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A Rapid Protocol for the Isolation of Polysaccharide- and Polyphenolic-Free Genomic DNA for RAPD Analysis of Threatened Medicinal Plants

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ABSTRACT

Many procedures in molecular biology require the isolation of high quality genomic DNA which is difficult in several plant species because of the presence of secondary metabolites that interfere with DNA isolation which effects downstream applications such as DNA restriction, amplification and cloning. Here we describe a modified procedure based on the hexadecyltrimethylammoniumbromide (CTAB) method to isolate DNA from leaf tissues containing high levels of polysaccharides, polyphenols, gums, tannins and secondary metabolites. This modified CTAB (2%) protocol includes the use of a combination of polyvinylpyrrolidone (PVP) and polyvinylpolypyrrolidone (PVPP), 0.2% β mercaptoethanol for *Pterocarpus marsupium* and use of 2 M NaCl, 1% PVPP, and 1% β-mercaptoethanol for other selected medicinal plants with an initial prolonged chloroform: isoamylalcohol (24:1) step (30 min shaking) repeated twice, RNAse treatment for 1 hr and avoiding the incubation and precipitation steps in isopropanol. The yield was approximately 20 µg DNA/200 mg of initial fresh leaf tissue. This method solved the problem of DNA degradation and co-precipitation of the secondary metabolites. The isolated DNA proved amenable to restriction digestion and PCR amplification. The technique is fast, reproducible and can be applied for further molecular approaches.

Keywords: *Ceropegia spiralis*, *Frerea indica*, *Holostemma ada-kodien*, polymerase chain reaction, *Pterocarpus marsupium*, *Tacca leontopetaloides*

INTRODUCTION

Pterocarpus marsupium Roxb. (Papilionaceae) is a medium-sized, deciduous tree distributed all over India whereas *Ceropegia spiralis* Wight. (herb, endangered to Peninsular India; Asclepiadaceae), *Frerea indica* Dalz. (endemic and endangered in Peninsular India; Asclepiadaceae), *Holostemma ada-kodien* Schult. (vulnerable; Asclepiadaceae) distributed in peninsular India, Tropical Himalayas, Srilanka, Myanmar and Western China), *Tacca leontopetaloides* L. (near-threatened; Taccaceae) distributed in Indo-Malaysia. Medicinally all the plant species are important as they help in curing various ailments. The leaves of these plants contain high levels of polysaccharides, polyphenols, phenolic compounds, gums, resins, tannins and other secondary metabolites.

The application of DNA technology in plant molecular research is progressing during the past ten years especially in the area of identification of elite genotypes which can yield pharmaceutical compounds at enhanced level for which the isolation of plant DNA for use in Southern blot analysis, Polymerase chain reaction (PCR), Restriction fragment length polymorphisms (RFLPs), Arbitary primed DNA amplifications (RAPD, SSR-PCR) and Genomic library construction is one of the most important and time-consuming steps. The quality and quantity of DNA needed varies between different applications.

The commonly encountered problems in the isolation and purification of high molecular weight DNA from certain medicinal plants is difficulty due to degradation of DNA because of endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weishing *et al*. 1995). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of extracted DNA (Loomis 1974; Porebski *et al*. 1997).

In our laboratory we are often engaged in studies related to conservational aspects of Red-listed medicinal and aromatic plants where diversity analysis is one of our objectives (Prasad *et al.* 2006, 2007). Different plant species concentrated for diversity analysis were *Oroxylum indicum* (Jayaram and Prasad 2008), *Pterocarpus santalinus* (Padmalatha and Prasad 2007a), *Rauvolfia serpentina* (Padmalatha and Prasad 2007b), *Celastrus paniculatus* (Raju and Prasad 2007b) and *Rauvolfia tetraphylla* (Padmalatha and Prasad 2006b). Therefore we found it is necessary to develop a common protocol for DNA isolation for herbs and tree species, which can be used for RAPD analysis. The protocol described here is relatively simple, efficient, rapid, provides high yield and is consistently restrictable and amplifiable by PCR. The yield of the genomic DNA using this protocol is higher than that obtained with commercial kits. Several experiments were carried out using different methods, however only the optimized protocol is described here.

