Full Length Research Paper

Genomic DNA extraction method from pearl millet (*Pennisetum glaucum*) leaves

Sghaier ZIDANI[1*], Ali FERCHICHI[1] and Mohamed CHAIEB[2]

[1]Laboratoire d'aridoculture et cultures oasiennes, Institut des Régions Arides, Elfjè, 4119 Médenine, Tunisie.
[2]UR-Biologie et écophysiologie des milieux arides, Faculté des Sciences de Sfax, BP 802, 3018 Sfax, Tunisie.

DNA extraction is difficult in a variety of plants because of the presence of metabolites that interfere with DNA isolation procedures and downstream applications such as DNA restriction, amplification, and cloning. Here we describe a modified procedure based on the hexadecyltrimethylammonium bromide (CTAB) method to isolate DNA from tissues containing high levels of polysaccharides. The procedure is applicable to both dry and fresh leaves of *Pennisetum glaucum*. This modified CTAB (2%) protocol include the use of 1.4 M NaCl, 1% polyvinylpyrrolidone (PVP), 1% β-mercaptoethanol and 100% ethanol in the extraction as well as reducing the centrifugation times during the separation and precipitation of the DNA. This method solved the problems of DNA degradation, contamination, and low yield due to binding and/or coprecipitation with starches and polysaccharides. The isolated DNA proved amenable to PCR amplification and restriction digestion. The technique is fast, reproducible, and can be applied for SSR-PCR markers identification.

Key words: *Pennisetum glaucum*, genomic DNA isolation, leaves.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) is a member of the Gramineae family, and it is the staple food and fodder crop of millions of poor rural families in the hottest and driest dryad agricultural environments of Asia and Africa. Although grain and stover of this crop are not commercially important commodities, as most are consumed in the homesteads where they are produced, crop losses are economically important. Indeed, in some of the hottest, driest regions of India and Africa, pearl millet is the only cereal that can be grown and so plays a critical role in food security. In these harshest of environments, grains yields is severely limited by drought

and disease (FAO and ICRISAT, 1996).

The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar identification (Nybom, 1990). Isolation of plant nucleic acids for use in Southen blot analysis, polymerase chain reaction (PCR) amplifications, restriction fragment length polymorphisms (RFLPs), arbitrary primed DNA amplifications (RAPD, SSR-PCR), and genomic library construction is one of the most important and time-consuming steps. The degree of purity and quantity varies between applications. A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. The procedure should also be quick, simple and cheap. The extraction process involves, first of all, breaking or digesting away cell walls in order to release the cellular constituents. This is followed by disruption of the cell membranes to release the DNA into the extraction buffer. This is normally achieved by using detergents such as sodium dodecyl sulphate (SDS) or cetyl-methyl

*Corresponding author. E-Mail: sghaier.zidani@fss.rnu.tn.

ammonium bromide (CTAB). The released DNA should be protected from endogenous nuclease. EDTA is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases, for this purpose. The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and pigments which may interfere with the extracted DNA and difficult to separate (Puchooa, 2004). Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat-treated RNase A. Polysaccharide-like contaminants are, however, more difficult to remove. They can inhibit the activity of certain DNA-modifying enzymes and may also interfere in the quantification of nucleic acids by spectrophotometric methods (Wilkie et al., 1993). NaCl at concentrations of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson et al., 1993). The concentration ranges mentioned in literature varies between 0.7 M (Clark, 1997) and 6 M (Aljanabi et al., 1999) and is dependent on the plant species under investigation. Some protocols replace NaCl by KCl (Thompson and Henry, 1995).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Various plants contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification. Antioxidants are commonly used to deal with problems related to phenolics. Examples include β-mercaptoethanol, Bovine Serum Albumin, sodium azide and PVP amongst others (Dawson and Magee, 1995; Clark, 1997). Phenol extractions when coupled with SDS are also helpful. However, with plants having a high content of polyphenolics, SDS-phenol tends to produce low yields of DNA (Rezaian and Krake, 1987). Several laboratories involved in the project performed side-by-side comparison of all four DNA isolation procedures. Two methods are based on classical principles of lyses and purification. The first one is the commonly used protocol of Doyle and Doyle (1990), which has been used successful in many plant species. The second one, from Guillemaut and Maréchal-Drouard (1992), originated from Dellaporta et al. (1983) and was modified according to Ziegenhagen et al. (1993).

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Three widely-used PCR-based markers are RAPDs (Williams et al., 1990), SSRs or microsatellites (Tautz, 1989), and AFLPs (Vos et al., 1995). Each marker technique has its own advantages and disadvantages. The choice of a molecular marker technique depends on its reproducibility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation has

low cost and labour requirements and high reliability. Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available (Zietkiewicz et al., 1994). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite.

MATERIALS AND METHOD

Several experiments were carried out, however, only the optimised protocol is described here.

