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A Simple and Efficient Method for High Quality DNA Extraction from Sweet Sorghum [*Sorghum bicolor* (L.) Moench]

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Abstract: Isolation of high quality, intact high molecular weight genomic DNA from plants which are rich in polysaccharides, polyphenols, secondary metabolites and chemical heterogeneity is an immense problem in the field of plant biology. Several protocols have been developed for eliminating these tricky elements during the extraction of DNA, but none is found to be universally applicable. The purpose of the present study was to develop a reliable protocol for extracting high quality genomic DNA from polyphenols, polysaccharides and secondary metabolites rich plants like sweet sorghum. We made seven critical modifications to the available CTAB method to isolate genomic DNA from 25- and 60-d-old transgenic sweet sorghum leaf tissues. The yield of DNA ranged from 9.2– 10.2 µg from 200 mg of leaf tissue. An absorbance value of 1.8 at A_{260}/A_{280} indicates that it's free from RNA and protein contamination. PCR analysis using *bar* primers shows a consistent and reliable amplification product at 475 bp. This method is highly suitable for extracting high quality genomic DNA from plants with high levels of polysaccharides and polyphenolics without blending commercial kits. Our protocol facilitates the processing of large number of plant samples for genomic analysis, mapping and next generation sequencing.

Key Words: Cereal, Ethanol, Genomic DNA, NaCl, Polysaccharides, Polyphenols

Introduction

DNA extraction from plant tissues, unlike DNA isolation from mammalian tissues, remains difficult due to the presence of a rigid cell wall surrounding the plant cells (Ghosh *et al*., 2009). Extraction of high quality genomic DNA from polysaccharide, polyphenolics and other secondary metabolites rich plants is a big challenge (Dhanya *et al*., 2007, Xin *et al*., 2012). Various plants contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification (Zidani *et al*., 2005). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications (Kazemi *et al*., 2012; Katterman *et al*., 1983; Peterson *et al*., 1997; Porebski *et al*., 1997; Hemphill *et al*., 2006). The age and type of tissue greatly affect the quality and yield of the DNA. Young and tender tissues are good for DNA extraction. During DNA extraction RNA, proteins, polyphenols and polysaccharides are co-extracted. Apart from polysaccharides another major often met problem with polyphenols (Kasajimaa *et al*., 2013). The Cetyl Trimethyl Ammonium Bromide (CTAB) method and its modifications (Doyle *et al*., 1987) were extensively used in different laboratories, but these methods are time consuming and most of the available procedures are based on the use of commercial kits, which make extraction economically difficult for large-scale genomic applications (Amani *et al*., 2011; Abdellaoui *et al*., 2011). A good isolation protocol should be simple, rapid and efficient enough, yielding appreciable levels of high quality DNA suitable for molecular studies (Sablok *et al*., 2009). *Sorghum bicolor* is an essential food crop in Africa, Central America and South Asia and is the "fifth" most important cereal crop grown in the world. We report an improved method of DNA extraction from sweet sorghum plants

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that is independent of commercial kits for DNA extraction.

Materials and Methods

Leaf tissues used for this study were harvested from 25 and 60 day old sweet sorghum transgenic plants grown in greenhouse. Leaf tissues were collected in self-sealing polyethylene covers, labeled and immediately flash freezed with liquid nitrogen, stored at -20°C.

Reagents and consumables

- CTAB Hexadecyl trimethyl-ammonium bromide (Sigma, Catalogue No. H6269)
- Absolute and 70 % Ethanol
- Sodium Chloride (Himedia, Cat# RM853)
- RNase A (Fermentas, Cat# EN0531)
- Polyvinyl polypyrrolidone (Himedia, Cat# RM10324)
- Sodium acetate (Himedia, Cat# MB048)
- Safe-Lock microfuge tubes 2.0 ml (Eppendorf, Cat# 22363352)
- PCR tubes 0.2 ml (Fisher scientific, Cat# 14230225)

Solutions

- DNA extraction buffer: 2 % CTAB, 1 M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl and 1 % β-mercaptoethanol. β-mercaptoethanol should be added before using the buffer.
- TE buffer: 10 mM Tris and 1 mM EDTA (pH 8.0).
- Chloroform: iso-amyl alcohol at 24:1 v/v.

