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ISOLATION AND PCR AMPLIFICATION OF GENOMIC DNA FROM DRY LEAF SAMPLES OF SUGARCANE

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ABSTRACT

Efficient method for isolation of high quality genomic DNA is the first step in the development of DNA based markers for fingerprinting and genetic diversity of crops. We have described a simple and user friendly protocol for extraction of DNA from dried sugarcane leaf samples, which dose not require any use of liquid nitrogen, making it advantageous over other common protocols. The DNA obtained using this procedure were consistently amplifiable, using both RAPD primers as well as ISSR primers, making it most useful in genetic diversity analysis of the sugarcane germplasm.

KEY WORDS

Sugarcane, genomic DNA, PCR, RAPD, ISSR

ABBREVIATIONS

CTAB, cetyl trimethyl ammonium bromide; EDTA, ethylene diamine tetra acetic acid; PVP, polyvinyl pyrrolidone; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; ISSR, inter simple sequence repeats.

INTRODUCTION

Sugarcane is a tropical plant and grows well under tropical conditions all over the world. However in India sugarcane is cultivated under wide range of agroclimatic conditions. It is an important cash crop in tropical and sub-tropical regions, accounts for over two third of world sugar production.

Genetic diversity had been studied in many crops as well as in sugarcane using molecular DNA markers for resolving phylogenetical relationship, variability among the species, hybrid population and tissue cultured raised plants to identify elite sugarcane varieties is important in sugarcane improvement program. Genomic DNA is the base material for molecular studies. Currently DNA

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isolation protocols vary from very simple extraction to slightly more complex procedures. Usually, plant tissues are ground in liquid nitrogen and extracted in CTAB buffer (Murray and Thomson 1980). Presence of polyphenolics in plant tissues, which are powerful oxidizing agents and co-isolation of highly viscous polysaccharides in plant tissues, hamper DNA isolation, restriction, amplification and cloning reactions in this case used a modification of a CTAB DNA extraction protocol (Khanuja *et al*., 1999; Kim *et al*., 1997; Porebski et al., 1997). These impart brown colour and reduce the yield and purity of extracted DNA (Aljanabi *et al*., 1999; Guillemaut and Maréchal-Drouard, 1992; Katterman and Shattuck, 1993). Several protocols for isolation of genomic DNA for plants containing high amounts of polyphenolics and polysaccharides have been described (Burr *et al*., 2001; Dilworth and Frey, 2000; John, 1992; Kim *et* al., 1997). Although these methods yield high purity DNA but are expensive and require liquid nitrogen. A Rapid DNA extraction protocol for sugarcane and its relatives (Honeycutt *et al*., 1992) and modified CTAB extraction protocol for DNA free from polysaccharides and polyphenols in sugarcane DNA (Aljanabi *et al*., 1999) have been published.

Our institute has more than 800 sugarcane germplasm at Sugarcane Breeding Centre at Amboli and produce more than 15 million tissue culture sugarcane plantlets for the farming community. Therefore it is essential for us to develop an easy, fast and efficient protocol for isolation of quality DNA from sugarcane varieties/germplasm for fingerprinting as well as to carry out genetic fidelity testing of the tissue culture raised plants to ensure quality material supplied commercial growers.