MATERIALS AND METHODS

Plant source

Ceropegia spiralis Wight, *Holostemma ada-kodien* Schult, *Pterocarpus marsupium* Roxb., *Tacca leontopetaloides* L. were collected from different districts of Andhra Pradesh, and *Frerea indica* Dalz. collected from the Field gene bank, Department of Botany, Kolhapur University, India and grown in a field experimental site as an act of *ex situ* conservation of Red-listed medicinal plants at the University of Hyderabad. Young leaves were harvested fresh before DNA isolation.

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Techniques Paper

Stock solutions

10% Hexadecyltrimethyl-ammonium bromide (CTAB) (w/v) (Sigma Aldrich, USA), 1 M Tris-HCl (pH 8.0) (Sigma Aldrich, USA), 0.5 M EDTA (pH 8.0) (Sigma Aldrich, USA), 5 M NaCl (Qualigens fine chemicals, India). In addition, chloroform: isoamylalcohol (24:1) (Qualigens fine chemicals, India), 80% ethanol (Qualigens fine chemicals, India) and a TE buffer consisting of 1 mM Tris (pH 8.0) and 0.5 mM EDTA (pH 8.0) (Sigma Aldrich, USA) were also needed (all solutions were sterilized by autoclaving).

Extraction buffer

An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 1% polyvinyl polypyrrolidone (PVPP-Mr 10,000) (added before grinding) and 1% β-mercaptoethanol (v/v) (added immediately before grinding) was prepared. Several standard protocols (Dellaporta *et al*. 1983; Doyle and Doyle 1987) including the plant genomic isolation kit DNAzol are available. However, only the optimized protocol is described here.

DNA isolation protocol

1. Fresh leaf sample (200 mg) was collected and ground in liquid nitrogen along with PVPP. The pulverized leaves were quickly transferred to microcentrifuge tubes (2 ml), containing pre-warmed (65°C) extraction buffer (600 µl) with β-mercaptoethanol (20 µl) and shaken thoroughly to form a slurry. The tube was incubated at 65°C for 60 min with frequent swirling every 10 min.
2. An equal volume of chloroform: isoamylalcohol (24:1) was added and mixed gently for 30 min and centrifuged at 12,000 rpm for 10 mins at room temperature (RT) to separate the phases. The supernatant was carefully decanted and transferred to a new microcentrifuge tube. The same step was repeated twice.
3. To the supernatant an equal volume of chilled isopropanol was added and DNA in the form of fibres was spooled and transferred into another tube containing 70% ethanol (300 µl) and washed by centrifuging at 8000 rpm for 10 min.
4. The pellet was air-dried and resuspended in Tris EDTA (TE) 500 µl and 2 µl of RNAse (Sigma Aldrich, USA) was added and incubated at 37°C for 1 h.
5. To the sample equal volumes of phenol: chloroform: isoamylalocohol (24:25:1, v/v/v) was added and centrifuged at 8,000 rpm for 12 min.
6. The supernatant was collected and an equal volume of chloroform: isoamylalcohol was added (24:1 v/v) and centrifuged at 12,000 rpm for 12 min.
7. To the supernatant an equal volume of chilled isopropanol was added and mixed gently.
8. The spooled DNA fibres were washed in 70% ethanol by centrifuging at 8,000 rpm for 8 min. The pellet was air-dried and the DNA was dissolved in TE depending on the size of the pellet.

Amount and purity of DNA

The yield of DNA per 200 mg of the leaf tissue was measured using a UV-Visible Spectrophotometer (Cintra 5, GBC Scientific, Australia) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Sambrook *et al*. 1989). 5 µl of stock DNA was added to 1 ml of double distilled water (a dilution factor of 1:200). The nucleic acid concentration was calculated using the formula A_{260} × 50 µg/ml × 0.001 µl/ml × dilution factor (1000/5). This provides the concentration of DNA in µg/ml which is diluted to 50 ng/µl using sterile double distilled water and used for RAPD reaction.

