A SIMPLE METHOD FOR ISOLATION OF DNA FROM PLANTS SUITABLE FOR LONG TERM STORAGE AND DNA MARKER ANALYSIS

E. Nalini and N. Jawali
Molecular Biology and Agriculture Division
Bhabha Atomic Research Centre

and

S.G. Bhagwat
Nuclear Agriculture and Biotechnology Division
Bhabha Atomic Research Centre

Abstract

DNA based markers have extensively been used in genome mapping. Restriction Fragment Length Polymorphism (RFLP) markers were the first to be used for this purpose. With the advent of Taq DNA Polymerase, PCR-based markers have become popular since they require less time, effort and expense for molecular mapping. PCR based studies such as in construction of a linkage map, estimating genetic diversity in a germplasm etc. involves a limited number of samples (50-200). Such studies require isolation of sufficiently pure DNA, which is suitable both for the purpose as well as for preserving the same for a considerable period of time. Although the DNA needed per reaction in PCR based markers is very low, the number of PCR reactions to be performed is large and hence a good amount of DNA would be needed for such studies. We have developed a simple method for isolation of DNA from plant tissue (leaf or seed). The method is suitable for isolating DNA from a small to medium number of plant samples. The DNA can be stored for a longer duration. The method involves extraction of DNA using a buffer (pH 8.0) containing Tris (100 mM), EDTA (20 mM), 7 M urea, 0.5 M NaCl and 0.1% β- mercaptoethanol, followed by purification of DNA with phenol, chloroform and Isoamlyalcohol and finally precipitation of DNA by sodium acetate and isopropanol. The protocol is simple and fast as it does not involve time consuming steps such as incubation at higher temperatures, does not require expensive chemicals such as proteinase K, liquid nitrogen etc., and no special equipment is needed.

Introduction

In recent years, considerable emphasis has been placed on the development and use of molecular markers for a variety of objectives. One of the most extensive uses of the molecular markers has been the development of genetic and physical maps of genomes. Molecular markers are also used for a wide range of purposes such as germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis, marker-assisted selection, mapping Quantitative Trait Loci (QTL) etc. (Gupta et al, 1999) Till recently this was achieved by Restriction Fragment Length Polymorphism (RFLP) analysis. However, the advent of thermo stable *Taq* DNA Polymerase has led to the development of several PCR- based markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR),

Sequence tagged Microsatellite Site (STMS), Amplified Fragment Length Polymorphism (AFLP), etc. Some of the advantages PCR based markers have over RFLP are that they are faster, easier, need less amount of DNA per assay and high purity of DNA is not an absolute requirement as compared to RFLP.

The quality and quantity of DNA required depends upon the objective. In marker assisted selection the number of samples are high but the number of PCR reactions to be performed are few and hence the amount of DNA needed is small and need not be stored for a long duration [McCarthy et al (2002), Guo-Liang Wang et al (1993)]. On the other hand in case of QTL mapping or population studies of RILS, F_2 population etc., the number of PCR reactions to be performed are large and therefore considerable amount of purified DNA would be needed which can be stored for a longer duration.

Although several protocols are available for DNA isolation in wheat [(Stein et al (2001), Dellaporta et al (1983), Sharp, et al (1988), Murray et al (1980), Chunwongse et al (1993), McCarthy et al (2002), Clarke et al (1989), Benito et al (1993), etc.)] and in other plant species [Krishna and Jawali (1997), Hong Wang et al (1993), Xiao-Dong Wong et al (1996), Lange et al (1998), Kamalay et al (1990), Francois Guidet (1994), etc.] all of them either involve elaborate and time consuming steps such as hours of incubation at 65ºC or 37ºC or use of expensive chemicals such as proteinase K, liquid nitrogen etc. Some methods involve use of specific equipment designed especially for DNA isolation. Although the method by McCarthy et al (2002) for DNA isolation does not involve incubation and specific equipment, it is suitable for isolation of DNA from seed material and not for leaf tissue.

