 kDa that specifically binds to the toxin produced by *H. sacchari* (HS-toxin) (Kenfield and Strobel, 1981). Plasmalemma of resistant sugarcane has a similar protein that differs by four amino acid residues and does not bind toxin (Strobel and Hess, 1974; Strobel et al., 1975). However, some nonhosts of *H. sacchari* (tobacco and mint) bind as much or more ^{14}C-labeled toxin preparation as does susceptible sugarcane (Kenfield and Strobel, 1977). Conclusive evidence for the role of toxin receptors in susceptibility of host plants to pathogens is still lacking. In fact, toxin receptors or affinity sites may be existing in most of the plant tissues and host specificity may be an event after the toxin binding (Akimitsu et al., 1992).

8.5.3 Susceptible Tissues May Be More Sensitive to Toxins

Susceptible tissues may be highly sensitive to certain (host-specific) toxins. Tomato cultivars resistant to *Alternaria alternata* f. sp. *lycopersici* are at least 1000-fold less sensitive to the AAL-toxin than susceptible cultivars (Gilchrist and Grogan, 1976). The toxins at lower concentrations induce disease symptoms in the susceptible plants, whereas at higher concentrations they induce disease symptoms in the resistant varieties also (Vidhyasekaran et al., 1986; Lalitha et al., 1989; Kohmoto et al., 1991). However, it should be noted that nonhost-specific toxins at low concentrations affect both susceptible and resistant plants almost equally and many pathogens are known to produce nonspecific toxins. Hence, sensitivity of

host tissues to the toxins may not be an important factor in their susceptibility to the pathogens.

8.5.4 Specific Protein Synthesized after Toxin Exposure May Confer Host Specificity

Treatment of sorghum seedlings with *P. circinata* toxin (PC-toxin) induces visible symptoms of the disease only in susceptible genotypes. These symptoms were associated with a loss of electrolytes from roots into the surrounding medium (Dunkle, 1978; Dunkle and Wolpert, 1981) and the enhanced synthesis of a family of proteins with M_r of 16 kDa and their respective mRNAs only in susceptible genotypes of the host (Wolpert and Dunkle, 1983; Traylor et al., 1988). Treatments which prevent the synthesis of 16 kDa proteins protect seedlings against the effects of PC-toxin (Wolpert and Dunkle, 1983). The treatments that induce the synthesis of 16 kDa proteins elicit the same disease symptoms as the toxin (Traylor et al., 1987). The results suggest that the 16 kDa proteins may confer host specificity.

However, treatment with *Colletotrichum graminicola* elicitor also enhanced the synthesis of the 16 kDa proteins in sorghum seedling root tips. Synthesis of the proteins was enhanced in seedlings pretreated with elicitor and then treated with toxin and in seedlings pretreated with toxin and subsequently treated with elicitor. The enhanced synthesis of the 16 kDa proteins was not observed in seedlings pretreated with toxin and transferred with water for 12 h before pulse-labeling with ^{3}H leucine. The results suggest that the seedlings recover from the effects of the toxin or that the effects are temporary and subside. The effects of the elicitor were genotype nonspecific; the enhanced synthesis of the 16 kDa proteins was induced in root tips of both susceptible and resistant seedlings (Ransom et al., 1992). The induction of enhanced synthesis of 16 kDa proteins may be a general stress response or a response to a generally toxic preparation. The elicitor is also phytotoxic like the toxin (Ransom et al., 1992). Further these 16 kDa proteins have been detected in corn, barley, oat, rice, water hyacinth (*Eichhornia crassipes*), and spider plant (*Chlorophytum* sp.) (Ransom et al., 1994). The results suggest that the 16 kDa proteins are conserved proteins in several plant species. These studies do not confirm the importance of 16 kDa proteins in conferring susceptibility in plants to pathogens.

8.5.5 Proteins of Susceptible Hosts May Enhance Potential of Pathogens to Produce Toxins

Presence of susceptible host proteins appears to enhance virulence of pathogens. *Cercospora kikuchii*, the soybean pathogen produces the important pathogenicity factor cercosporin in infected soybeans. The pathogen responds to soybean proteins with increased cercosporin production and changes in transcript accumulation. It suggests an alteration in fungal gene expression in the presence of host plant substances (Ehrenshaft and Upchurch, 1993).