Plant material

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) Seeds were collected from governorate of Medenine in Southern Tunisia and were aseptically germinated at 30°C for 7 days. The tissue used is fresh or dried leaves.

Solutions

An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidone (PVP), 1% β-mercaptoethanol (v/v), and 3 M sodium acetate (pH 5.2), was prepared. In addition, chloroform : isoamylalcohol (24:1), 75% and 100% ethanol and a TE buffer consisting of 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0) were also prepared.

DNA isolation protocol

Leaves were harvested and frozen immediately in liquid nitrogen. The use of lyophilized tissues offers several advantages. Dry tissue can be efficiently disrupted while the DNA is unhydrated and can be stored for several years with little loss of DNA quality. A 0.3 g of leaf sample was ground in liquid nitrogen using a mortar and pestle. The pulverized leaves were quickly transferred to liquid nitrogen. 2% of CTAB buffer (1 ml) containing 1% (v/v) β-mercaptoethanol and 1% PVP was quickly added to the microcentrifuge tube (2 ml) and stirred with a glass to mix. The tube was incubated at 60°C for 30 min with frequent swirling. An equal volume of chloroform : isoamylalcohol (24:1) was added and centrifuged at 10 000 rpm and 4°C for 15 min to separate the phases. The supernatant was carefully decanted and transferred to a new tube. The above steps, beginning with the addition of chloroform: isoamylalcohol (24:1) and ending with decanting of supernatant, were repeated twice. The supernatant was precipitated with 2/3 volume of ethanol. The precipitated nucleic acids were collected and washed twice with the buffer (75% ethanol, 3 M sodium acetate, TE) (The tubes should not be shaken vigorously because DNA is very vulnerable to fragmentation at this step). The pellets were air dried and resuspended in TE. The dissolved nucleic acids were brought to 1.4 M NaCl and reprecipitated using 2 volumes of 75% ethanol[3] (If the pellet obtained was hard to re-suspend, this step was repeated one more time. Also, when colored DNA pellet was obtained, the color can be removed using 2-3 extractions with ethanol.). The pellets were washed twice using 100% ethanol[4], dried and re-suspended in 100 µl of TE buffer. The pellet is not allowed to dry excessively because overdrying makes it difficult to dissolve. The tube was incubated at 37°C for 30 min to dissolve genomic DNA, and RNase was then added.

Amount and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a UV-VIS Spectronic Genesys 5 (Milton Roy) spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA samples from the leaf tissues were digested with Sau3A and electrophoresed on a 0.8% agarose gel, according to Sambrook et al. (1989).

PCR reactions and electrophoresis

The primer used is $(GACA)_5$: 5'GACAGACAGACAGACAGACA-3'. µl. Specific annealing temperature (Ta) determined $(GACA)_5$ is 62°C. PCR reactions were performed with the Gene Amp PCR System 2400, Perkin Elmer. The PCR conditions must be optimised for other thermo-cyclers and annealing temperatures must be optimised for each primer set.

Each 25 µl reaction volume contains 2.5 µl reaction buffer (10x), 2.5 µl $MgCl_2$ (25mM), 2 µl dNTP mixture (2.5 mM), 4 µl of primer (10 pmol l^{-1}), 0.5 µl Taq DNA polymerase (Red Goldstar™ DNA polymerase, Eurogentec, 5 units/µl) and 1 µl of DNA (40 ng). PCR consists of one cycle of 94°C, 2 min, which was followed by 27 cycles of 94°C, 1 min; 62°C , 1 min; 72°C, 2 min, and finally one cycle of 72°C, 7 min.

The PCR products were analyzed by electrophoresis using a 2% agarose gel in TBE buffer. DNA was stained by soaking the gel in a 0.5 mg/ml ethidium bromide solution.

Figure 1: (b) Restriction enzymes digestion of pearl millet genomic DNA. Lanes 1-3: DNA digested with Sau3A.

Figure 1: (a) Electrophoresis of pearl millet DNA on 0.8% agarose gel following RNase treatment. Lanes 1-3: pearl millet DNA leaves. 4 µl DNA was loaded per lane.

RESULTS AND DISCUSSION

To test the effect of various modifications to our DNA extraction protocol, we used fresh or dried leaves of pearl millet. We first investigated the effect of detergents in the DNA extraction buffer. Detergents, SDS and CTAB, were added to the solution containing 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 50 mM EDTA, and 1% β-mercaptoethanol. During the addition of preheated CTAB containing β-mercaptoethanol, moving quickly at this stage was critical in getting good quality DNA. To aid in minimizing time spent doing this step, the 1 ml of 2% CTAB was measured in a 2 ml microcentrifuge tube to which 100 µl of β-mercaptoethanol (1%, v/v) was added and the tube placed in a 60°C water bath until ready for use. Addition of the prewarmed, premeasured CTAB buffer to the frozen leaf tissue contained in the prechilled conical tube saves precious time in bringing the tissue from -80°C to 60°C as rapidly as possible resulting in DNA of higher quality (Puchooa, 2004). Using 1% β-mercaptoethanol produced nucleic acid pellets that were not nearly brown. Inclusion of PVP improved the colour of the nucleic acid obtained. DNA could only be extracted with the solution containing CTAB. The addition of β-mercaptoethanol to the CTAB extraction buffer prior to incubation is also a critical factor (Figure 1a).