In this paper we describe a DNA isolation method suited for isolation of reasonably pure DNA in sufficient amount from wheat leaves that can be stored for a longer duration and lasting for several PCR reactions. The method is rapid involving no incubation steps, expensive chemicals and specific equipment.

Materials and Methods

Materials

Segregating F_2 population obtained from a cross Sonalika X Kalyansona (bread wheat: *Triticum aestivum L* .) was used for this study. Fresh as well as frozen leaf tissue was used in this method. Leaves from one-month-old field grown plants were harvested and brought on ice to laboratory. The leaves were thoroughly washed with tap water and rinsed with distilled water, blot dried and weighed. The leaves were either stored at –70˚C or used directly for extraction.

The chemicals needed for isolation of DNA viz. Tris, EDTA were obtained from Sigma and Sodium chloride, urea, SDS, Isopropanol, sodium acetate, chloroform, Isoamlyalcohol and phenol were of the analytical grade. PCR grade dNTPs were procured from Roche chemicals. Enzyme Taq DNA Polymerase, 10X-assay buffer for Taq DNA Polymerase and Magnesium chloride were obtained from Bangalore Genei Pvt. Ltd. The primers were obtained from Gibco BRL. The agarose used for gel electrophoresis was also obtained from Sigma. The Hoechst dye used for DNA estimation was procured from Amersham Pharmacia biotech.

Methods

DNA from the samples was isolated as follows. Around 0.5 g of leaf tissue was placed in a mortar and homogenized with 2 ml of extraction buffer. The extraction buffer (pH 8.0) consisted of 100 mM Tris, 20 mM EDTA, 0.5 M NaCl, 7 M Urea, 0.1% β-mercaptoethanol and 2% SDS. Long fibers of the tissue were retained back after crushing and the homogenate was transferred to a 2 ml-microfuge tube. An equal volume of phenol:chloroform :Isoamlyalcohol (25:24:1) was added to the tubes and mixed well by gently

shaking the tubes. The tubes were centrifuged at room temperature for 15 min at 15,000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of chloroform: Isoamlyalcohol (24:1) was added and mixed.

The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 15,000 rpm was transferred to a new tube. The DNA was precipitated from the solution by adding 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol. After 15 min of incubation at room temperature the tubes were centrifuged at 4ºC for 15 min at 15,000 rpm. The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air-dried. The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM). To remove RNA 5 µl of DNAse free RNAse A (10 mg/ml) was added to the DNA.

Estimation of DNA

The amount of DNA in the RNAse treated mixture was estimated by measuring the fluorescence emission using the dye Hoechst. Since the dye binds specifically to double stranded DNA, the presence of small fragments of degraded RNA in the sample did not interfere with the measurements. The fluorescence intensity was measured at emission maximum of 546 nm using the fluorimeter Hoefer DyNA Quant 200. The amount of DNA was estimated by comparing the emission intensity obtained using a known amount of standard DNA (100 µg/ml) (provided by Amersham) which acts as the reference. The DNA was also observed on 2% agarose gel by electrophoresis using 1X TBE buffer. The DNA fragments were stained with ethidium bromide and viewed under UV light and subsequently photographed. The quantity of the DNA was estimated against a marker (λHindIII, 100 ng/µl) which was also loaded in the gel along with the DNA. The DNA was further used for PCR.

PCR Analysis

AP-PCR ANALYSIS: AP-PCR amplification was carried out in a volume of 25 µl containing 100 ng of template DNA, 2 mM $MgCl_2$, 25 pmoles of primer SS26.1 (5'-GAA\GGG\TAA\TTC\ AGA\GCC\A -3'), 2.5 µl of 10X assay buffer which includes (10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% Gelatin), 0.2 mM each of dNTPs and 1 unit of Taq DNA Polymerase. The PCR amplification was carried out in an Eppendorf Mastercycler-Gradient thermal Cycler as follows: 1 cycle of 5 min at 94ºC, 5 min at 45ºC and 5 min at 72ºC, and 35 cycles of 1 min at 94ºC, 1 min at 45ºC and 1 min at 72ºC, followed by a final 10 min extension at 72ºC.

