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A MODIFIED CTAB DNA EXTRACTION PROCEDURE FOR PLANTS BELONGING TO THE FAMILY SAPINDACEAE

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ABSTRACT

A good DNA extraction procedure for the isolation of genomic DNA should yield adequate and intact DNA of reasonable purity. The procedure should also be quick, simple, and cheap and if possible should avoid the use of dangerous chemicals. This paper describes a simple, rapid and efficient method for isolating genomic DNA extraction methods for mature leaves, resting buds and seedling leaves of 5 different genera namely *Sapindus, Cardiospermum, Litchi, Allophilus, Dodonea* of Sapindaceae. The plants belonging to this family is considered to be a "difficult" for DNA isolation due to its high polyphenolic content, which may interfere with the DNA purity especially for subsequent manipulations. This modified CTAB protocol include the use of 2M NaCl, 2% Polyvinyl pyrrolidone (PVP), 5% mercaptoethanol and 80% ethanol in the extraction as well as reducing the centrifugation times during the separation and precipitation of the DNA. Isolated genomic DNA showed high purity and high quantity. The DNA was digested by resctriction endonuleases and it was suitable for subsequent PCR amplification.

INTRODUCTION

Sapindaceae also known as the soapberry family, is a family of flowering plants in the order Sapindales. There are about 140-150 genera with 1400-2000 species, including maple, horse chestnut and lychee. Sapindaceae members occur in temperate to tropical regions throughout the world. Many are lactiferous, i.e. they contain milky sap and many contain mildly toxic saponins with soap-like qualities in the foliage or the seeds, or roots. The applications of currrent nucleic acid technologies to crop improvement include gene mapping, genetic finger printing, population studies and phylogenetic analyses. These technologies have application for the improvement crop varieties of sapindaceae. The development of efficient DNA extraction techniques that yield DNA of a purity adequate for restriction enzyme digest and PCR has proven difficult from plant materials in these genera. After experimenting with variety of techniques, we have developed simple efficient procedures for the extraction of DNA from *Sapindus* and other genera in the family Sapindaceae. 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 cetylmethylammonium 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. Most proteins are removed by denaturation and precipitation from the extract using chloroform and phenol. RNAs on the other hand are normally removed by treatment of the extract with heat treated RNAase 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

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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 Species of Sapindaceae are considered to be difficult for DNA isolation due to its polyphenolic content, which may interfere with the DNA purity especially for subsequent manipulations. Antioxidants are commonly used to deal with problems related to phenolics. Examples include 2-mercaptoethanol, ascorbic acid, 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). Although there are several published protocols on plant DNA isolation (Dellaporta *et al.,* 1983; Rogers and Bendich, 1988; Draper and Scott, 1988), the production of large quantities of purified members of sapindaceae genomic DNA is still difficult. The optimized protocol described here is completely different from that proposed by Anuntalabhochia *et al.,* (2002) and is specifically designed to isolate genomic DNA of members of Sapindaceae within a short period of time using small amounts of plant tissues and yielding a high quantity of purified genomic DNA.

MATERIALS AND METHODS

DNA was extracted from plant materials of *Sapindus*, *Cardiospermum*, *Litchi*, *Allophilus*, and *Dodonea* (Family Sapindaceae). Several experiments were carried out, however, only the optimized protocol is described here.

Plant material

Samples of Young, tender leaves were collected early morning. These were kept between moist tissue paper in a plastic bag and kept away from sunlight. The leaves were de-starched by covering them for 24 to 48 hours before use.

Solutions

An extraction buffer consisting of 2% Cetyl trimethyl-ammonium bromide (CTAB) (w/v), 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 2 M NaCl, 2% polyvinylpyrrolidone (PVP - Mr 10,000), 5% ß- mercaptoethanol (v/v), and 10 mM Ammonium acetate was prepared. In addition, chloroform: octanol (24:1), 75% and 80% alcohol and a TE buffer consisting of 1 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0) were also needed.

