Electronic Journal of Plant Breeding, 6(4): 1164-1168(Dec- 2015)

ISSN 0975-928X

Research Note

Simple, Rapid, Economical and High yielding method for extracting genomic DNA from cotton (*Gossypium spp*.)

Gulamnabi L.Vanti[1], Ishwarappa S. Katageri[2], Bale M. Swamy[1], Prashanth A. Sangannavar[2], Ramesh Methre[2], Vamadevaiah Hiremathada[2]

1. Lectins and Glycobiology laboratory, Department of Biochemistry, Karnatak University, Dharwad, 580003, India
2. Agricultural Research Station, University of Agricultural Science, Dharwad 580007, India.

(Received: 16th Sep 2015; Accepted: 10th Jan 2015)

Abstract

The complex allotetraploid nature of the cotton genome makes genetic, genomic and functional analyses extremely challenging. Until now, several methods have been published for the purpose, but none is found to be universally applicable. To overcome these difficulties, we have designed protocol for the isolation of high quality and quantity genomic DNA from young as well as older leaves, which is suitable for molecular biology applications. We modified and optimized the CTAB method for plant genomic DNA extraction in large quantity and described protocol takes less than 80 min and does not require RNase treatment and purification step. The DNA yield ranged between 700–800 µg per gram of leaf tissue. Quantification of isolated DNA from spectral (A260/A280) measurements as well as agarose gel electrophoresis showed negligible polysaccharide and protein contamination. Thus the extracted DNA was very much suitable for southern hybridization and PCR based applications

Keywords

Cotton · DNA extraction · PCR · Restriction digestion

Cotton is a principal commercial crop, being the major raw material for textile industry, which is cultivated in about 80 countries (Palle *et al.,* 2013). It provides livelihood for more than 180 million people with an annual contribution of $500 billion (Rahman *et al*,. 2012). *Gossypium* genus comprises of many species, among them four species such as *G.hirsutum* (4n), *G.barbadense* (4n), *G.arboreum* (2n) *and G.herbaceum* (2n) are cultivated for commercial purpose. Significant advancement in molecular biology techniques and genetic engineering during the past few decades led to manipulation of genetic material for crop improvement. However, the problem with the recalcitrant targets, which plant molecular biologists are facing daily in obtaining contamination free, high-quality genomic DNA, without which any further analysis or manipulations are not possible (Varma *et al.,* 2007). The standard protocols fails to obtain a high-quality DNA required for further molecular work from cotton plants due to manifestation of secondary metabolites. To overcome this difficulty, researchers have developed several protocols to isolate genomic DNA from cotton for various purposes (Dabo *et al.,* 1993; Paterson *et al.,* 1993; Permingeat *et al.,* 1998; Chaudhry *et al.,* 1999). However these procedures are more laborious, time consuming and not appropriate for screening analyses of transgenic populations. Several biotechnology companies have made advancement in these directions to provide fast and better results, but these kits are not cost effective enough to be used on a general laboratory scale (Varma *et al.,* 2007).

In this investigation, we have addressed the factors that are imperative during genomic DNA isolation from plants containing high amount phenolics and polysaccharides and report a modified CTAB procedure for rapid cotton DNA isolation suitable for southern hybridization and polymerase chain reaction (PCR) based downstream applications.

All the molecular grade chemicals and reagents required for DNA isolation were procured from Sigma Chemical Co., St. Louis, MO, USA. Restriction enzymes, Standard DNA markers and PCR reagents were procured from New England Biolabs (NEB; USA). The present study was conducted at Agricultural Research Station, University of Agricultural Sciences (ARS-UAS), Dharwad, India.