ISSR ANALYSIS: PCR amplification of template DNA using a 3′ anchored ISSR primer was carried out in a volume of 25 µl. The reaction mixture contained 100 ng of template DNA, 2 mM $MgCl_2$, 25 pmoles of the primer SSR11.2 (5'-GAC\AGA\GAC\AGA\GAC\A (C\T)C -3') 2.5 µl of 10X assay buffer which included (10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% Gelatin), 0.2 mM each of dNTPs and 1 unit of Taq DNA Polymerase. The PCR amplification was carried out on an Eppendorf Mastercycler-Gradient thermal Cycler as follows: 1 cycle of 5 min at 94ºC, 5 min at 50ºC and 5 min at 72ºC, 45 cycles of 1 min at 94ºC, 1 min at 50ºC and 1 min at 72ºC followed by a final 10 min incubation at 72ºC.

The PCR products obtained by AP-PCR and ISSR were separated on 2% agarose gel by electrophoresis using 1X TBE buffer. The DNA fragments were stained with ethidium bromide and viewed under UV light and subsequently photographed (Sambrook et al 1989).

Results and Discussions

To check the efficiency and reliability of the procedure, the DNA was isolated from leaves of 150 wheat plants. The amount of the leaf sample

used varied from 0.5 g to 0.6 g. By using mortar and pestle one person could easily isolate DNA from 48 samples in a day. On an average 200 µg of DNA was obtained and significant positive correlation was observed between the leaf weight in the range 0.5 g to 1.0 g and the DNA isolated indicating the reliability of the protocol. The efficiency of the protocol is indicted by the quantity of the DNA obtained which is comparable to the yield obtained by other extraction protocols [Stein et al (2001), Clarke et al (1989)]. Considering that 100 ng is required for one PCR reaction the amount of DNA obtained is sufficient for 2000 reactions.

The suitability of the DNA isolated as a template in PCR amplification reactions such as AP-PCR and ISSR was analyzed. The AP-PCR amplification product profiles using 19mer primers using DNA as template from F_2 plants was analyzed. The DNA isolated from all the 150 plants yielded consistently amplified products. As an example the products obtained from the parents and some F_2 plants using primer SS26.1 are shown in Fig.1. A polymorphic band was detected between the parents which segregated among the F_2 individuals in the ratio 2.75:1 that is close to the expected value of 3:1 with a chi-square value of 0.32 at significance level of 1%. The results showed that the DNA isolated by the above method is reliable and sufficiently pure to be used in AP-PCR analysis to study genetic segregation.

The results also showed that the DNA isolated by this protocol produced consistent and reliable ISSR profiles with several 3′ anchored ISSR primers. Fig.2 shows an example of ISSR profiles obtained from F_2 plants using the primer SSR11.2 As in the case of AP-PCR analysis, the bands that were polymorphic between the parents showed segregation in the F_2 population. DNA isolated using the above method and stored at 4ºC for over a year was used in AP-PCR and ISSR analysis to check its quality. The results showed that all the 150 samples yielded amplification products with their profiles being the same as obtained earlier.