The purity of genomic DNA was dependent on the number of washes. A three-time wash combined with a short-run centrifugation was sufficient for DNA purification and removal of endogenous nucleases or other proteins. As CTAB is soluble in ethanol, residual amounts are removed in the subsequent wash. During ethanol precipitation of nucleic acids from 1.4 M NaCl, polysaccharides remain dissolved in the ethanol (Fang et al., 1992). The freer the nucleic acids are from
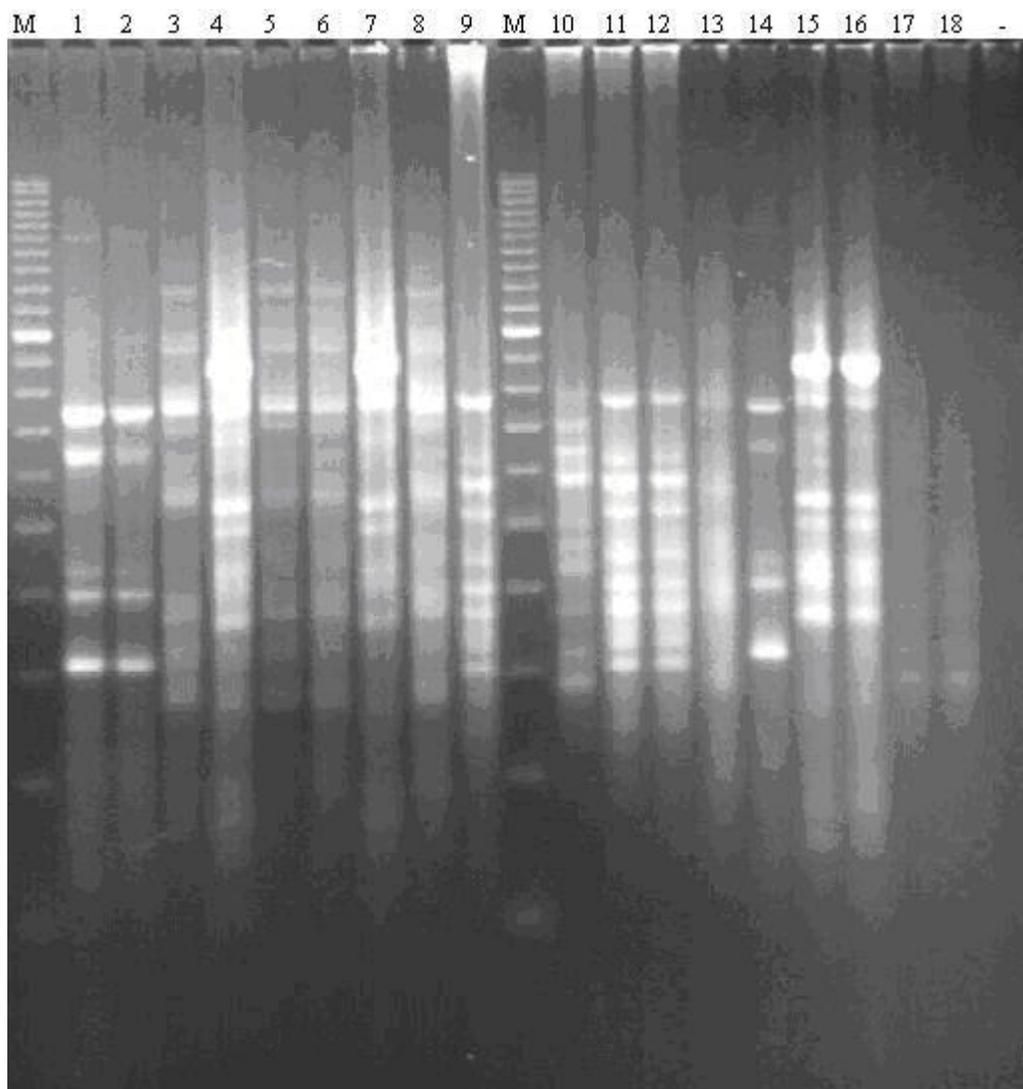
Figure 2. Amplification of purified DNA with SSR-PCR. DNA was purified using the method described. The purified DNA was amplified using SSR-PCR, and the amplification products were separated on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. Lanes 1-18: pearl millet cultivars amplified using SSR-PCR primer $(GACA)_5$, For reference, a negative control (-) was included. Lane M: contains a 100 bp DNA size marker.

contaminants, the easier it is to re-suspend the pellet. If the pellet obtained from the first ethanol precipitation from 1.4 M NaCl was found to be hard to resuspend, two such precipitations were done and the pellet obtained from the second precipitation usually goes into solution very easily. It was found that washing in 80% ethanol gave better DNA as a result of the removal of any residual NaCl and/or CTAB. The DNA extracted can be digested with restriction enzymes such as Sau3A (Figure 1b).