8.5.6 Sucrose Influx May Have Correlation with Sensitivity to Toxin

Sucrose transport system may also be involved in conferring sensitivity to toxin in susceptible tissues. Sucrose transport system in tomato isolines resistant or susceptible to *A. alternata* f. sp. *lycopersici* varied significantly. The young leaflets of the susceptible variety possessed an endogenous sucrose uptake rate three times higher than the young leaflets of the resistant variety. Thus a physiological difference in sugar transport associated with disease resistance mechanism has been identified in tomato (Moussatos et al., 1993). The pathogen produces a host-specific toxin, AAL-toxin. AAL-toxin significantly affected sucrose transport. AAL-toxin treatment reduced sucrose influx to discs of the young tomato leaves. The resistant

isoline exhibited a recovery after 4 h, whereas the susceptible isoline continued to decline. The toxin moves in the phloem and one of the disease symptoms associated with AAL toxin and *A. alternaria* f. sp. *lycopersici* in whole plants is browning of phloem tissue. High initial or stimulated sucrose transport rates would function to increase uptake of AAL-toxin. The susceptible isolines showed higher initial sucrose transport (Moussatos et al., 1993). In general, sensitivity to AAL-toxin involved a dramatic perturbation of sucrose influx, whereas resistance had a reduced perturbation from a lower initial sucrose influx rate. The toxin causes reduction in sucrose transport and a higher initial uptake of the toxin along with the sucrose transport system in the susceptible isoline would have caused more perturbation in the sucrose transport (Moussatos et al., 1993).

p-Chloromercuribenzene sulfonic acid (PCMBS) is a sucrose transport inhibitor. PCMBS treatment resulted in more interveinal necrosis in the susceptible isoline than in the resistant isoline in the absence of AAL-toxin. The results suggest that sucrose transport and factors that regulate sucrose retrieval are directly involved in AAL-toxin-induced metabolic changes that culminate in cell death and the sensitivity to the toxin depends upon the sucrose transport system (Moussatos et al., 1993). Thus a physiological process may be involved in the host specificity. Sucrose transport system may be linked to ethylene biosynthesis (Moussatos et al., 1994). AAL-toxin induced ethylene biosynthesis in susceptible tomato cultivar and not in resistant isoline. Dihydroorotic acid, an intermediate in pyrimidine biosynthesis, abolished the AAL-toxin-induced increase in 1-aminocyclopropane-1-carboxylic acid, the precursor of ethylene. Addition of *N*-(phosphonacetyl)-L-aspartate, a specific inhibitor of pyrimidine biosynthesis, elicited interveinal necrosis resembling AAL-toxin treatment (Moussatos et al., 1994). These results suggest a metabolic linkage between the action of sucrose transport, ethylene biosynthesis, pyrimidine metabolism, and cell death and the toxin had an important role in the metabolic linkage. It has been suggested that this cascade of reactions induced by the toxin may be involved in determination of host specificity.

8.5.7 Transport of Toxin to Cytoplasm May Occur Only in Susceptible Interactions

Toxins may cause cell death resulting in development of necrotic symptoms. Toxin has to be transported to cytoplasm to cause cell death. This type of transport may occur only in susceptible interactions. Ptr ToxA, the toxin produced by the wheat tan spot pathogen (*Pyrenophora tritici-repentis*), was found to be internalized in susceptible wheat varieties, not in resistant ones (Manning and Ciuffetti, 2005). Once internalized, ToxA localized to cytoplasmic compartments and to chloroplasts. ToxA protein was capable of crossing the plant plasma membrane from the apoplastic space to the interior of the plant cell in the absence of a pathogen. It has been suggested that ToxA sensitivity gene present in only susceptible plants is most likely related to protein import, which may be absent in resistant wheat cultivars (Manning and Ciuffetti, 2005).

8.6 CONCLUSION

Necrotrophic pathogens are known to cause different types of diseases, such as leaf spots, leaf blights, wilts, root rots, stem rots, and ear rots. These pathogens produce host-specific and nonspecific toxins and these toxins reproduced typical symptoms of these diseases. The important function of these toxins appears to suppress defense mechanisms of host plants, which may facilitate invasion of the host tissues by pathogens. Loss of defense response helps even nonpathogens to cause infection.