Equipment

- Extraction water bath (Labtech, India)
- Sorvall refrigerated centrifuge (Thermo scientific, USA)
- Nanodrop ND-1000 Spectrophotometer (Thermo scientific, USA)

DNA Isolation

1. Grind 200 mg of leaf tissues in 2 ml microfuge tube flash freezed for 15 min in liquid nitrogen using micro pestle with 2 % PVPP until fine, homogenous powder is obtained.
2. Transfer pulverized tissue to labeled 2 ml eppendorf tube.
3. Add 1.0 ml CTAB buffer warmed at 65 °C and 5 µl, 1 % β- Mercaptoethanol.
4. Cap the tubes and invert 6-7 times and incubate at 65 °C for 1 hour with occasional gentle swirling.
5. Cool down tubes at room temperature for 10 min, then centrifuge at 12000 rpm for 10 min and transfer aqueous layer to a new-labeled fresh 2.0 ml tube without disturbing the cell debris.
6. Add 0.7 ml of chloroform to aqueous phase, mix by gentle inversion and incubate at room temperature for 5 min and centrifuge at 12,000 rpm for 10 min.
7. Transfer aqueous phase to a new-labeled fresh 2 ml tube.
8. Add 0.7 ml absolute ethanol to aqueous phase and incubate at RT for 5 min for precipitating DNA.
9. Centrifuge at 10,000 rpm for 10 min and discard the supernatant.
10. Dissolve DNA pellet in 100 µl TE buffer.
11. Add 50 units RNase A to each tube, mix twice for 30 s and incubate at 37 °C for 1 h.
12. Add equal volume of chloroform: iso-amyl alcohol (24:1, v/v) to each tube, mix gently by inversion and incubate at RT for 15 min.
13. Centrifuge at 12,000 rpm for 10 min and transfer aqueous phase to a new-labeled fresh 2 ml tube.
14. Add 0.5 volumes of 0.3 M sodium acetate and 1/10 volume absolute ethanol to each tube and mix by gentle inversion.
15. Centrifuge at 12,000 rpm for 10 min and discard supernatant.
16. Wash DNA pellet with 70 % ethanol at 4000 rpm for 10 min.
17. Air dry DNA pellet for 10 min and add 50 µl TE buffer to each sample well to dissolve DNA.

Assessment of DNA quality and quantity

The quality and quantity of extracted DNA was examined through Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA) at A_{260} /A_{280}. The quality of DNA was further tested by agarose gel electrophoresis, on 0.8% agarose gel with 1X TAE buffer. The DNA was compared on gel with 1 kb Gene Ruler™ DNA Ladder Mix (Fermentas, USA). The suitability of extracted DNA for molecular techniques was assessed through PCR. The total reaction volume 20 µl contained nuclease free water, 100 ng template DNA, 10 X buffer, 15 mM $MgCl_2$, 10 mM dNTP, 10 pM of forward and reverse primers and 1U Taq DNA polymerase and amplified through the following programme in Applied Biosystems (USA) PCR machine. Initial denaturation for 1 min at 94 °C, final denaturation for 30 sec at 94 °C, annealing for 30 sec at 59 °C, and elongation for 30 sec at 72 °C, followed by a final extension for 10 min at 72 °C. Primers used in this study were *bar* forward 5′ CCGTACCGAGCCGCAGGAAC 3′ and *bar* reverse 5′ CAGATCTCGGTGACGGGCAGGAC 3′.

Results

This method modified from basic CTAB DNA extraction protocol with seven critical modifications (Table 1) yielded high quality of DNA from both young and old sweet sorghum leaf tissues (Fig. 1). The total DNA yield was

10.2 and 9.2 µg (50 µl final volume) from 200 mg of 25 and 60-d-old transgenic sweet sorghum leaf tissues. Quality and quantity of DNA was determined based on the absorbance at A_{260} and A_{280} ratio and 0.8% agarose gel electrophoresis. The absorbance of isolated DNA was 1.8. at A_{260}/A_{280} indicating without RNA and protein contamination (Varma *et al*., 2007). The agarose electrophoresis results suggest that our improved DNA extraction method produces high quality genomic DNA without shearing and RNA contamination (Fig. 2). The suitability of the DNA isolated as a template in PCR amplification reactions was analyzed with *bar* primers and results showed that consistent and reliable amplification product at 475 bp with bar primers (Fig. 3).