DNA isolation and Purification

Leaves were harvested and frozen immediately in liquid nitrogen. They were frozen at –80°C until required. A 4.0 g of leaf sample was ground in liquid nitrogen using a pre-chilled pestle and mortar (either –20° C or –80° C). Additional liquid nitrogen was added as required. The pulverized leaves were quickly transferred to a pre-chilled 50-mL Falcon tube. 2% CTAB buffer pre-heated (65° C for 5 min) was added with frequent swirling. An equal volume of chloroform: octanol (24:1) was added and the sample centrifuged for not more than 5 min in a bench-top centrifuge (Biofuge 13, Heraeus) at room temperature to separate the phases. The supernatant was carefully decanted and transferred to a new tube. The above steps, beginning with the addition of chloroform/octanol (24:1) and ending with decanting of supernatant, were repeated twice. The supernatant was precipitated with 2/3 volume of isopropanol. The precipitated nucleic acids were collected and washed twice with the buffer (75% ethanol, 10 mM ammonium acetate, TE). The pellets were air dried and re-suspended in TE Buffer. The dissolved nucleic acids were brought to 2 M NaCl and re-precipitated using 2 volumes of 70% ethanol (If the pellet obtained was hard to re-suspend, this step was repeated one more time). The pellet was washed twice using 80% ethanol, dried and re-suspended in 100 µL of TE buffer. The tube was incubated at 65° C for 5 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. The ration was 1.8 and DNA samples from the leaf tissues were digested with EcoRI and HindIII and electrophoresed on a 0.8% agarose gel.

RESULTS AND DISCUSSION

Experiments where the CTAB method of extraction without modification was used, gave a DNA yield of 335.02 µg per 1.0 g of leaf material. However, it was contaminated with polysaccharides and phenolics as shown by the spectrophotometer readings which gave a A_{260} nm/A_{230}nm ratio of 1.11 and a A260nm/A280nm ratio of 1.48. The samples were very viscous and pinkish in color. Upon electrophoresis, fire type bands were obtained confirming the presence of polysaccharides. In contrast from the next set of experiments where the NaCl concentration was increased from 1.4 M to 2.0 M and use of PVP (Mr 40000) during extraction, the quality of DNA was improved. The bands following electrophoresis were sharper, bright and clear (Figure 1a). However, the yield was reduced to 240.12µg DNA per 1.0 g tissue. Several modifications were made with one parameter tested at a time. Modifications include the ratio of buffer to tissue, use of 5% mercaptoethanol rather than 2%, reduction in the incubation time, washing in 80% ethanol and use of PVP (Mr 10000). Isolation of genomic DNA using the described method was quite easy and did not take more than 15 min for each sample. The yield was 360 µg per 1.0 g of leaf material. The A_{260} nm/A_{230} nm ratio was 1.80 while A_{260}nm/A_{280}nm ratio was 1.90. From the different steps followed and modifications made, it was found that using younger leaves instead of older ones reduces nucleic acid contamination by plant metabolites that interfere with solubilisation by precipitated nucleic acids. In a set of experiments using different amount of starting material, it was observed that the ratio of buffer to leaves should always be 4:1 v/w or greater to obtain sufficient amount of pure form of DNA. During the addition of preheated CTAB containing β-mercaptoethanol, moving quickly at this stage was critical in getting good quality DNA.