Toxins cause changes in plant cell membrane permeability. Increase in the electrolyte leakage is the earliest symptom induced by fungal pathogens and this leakage leads to

necrosis. The toxin may not cause general membrane disruption but may inhibit specific membrane transport system. The leakage may occur through the membrane matrix possibly through shuttle-type carrier.

H^+-ATPase pumps protons across the plasma membrane. Some toxins stimulate the activity of H^+-ATPase. This stimulation may not be due to de novo synthesis of H^+-ATPase but due to proteolytic removal of a part of the C-terminal end of the 100 kDa H^+-ATPase. The toxins may inhibit calmodulin activity affecting calcium transport. Depolarization of membrane potential occurs due to toxin application. Depolarization may be due to reduced ATP levels.

The pathogens may induce changes in phospholipid contents of the host cell membrane. The toxins activate phospholipases resulting in reduction in phospholipid content. The removal of phospholipids from membranes results in cell death. Hydroxyl radicals and superoxide anions are induced to accumulate due to fungal infection. They may cause disruption of lysosome membrane. Some toxins cause peroxidation of plant lipids. They release singlet oxygen and superoxide anions which are toxic to plant cells.

Mitochondrial membranes are also affected by toxins. The electron transfer occurs across the inner membrane of mitochondrion, which drives oxidative phosphorylation producing cellular energy. The toxins inhibit ATP synthesis which results in suppression of defense mechanisms of the host.

Membrane dysfunction generally leads to suppression of defense mechanisms of the host. It also induces efflux of nutrients to the pathogen. In resistant and incompatible host–pathogen interactions, the action of the toxins is suppressed. Detoxification of the toxins is an important host-defense mechanism. Some host enzymes detoxify the toxins. A resistance gene in maize, *Hm1*, encodes a toxin-degrading enzyme. Absence of toxin-binding sites in resistant varieties has been suggested; but convincing evidences are lacking. The host specificity to the toxins may be due to an event after the toxin binding.

Membrane dysfunction followed by cell death may nullify all defense mechanisms of the host favoring development of the pathogen in host tissue. Toxins may play an important role in fungal pathogenesis. Detoxification of these toxins may be a good approach to manage these diseases (Vidhyasekaran, 2007).

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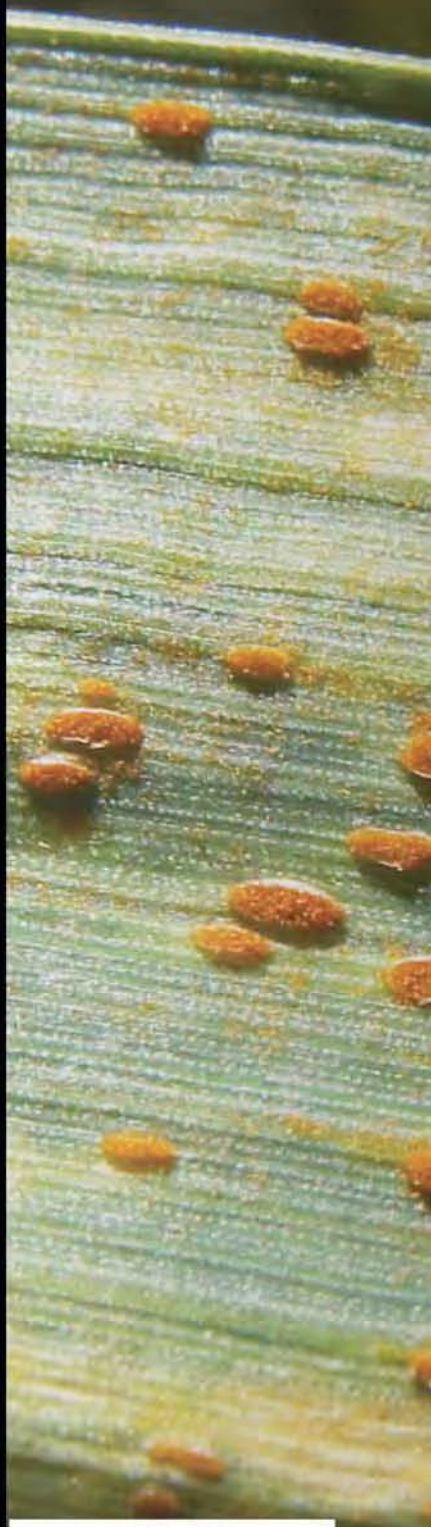

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