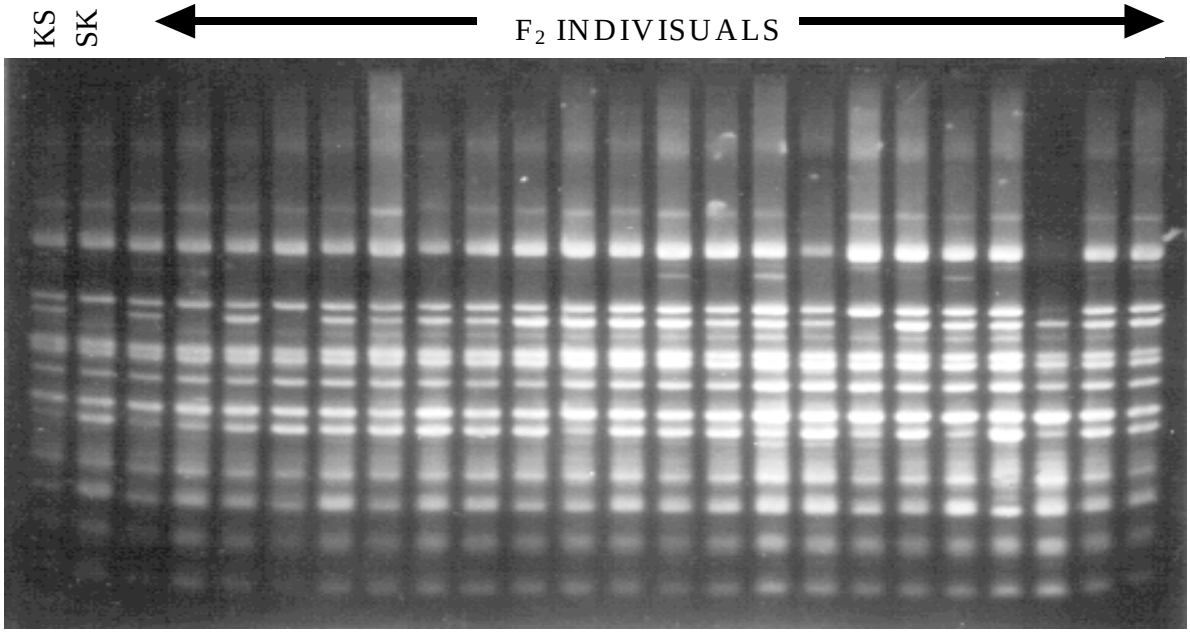

Fig. 1 AP- PCR analysis of 22 F_2 plants of wheat (Triticum aestivum) derived from a cross between Sonalika (Sk) X Kalyansona (Ks). The PCR products were analyzed as indicated in Materials and Methods. The polymorphic band showing segregation has been indicated.