Table 1: Modifications in the current protocol in comparison to Doyle., *et al*., (1987) protocol

Modified current protocol	Doyle., *et al* (1987) protocol
5 M NaCl 0.5 M EDTA 1 M Tris-HCl	1.4 M NaCl 20 mM EDTA 100 mM Tris-HCl
Addition of chloroform prior to the DNA precipitation for efficient removal of proteins and polysaccharides	Direct DNA precipitation after extracting with extraction buffer
Precipitation of DNA with absolute ethanol	Precipitation of DNA with cold isopropanol
60 min RNase A treatment for DNA	30 min RNase A treatment for DNA
Addition of chloroform: iso-amyl alcohol (24:1, v/v) after RNase A treatment of DNA	Addition of chloroform : iso-amyl alcohol (24:1, v/v) before RNase A treatment of DNA

Figure 1: Sweet sorghum transgenic plants (A) 25-d-old and (B) 60-d-old.

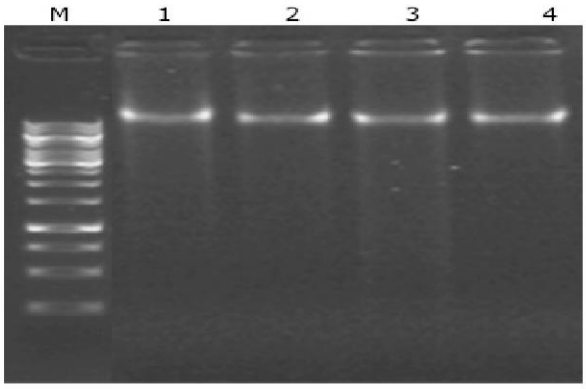

Figure 2: Electrophoresis of total genomic DNA extracted from sweet sorghum transgenic plants resolved in 0.8 % agarose gel. (Lanes: M-Marker, 1-2: DNA from 25-d-old sweet sorghum transgenic plants; 3-4: DNA from 60-d-old sweet sorghum transgenic plants).

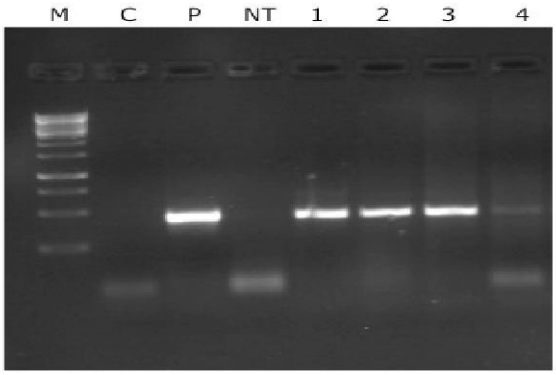

Figure 3: PCR amplication of *bar* gene using the DNA extracted by modified method as template DNA with *bar* primers. (Lanes: M-Marker, C-Control, P-Plasmid, NT-Non-transformed, 1-2: 25-d-old sweet sorghum transgenic plants and 3-4: 60-d-old sweet sorghum transgenic plants).

Discussion

We made seven critical modifications to basic CTAB (Doyle *et al*., 1987) DNA extraction method. Firstly, we incorporated a higher concentration of NaCl (5 M) in CTAB extraction buffer that improved the DNA yield and quality by preventing the sample from becoming viscous in nature during sample grinding. Second improvisation is to increase the amount of EDTA (0.5 M) that efficiently neutralizes the divalent cations required for DNAse activity and thus protects the DNA from degradation. Thirdly, addition of a higher concentration of Tris-HCl (1 M) facilitates constant pH during extraction with CTAB buffer. Further, the chloroform extraction step is performed before precipitating the DNA, which improves the DNA quality and yield by efficient removal of polysaccharides, lipids and other non-polar

substances. The fifth modification is the precipitation of DNA with ethanol instead of cold isopropanol. Ethanol being volatile useful for DNA precipitation and ideal for small- and/or medium-scale DNA extraction. DNA precipitation with isopropanol leaves a high salt concentration that is very difficult to remove. Enhancing the time-period of RNase A treatment for DNA from 30 to 60 min facilitates proper removal of RNA from the sample. Finally, addition of chloroform and iso-amyl alcohol (24:1, v/v) after RNase A treatment of DNA improves the DNA quality by efficiently removing proteins from the DNA sample. Incorporating the above-mentioned modifications led to the extraction of high quality genomic DNA without contaminants like RNA and protein from sweet sorghum young and old leaf tissues.

Conclusion

A simple and efficient high quality DNA extraction method from sweet sorghum leaves was established and validated. The main advantages of this protocol are it's short and time saver, generates high quality DNA and is not exorbitant as it is independent of the use of costly commercial kits. Moreover, this method is highly suitable for extracting high quality DNA from plants with high levels of polysaccharides and polyphenolics and for processing large number of plant samples for genomic analysis, mapping and next generation sequencing. An individual worker can process 100 samples in a day.

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