DNA analysis

Restriction digestion

The susceptibility of genomic DNA to cleavage by enzymes was determined by digesting the DNA (1 µg) with 2 units of *Bam*HI (MBI Fermentas, Germany) separately and subjected to 0.8% agarose gel electrophoresis. Initially the reagents were mixed carefully, centrifuged for few seconds and incubated overnight at 37°C.

Polymerase chain reaction

The genomic DNA was analyzed for amplification by using the Operon primer OPA-04 (5′-AATCGGGCTG-3′). The reactions were carried out in a DNA Thermocycler (MJ Research, California, USA). The RAPD protocol was followed according to Padmalatha and Prasad (2006a).

RESULTS AND DISCUSSION

Plant species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, the biochemical composition in plant tissues of different species is expected to vary considerably. The chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing *et al*. 1995). The protocol developed here is a modification of the CTAB method of Khanuja *et al*. (1999) and Pirttilä *et al*. (2001) for the isolation from fresh samples of plants producing large amounts of secondary metabolites.

Forest trees in general and Fabaceae in particular have proved to be recalcitrant; *P. marsupium*, a tree species (Anis *et al*. 2005), is one among them and the remaining selected species also are rich in secondary metabolites. DNA extraction is very difficult due to the above mentioned reasons. Moreover many impurities like tannins, gums, polysaccharides, resins etc., interfere with the DNA but our procedure described here can be used to obtain consistently high quality, low polysaccharide genomic DNA. Although large quantities of DNA was obtained by other standard methods like Dellaporta *et al*. (1983) and Doyle and Doyle (1987), the DNA was contaminated with high levels of polysaccharides which interfered, making it difficult to dissolve nucleic acids from complexes with secondary compounds such as polysaccharides or polyphenols, released by cell disruption, leading either to the embedding of DNA in a sticky gelatinous matrix or brown coloured products (Guillemaut and Marachel-Drouard 1992). The presence of contaminants or inhibitors in the DNA might lead to appearance of a smear or absence of amplification during PCR reaction.

Hence to test the effects of various modifications in DNA extraction protocol, young leaves were used for our experiments wherein nucleic acid contamination by plant metabolites that interfere with solubilisation and precipitation is reduced to some extent. We first investigated the effect of detergents in the DNA extraction buffer. Detergents such as sodium dodecylsulphate (SDS), CTAB and a combination of both were added in the extraction buffer and it was found that CTAB was comparatively a better detergent. Inclusion of a combination of both PVP and PVPP improved the colour of the nucleic acid in *P. marsupium* when compared to their individual usage. Use of β-mercaptoethanol in the extraction buffer prior to incubation is also a critical factor, which helped in the removal of brownish colour to some extent. Addition of pre-warmed CTAB buffer to the frozen leaf tissue saves precious time in bringing the tissue from –80°C to 65°C as rapidly as possible resulting in DNA of higher quality (Puchooa 2004). During the incubation at 65°C, it was found that there were no significant differences in the yield of DNA when compared to 90 min, 120 min and overnight incubation. In our protocol the extraction buffer contained high amounts of PVPP and β-mercaptoethanol, helpful in removing polyphenols and also to prevent oxidation of secondary metabolites in the disrupted plant material. The extraction of DNA was repeated twice with chloroform: isoamylalcohol because of the presence of many pigments, gums and polyphenolics, which enter into the orga-

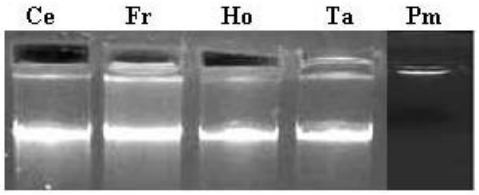

Fig. 1 Electrophoresis of total genomic DNA of *C. spiralis* (Cs), *F. indica* (Fi), *H. ada-kodien* (Ha), *T. leontopetaloides* (Tl), *P. marsupium* (Pm) on 0.8% agarose gel stained with ethidium bromide and visualized under UV light.