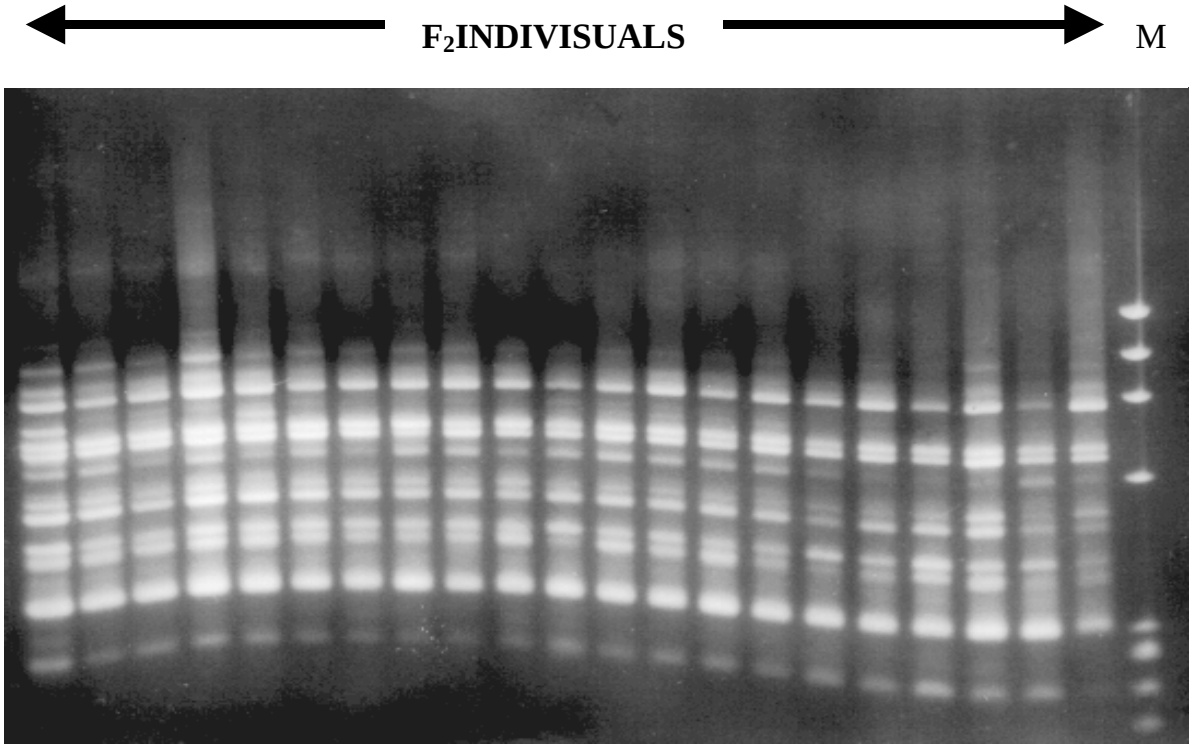

Fig. 2 ISSR analysis of 21 F_2 plants of wheat (Triticum aestivum) derived from a cross between Sonalika X Kalyansona. Lane M: Φx174 DNA digested with Hae III. The polymorphic band showing segregation has been indicated.

DNA isolated from plants often contains certain compounds that inhibit PCR amplification reactions (Krishna and Jawali 1997, McCarthy et al 2001, etc.). In this method Sodium chloride and β-mercaptoethanol were added in the extraction buffer to take care of the polysaccharides and the polyphenols in the leaf tissue which are the compounds which could contribute to the inhibition of the DNA amplification during PCR reactions. Hence there were no additional steps needed for the removal of these compounds [McCarthy et al (2002), Maliyakal, E.J et al 1992, Oard, J.H. et al (1992) Shirzadegan et al (1991)]. The presence of the enzyme RNAse A in the DNA solution does not hamper the amplification. Hence repurification of the DNA is not needed. (Sharp et al 1988).

While this method was designed especially for isolating DNA from leaf tissue of hexaploid wheat for marker analysis, it was also found to be suitable for wheat seeds, and from leaf and seed material of mungbean, mustard, sesbania. (data not shown). Our experience showed that the DNA isolation protocol could be successfully applied to a broad range of plant species.

The advantages of this procedure over existing methods are its simplicity, rapidity of isolation and reduction in the cost. DNA could be isolated from 48 samples by one person within a day as the protocol did not involve time consuming steps such as incubation at 65ºC or 37ºC. In addition the protocol did not require liquid nitrogen that may not be easily available in many laboratories. All chemicals used are inexpensive and equipments used are available in the laboratories. The method is best suited for isolating DNA from a small to medium sized population, and where storing for a longer duration of time is required.