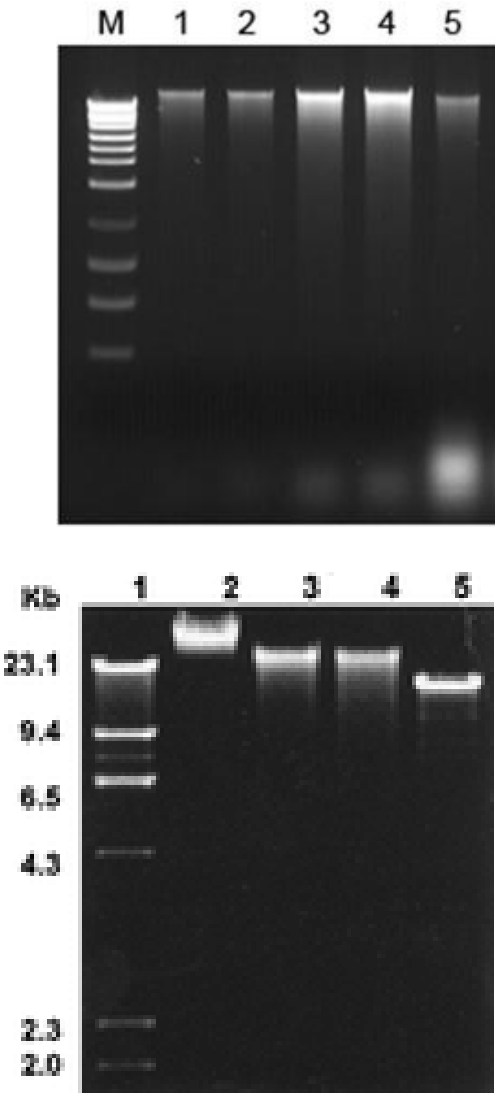

Fig. 1(a). Electrophoresis of different plant samples of DNA on 0.8% agarose gel. Lane M: Molecular marker, Lane 1: *Sapindus* DNA, Lane 2: *Cardiospermum* DNA, Lane 3: *Litchi* DNA, Lane 4: *Allophilus* DNA, Lane 5: *Dodonea* DNA samples; 5 µg DNA was loaded per lane. (b) DNA digested with *Eco*RI and *Hind*III

To aid in minimizing time spend doing this step, the 16ml of 2% CTAB was measured in a 50 ml conical tube to which 836µl of β-mercaptoethanol (5% v/v) was added and the tube placed in a 65°C water bath until ready for use. Addition of the pre-warmed, pre-measured CTAB buffer to the frozen leaf tissue contained in the pre-chilled conical tube saves precious time in bringing the tissue from -80°C to 65°C as rapidly as possible resulting in DNA of higher quality using 5% β-mercaptoethanol instead of the 2% used in the Doyle and Doyle (1987) procedure, produced nucleic acid pellets that were not nearly brown. Inclusion of PVP improved the color of nucleic acid obtained. From the spectrophotometer readings, PVP 10 (Mr 10000) gave better results than PVP 40 (Mr 40000). This may be explained by the fact high molecular weight PVP might have precipitated together with the nucleic acid, thereby being present as a contaminant. During incubation at 65°C, it was found that there were no significant differences in the yield of DNA when comparing the incubation time of 4 h, an overnight incubation and an incubation of 5 min. The latter was adopted for convenience. Once the nucleic acids were collected, they were washed in the wash buffer. The purity of genomic DNA was dependant 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 2M NaCl, polysaccharides remain dissolved in the ethanol (Fang *et al.,* 1992). The free nucleic acids are from contaminants, the easier it is to resuspend the pellet. If the pellet obtained from the first ethanol precipitation from 2M NaCl was found to be hard to resuspend, to such precipitations were done and the pellet obtained from the second precipitation usually goes in to the 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 was digested with restriction enzymes such as *Eco*RI and *Hind*III (Fig 1b). Our results supported by the earlier works done by Dellaporta *et al.* (1993), Rogers and Bendich (1988), Draper and Scott, (1988) and latest by Anuntalabhochia *et al.* (2002). Hence the current protocol is efficient and less time taking for isolation of DNA from the difficult plants like Sapindaceae members.

Conclusion

The development of efficient DNA protocol that yield pure form of DNA with out any contamination is a great challenge for molecular biologists. The present procedure; the modified CTAB method gave pure form of DNA as well as yield was also very high i.e. 360 µg per 1.0 g of leaf material. This is very simple and efficient protocol for plants belonging to Sapindaceae for further studies in the field molecular studies.

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