MATERIAL AND METHODS

DNA isolation

Young leaves from sugarcane were chosen as source material to facilitate easy cell disruption and to avoid high phenolic content, which interfere in DNA extraction. Leaves were dried uniformly in hot air oven at 40°C for 48hr. We observed that prolonged drying of leaves resulted in poor DNA yield and quality. The 10 ml of preheated extraction buffer (100mM Tris-HCl pH 8.0, 20mM EDTA pH 8.0 2M NaCl, 2% CTAB, 1% β- mercaptoethanol containing 0.2g PVP per g of leaf powder) at 65°C was added per gram of leaf powder. The contents were incubated for one hour at 65°C with intermittent shaking. It was brought to room temperature and 10ml of Chloroform:Isoamyl alcohol was added, mixed well by gentle inversion. Centrifuged at 4350 *g* for 15 min at 4°C. The supernatant was transferred to a fresh tube. This step was repeated till the aqueous phase is clear. Then 1/5th volume of 5M NaCl was added, mixed thoroughly and incubated with one volume of propanol at -40°C overnight to precipitate DNA. Centrifuged at 12000 *g* for 20 min and DNA pellet was washed with 70% aqueous ethanol and air-dried. DNA was dissolved in 1/10th volume of TE. RNase (10µg/ml) was added and incubated at 37°C for 30 min. Equal volume of phenol (Tris-saturated phenol, pH 8.0) was added, mixed well by inversion and centrifuged at 3000 *g* for 3 min at room temperature. The aqueous phase was washed twice with equal volume of Chloroform: isoamyl alcohol (24:1). The DNA was precipitated with 1/10 volume of 3M Na acetate pH 5.2 and 2.5 volume of chilled absolute alcohol to precipitate DNA. Centrifugation at 12000 *g* for 20 min at 4°C, DNA pellet was washed with 70% aqueous ethanol, air-dried and suspended in 300µl sterile water or TE

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(10 mM Tris HCl, 1mM EDTA, pH. 8.0). The quality of DNA was determined by 260/280nm absorption ratio and by agarose gel electrophoresis.

RAPD analysis

PCR amplification was carried out with 50ng template DNA, 2.5µl of 10x PCR buffer, 2.5 mM $MgCl_2$, 2 mM dNTPs, 15ng of primer and 1.0U Taq polymerase (Bangalore Genei, India) in a total volume of 25µl. DNA amplification was performed in BIO-RAD iCycler with initial denaturation at 92°C for 4.30 min, followed by 44 cycles each of 1 min at 92°C for denaturation, 1 min at 35°C for annealing, 2 min at 72°C for extension, with final extension at 72°C for 15 min. The amplicons were size-separated on 1.4% agarose gel electrophoresis in 1x TBE and stained with ethidium bromide. All the reactions were repeated at least twice for the consistent reproducible results.

ISSR analysis

As like RAPD, PCR amplification was done in 25 µl reaction mixture containing 30 ng template DNA, 2.5 µl of 10x PCR buffer, 2.5 mM $MgCl_2$, 2 mM dNTPs, 1.5 µM of primer and 0.5 U of Taq polymerase (Bangalore Genei, India). DNA amplification was performed in BIO-RAD icycler with initial denaturation of 5 min at 94°C, followed by 35 cycles each of 30 sec at 94°C for denaturation, 45 sec annealing at appropriate annealing temp for annealing of primers used, 2 min at 72°C for extension followed by final extension cycle of 7 min at 72°C. The amplicons were size-fractionated on 1.6% agarose gel in 1x TBE.

RESULTS AND DISCUSSION

The quantity and quality of the DNA isolated from dried plant material was on par with the DNA isolated by the conventional CTAB method for liquid nitrogen frozen fresh leaf samples of sugarcane. The 280/260nm absorption ratio ranged from 1.7 – 1.8. The yield of DNA ranged from 25 to 100µg per gram dry leaf. CTAB is a cationic detergent that precipitates DNA leaving the neutral polysaccharides in solution during DNA isolation. The DNA-CTAB pellets were treated with 1.4M NaCl, so that Na exchanges with the CTAB resulting in clean DNA. Use of PVP in extraction buffer has been reported earlier to remove polyphenolics (Kim *et al*., 1997; Porebski *et al*., 1997). PVP binds to the proteins, especially the polyphenol oxidase, which oxidizes the phenolic compounds in the samples resulting in the formation of polyphenol/protein/DNA complexes that is brown in colour, leads to degraded DNA. Addition of PVP in the extraction buffer binds to the phenolic compounds and co-precipitate them. Addition of β-mercaptoethanol up to 1% was found optimal to keep the nucleic acids in reduced environment. RNase treatment followed by repeated washing with phenol-chloroform and chloroform-isoamyl alcohol ensured the removal of RNA, histone proteins from DNA, traces of phenol in the preparation.