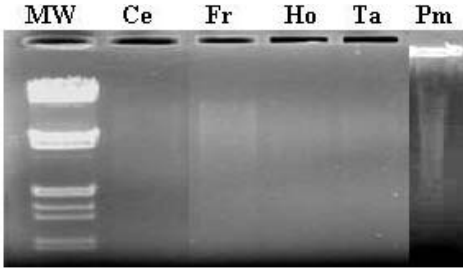

Fig. 2 Restriction digestion of total genomic DNA of *C. spiralis* (Cs), *F. indica* (Fi), *H. ada-kodien* (Ha), *T. leontopetaloides* (Tl), *P. marsupium* (Pm) and resolved on 0.8% agarose gel after digestion with *Bam*H1 restriction enzyme.

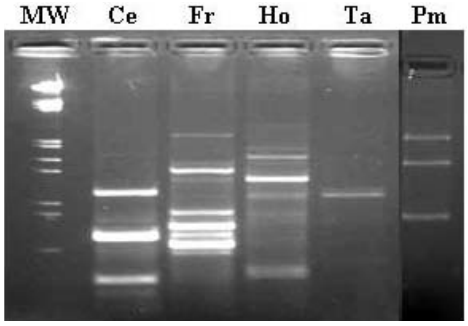

Fig. 3 RAPD profile of *C. spiralis* (Cs), *F. indica* (Fi), *H. ada-kodien* (Ha), *T. leontopetaloides* (Tl), *P. marsupium* (Pm) genomic DNA samples using the OPA-04 (5′-AATCGGGCTG-3′) on 2% agarose gel.

nic layer. Once the nucleic acids were precipitated in isopropanol, incubation and centrifugation steps were avoided because of the interference of gums and resins which precipitate along with the DNA. The spooled DNA was very efficiently washed in 70% ethanol, since CTAB is soluble in ethanol, and most of the residual impurities like polysaccharides were removed (Demeke and Adams 1992; Fang *et al*. 1992). Purification of DNA from proteins was achieved by using equal volumes of phenol, chloroform and isoamyl-alcohol.

Yield was approximately 20 µg DNA/200 mg of initial fresh leaf tissue. The DNA quality was estimated by measuring the 260/280 UV absorbance ratio, which varied between 1.8 and 2.0. Isolation of genomic DNA using the described method was quite easy and amenable for future works. To evaluate the suitability of the isolated DNA in downstream applications, we subjected the genomic DNA to amplification through PCR and digestion by using restriction enzymes. **Fig. 1** shows the result of the extracted DNA run on a 0.8% agarose gel, stained with ethidium bromide and visualized under UV light wherein there are no impurities like polysaccharides, proteins and RNA on the gel visually. **Fig. 2** shows digested DNA samples with *Bam*H1 restriction enzyme that produced good restriction patterns. Effective digestion of DNA with restriction enzymes would indicate absence of polysaccharides (Do and Adams 1991). PCR amplification of genomic DNA using Operon primer OPA-04 resulted in clear amplification as indicated by ethidium bromide staining of a 2% agarose gel (**Fig. 3**). It shows that the PCR products from successful amplification consists of 3 and 6 major bands with the Operon primer and some minor bands which are not seen visually. However minor differences in band size were also present which may be due to slight sequence length differences in the amplified spacer regions. Therefore it demonstrates that isolated DNA is suitable for any diagnostic purpose employing PCR as a technique.

Even though there is a wealth of protocols for optimization of DNA extraction in several plant species (Teixeira da Silva and Tanaka 2006) to our knowledge this is the first report of DNA isolation from leaves of selected woody medicinal plant species which is simple, rapid and efficient for PCR amplification and restriction endonuclease digestion. Moreover we found that the method described in this paper is functional for plants that were otherwise recalcitrant to DNA isolation hence we believe that this method will be helpful for molecular biological studies of many medicinal and aromatic plant species of economic importance.

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