References

1. Benito, c., Figueiras, A.M., Zaragoza, C., Gallego, F.J. and de la Pena, A. 1993. Rapid identification of Triticeae Genotypes from single seeds using the polymerase chain reaction. Plant Mol. Bio. 21:181-183.
2. Chunwongse, J., Martin, G.B. and Tanksley, S.D. 1993. Pre-germination genotypic screening using PCR amplification of half-seed. Theor. Appl. Genet. 86:694-698.
3. Clarke, B.C., Moran, L.B. and Appels, R. 1989. DNA analysis in wheat breeding. Genome. 32:334-339.
4. Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983. A plant DNA Minipreparation version 2. Plant Mol. Bio. Rep. 1: 19-22.
5. Francois Guidet. 1994. A powerful new technique to quickly prepare hundreds of plant extracts for PCR and RAPD analyses. Nucl. Acid Res. Vol 22, No. 9: 1772-1773.
6. Guo-Liang Wang, Rod, A.W. and Paterson, A.H. 1993. PCR amplification from single seeds, facilitating DNA marker-assisted breeding. Nucl. Acid Res. Vol 21, No 10: 2527.
7. Gupta, P.K., Varshney, R.K., Sharma, P.C. and Ramesh, B. 1999. Molecular markers and their applications in wheat breeding. Plant Breeding. 118: 369-390.
8. Hong Wang, Meiqing Qi, and Cutler, A.J. 1993. A simple method of preparing plant samples for PCR. Nucl. Acid Res. Vol21, No 17: 4153-4154.
9. Kamalay, J.C., Tejwani, R. and Rufener II, K.G. 1990. Isolation and analysis of Genomic DNA from single seeds. Crop. Sci. 30:1079-1084.
10. Krishna, T.G. and Jawali, N. 1997. DNA Isolation from single or half seeds suitable for Random Amplified Polymorphic DNA Analyses. Anal. Biochem. 250: 125-127.
11. Lange, D.A., Penuela, S., Denny, R.L., Mudge, J., Concibido, V.C., Orf, J.H. and Young, N.D. 1998. A plant DNA isolation protocol suitable for polymerase chain reaction based marker-assisted breeding. Crop. Sci. 38: 217-220.
12. Maliyakal, J.E. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucl. Acid Res. Vol 20, No 9: 2381.
13. McCarthy, P.L. and Berger, P.H. 2002. Rapid identification of transformed wheat using a half-seed PCR assay prior to germination. Biotechniques. 32: 560-564.
14. Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucl. Acid Res. Vol 8, 19: 4321-4325.
15. Oard, J.H. and S. Dronavalli. 1992. Rapid isolation of rice and maize DNA for analysis by random-primer PCR. Plant Mol. Biol. Rep. 10:236-241.
16. Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: A laboratory Manual. II ed^{n}. Cold Spring Harbour Laboratory. Press, Cold Spring Harbour, N.Y.
17. Sharp, P.J., Kreis, M., Shewry, P.R. and Gale, M.D. 1988. Location of β-amylase sequences in wheat and its relatives. Theor. Appl. Genet. 75: 286-290.
18. Shirzadegan, M., Christie, P. and Seemann, J.R. 1991. An efficient method for isolation of RNA from tissue cultured plant cells. Nucl. Acid Res. 19:6055.

19. Stein, N., Herren, G. and Keller, B. 2001. A new DNA extraction method for high-throughput marker analysis in a large genome species such as *Triticum aestivum*. Plant Breeding. 120: 354-356.
20. Xiao-Dong Wang, Zi-Ping Wang, and Yu-Ping Zou.1996. An Improved Procedure for the Isolation of Nuclear DNA from Leaves of Wild Grapevine Dried with Silica Gel. Plant Mol. Bio. Rep. 14(4)

This paper was awarded the second prize for Best Poster presentation at the National Symposium on "The Role of Biotechnology in the Development of the Indian Economy" at Vaze College, Mumbai, during February 22-23, 2003

About the authors ...

Ms Nalini Eswaran, *SRF, DAE-Mumbai University collaborative fellowship, is a first rank holder in M.Sc. degree in Life Sciences (Biomacromolecules, specialization) from Mumbai University in the year 2001. She was awarded the DAE-Mumbai University collaborative fellowship for doing her Ph.D., in 2001. She is carrying out her research project entitled "Genetic Linkage Map of Bread Wheat with an emphasis on Genes Controlling Plant Morphology" in the Molecular Biology Division of BARC under the guidance of Dr Narendra Jawali. She was presented with a 'Certificate of Appreciation' and a memento at the above symposium.*

Dr Narendra Jawali *obtained M.Sc., Degree in Biochemistry from Central College, Bangalore University, Bangalore in 1975. He joined the 19th batch of Training School in the Biology and Radiobiology discipline. He obtained his Ph.D. in 1988 from Mumbai University. He is working in the Molecular Biology Division of the Bioscience group, BARC. His research interests are plant and microbial molecular genetics.*

Dr S.G. Bhagawat *did his M.Sc. in Botany from Poona University, Pune, in the year 1975. He joined the 19th batch of Training School in the Biology and Radiobiology discipline. He did his PhD. on "Grain protein variation in wheat". Currently, he is working in the Nuclear Agriculture and Biotechnology Division of the Bioscience group, BARC. His research interests are genetics and genetic improvement of wheat.*