Rapid DNA extraction methods for sugarcane and other crops (Burr *et al*., 2001; Dilworth and Frey, 2000; Honeycutt *et al*., 1992) have been reported. Although these methods ensure quality DNA, the drawback is the use of liquid nitrogen, which is difficult to obtain in remote regions during sample collection (Aljanabi *et al*., 1999). Since, our protocol is developed to use dry leaf samples of leaves, no such equipments and chemicals like liquid

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nitrogen are required to get DNA which can be subjected to downstream processes like PCR amplification.

The DNA isolated from dried as well as liquid nitrogen frozen samples was amplified using 8-10 different RAPD random decamer primers (Operon, Inc, USA) as well as specific ISSR primers. The amplification patterns observed were good (Fig. 1). When compared with the DNA samples extracted from fresh samples of the same germplasm using liquid nitrogen, no difference in the banding pattern was observed indicating the utility of DNA isolated from dry samples without the use of liquid nitrogen for use in PCR amplification for RAPD and ISSR profiling. Further, dried leaves can be stored in sealed polythene covers over 6-8 months without compromising on the quality of DNA

Similar approach for storing leaf samples for DNA isolation have been reported earlier (Sharma *et al.*, 2003; Shalini *et al.*, 2007). The present study describes a simple, cheap and efficient method for extracting PCR amplifiable genomic DNA from sugarcane.

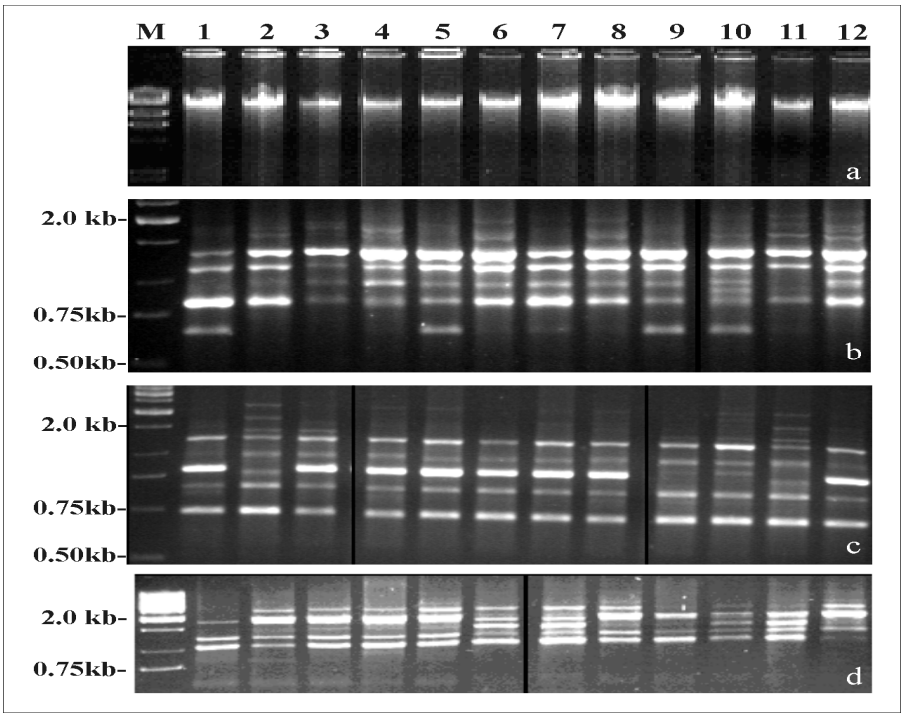

Fig. 1 a. Genomic DNA was extracted from dry leaves and fresh leaves of different sugarcane genotypes, b. Samples amplified with RAPD primer OPA 20, c. Samples amplified with RAPD primer OPG 02, d. Samples amplified with ISSR primer UBC 826. Lanes 1, 3, 5, 7, 9, 11 DNA isolated from fresh leaf samples fresh s and Lanes 2, 4, 6, 8, 10, 12 DNA isolated from dry leaf samples

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