DNA quality was estimated by measuring the 260/280 UV absorbance ratio which varied between 1.8 and 2. In only a few samples with extremely low DNA contents was the ratio lower than 1.8.

We evaluated the quality of the extracted DNA through two procedures: agarose gel electrophoresis and SSR-PCR. Figure 1 shows the result of the extracted DNA run on a 0.8% agarose gel, stained with ethidium bromide and visualized with UV light. In order to check the efficiency and reliability of the method, we first amplified the fresh leaves DNA of pearl millet cultivars using the primer, $(GACA)_5$. The amplified PCR products of leaf DNA showed identical band patterns and similar intensity to that of leaf tissue. However, different PCR patterns were obtained between pearl millet cultivars (Figure 2). We performed SSR-PCR amplification tests on all samples using primer and protocols previously

optimized in the agarose gel. Figure 2 shows amplification products from pearl millet leaves.

DNA purification from plant leaves has become the bottleneck in sample processing from plant tissue to PCR result. This procedure can be used to purify high-quality DNA from plant material using a walkway protocol. Purified DNA performed well in SSR-PCR and gave good yield. This will allow plant molecular biologists to achieve increased productivity when purifying plant genomic DNA in low to moderate throughput systems.

REFERENCES

Aljanabi SM, Forget L, Dookun A (1999). An improved rapid protocol for the isolation of polysaccharide and polyphenol-free sugarcane DNA. Plant Mol. Biol. Rep. 17: 1-8.
Clark MS (1997). In: Plant Molecular Biology - A laboratory manual; Springer-Verlog Berlin Heidelberg, New York. pp. 305-328.
Dawson CR, Magee RJ (1995). Plant tyrosinase (polyphenol oxidase). In: Colowick SP, Kaplan NO (Eds) Methods in Enzymology (Vol 2). Academic Press New York. pp. 817-827.
Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19-21.
Heliport SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19-21.
Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Focus 12: 13-15.
Doyle JJ, Doyle JL (1990). Isolation of Plant DNA from fresh tissue. Focus 12:13-15.
Fang GS, Hammer S, Grumet R (1992). A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biotechniques 13: 52-57.
FAO and ICRISAT, (1996). The world sorghum and millet economies: Facts, trends and outlook. Food and Agricultural Organization of the United Nations: Rome, Italy and International Crops Research Insitute for the Semi-Arid Tropics: Patanchera 502 324, Andhra Pradesh, India.
Guillemaut P, Maréchal-Drouard L (1992) Isolation of plant DNA: A fast, inexpensive and reliable method. Plant Mol. Biol. Rep. 10: 60-65.
Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8: 4321-4325.
Nybom H (1990). Application of DNA fingerprinting in plant breeding. Proc. Intern. DNA Fingerprinting Symposium. Bern. pp. 32-45.
Paterson AH, Brubaker CL, Wendel JF (1993). A rapid method for extraction of Cotton (Gossypium spp) genomic DNA suitable for RFLP or PCR analysis. Plant Mol. Biol. Rep. 11: 122-127.
Puchooa D (2004). A simple, rapid and efficient method for the extraction of genomic DNA from lychee (Litchi chinensis Sonn.). Afr. J. Biotechnol. 3: 253-255.
Rezaian MA, Krake LR (1987). Nucleic acid extraction and virus detection in grapevine. J. Virol. Methods 17: 277-285.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.
Tautz D (1989). Hypervariability of simple sequences as a general Source for polymorphic DNA markers. Nucleic Acids Res. 17: 6463-6471.
Thompson D, Henry RJ (1995). Single step protocol for preparation of plant tissue for analysis by PCR. Biotechniques 19: 394-397.
Vos P, Hoger R, Bleeker M, Reagan M, Lee TV, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414.
Wilkie SE, Issac PG, Slater RJ (1993). Random amplification polymorphic DNA (RAPD) markers for genetic analysis in Allium. Theor. Appl. Genet. 86: 497-504.
Williams JGK, Kubelic AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitray primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.
Ziegenhagen B, Guillemaut P and Scholz F (1993). A procedure for mini-preparations of genomic DNA from needles of silver fir (Abies alba Mill.). Plant Mol. Biol. Rep. 11: 117-121.
Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183.