Reagents Used:

Suspension buffer

50 mM Ethylenediaminetetraacetic acid (EDTA)
100 mM Tris-Hydro Chloride
0.8 M Sodium Chloride
0.5 M Sucrose

2% Triton ×100
0.1% β-Mercaptoethanol
Extraction buffer
20 mM Ethylenediaminetetraacetic acid (EDTA)
100 mM Tris-Chloride
1.5 M Sodium Chloride
2% Cetyltrimethylammonium bromide (CTAB)
1% β-Mercaptoethanol

Plant material and DNA extraction:Seeds of *Gossypium* species (*Gossypium hirsutum* and *Gossypium arboreum*) were obtained from ARS-UAS, Dharwad, India. Young leaves (first opened leaf from the top) as well old cotton leaves (Fourth or lower healthy nodal leaf) were collected and about 100 mg of each sample was gently ground in 2 mL sterile micro centrifuge tubes (Eppendorf; UK) using liquid nitrogen. Followed by grinding, pre chilled 1.5 mL suspension buffer was added and incubated at 60 °C for 30 min with gentle mixing. After incubation, tubes were cooled to room temperature (RT) and centrifuged at 6000 ×*g* for 10 min at RT. Supernatant was discarded and the pellet was gently mix in 1mL extraction buffer prior to incubation at 60 °C for 45 min.

Further the samples were mixed slowly with equal volume of chloroform: isoamyl alcohol (24:1) for 5 min and centrifuged at 6000 ×*g* for 10 min at RT. After centrifugation, supernatant was collected in 2 mL centrifuge tube, to the supernatant two volumes of chilled ethanol (100%) was added and the tubes were slowly mixed by inversion for 5 min. Precipitated DNA was spooled out using sterile glass rod in a fresh 1.5 mL tube containing alcohol, further the tubes containing DNA was centrifuged at 4000 ×*g* for 5 min at RT. Pellet was dried in vacuum and dissolved in 100 µl of TE buffer (pH 8, 10 mM Tris-HCl, 1 mM EDTA) or sterile double distilled DNase free water and stored at -20 °C until use.

Determination of genomic DNA quantity and quality: DNA quantity and purity was determined by agarose gel electrophoresis and spectrophotometric analyses. 1 µL of DNA samples were run on a 0.8% agarose gel and compared with known concentration of DNA standards. Further the quantity of DNA was measured by Nanodrop spectrophotometer (Thermo scientific; USA) at 260 nm and the DNA purity was determined by the absorbance ratio at $A_{260/280}$.

Restriction digestion:Cotton genomic DNA (1 µg) from tetraploid and diploid species were digested with *Kpn* I, *Xba* I and *Hind* III (NEB) restriction enzymes and the samples were incubated at 37 °C along with DNA without restriction enzymes. Followed by incubation, restricted DNA samples were separated by agarose gel electrophoresis (0.8 % w/v) in Tris-acetate-EDTA for 8 h at 40 V. Further the gel was stained with ethidium bromide (10mg/mL) and visualized after 20 min in UV transilluminator. In another experiment, 10 ug of DNA from cotton transgenic plants were restricted with *Xba* I restriction enzyme (NEB) and transferred onto a nylon membrane (Hybond^{TM} N^+; GE Health care UK). A fragment from plasmid pBI121 (Clontech USA) containing approximately 430 bp transgene was used to synthesis probe by random-primed labeling of DNA probes with DIG-11-dUTP, using non radio labeling southern kit as per the manufactures instruction (Roche; UK). Hybridization was performed for 16 h at 54 °C, and high-stringency washes were carried out according to the manufacturer's instructions. Colour developed by the addition of substrate, was exposed to X-ray film and recorded.

PCR analysis:Isolated genomic DNA was subjected to molecular studies. PCR amplifications were carried out using 20 µl reaction mixture containing 40 ng of template DNA, 1× PCR buffer, 1.0 mM of magnesium chloride ($MgCl_2$) 200 µM of deoxynucleotide triphosphates (dNTPs), 10 picomol of each primer, and 1 U of *Taq* polymerase. PCR amplification was carried out in a thermal cycler (Eppendorf Mastercycler). The following PCR conditions were used; 94 °C for 5 min; followed by 32 cycles of 94 °C for 45 s, 60 °C for 30 s, and 72 °C for 45 s; with a final extension at 72 °C for 10 min. All PCR components were obtained from NEB. Amplicons were separated by 1% agarose gel electrophoresis, visualized using ethidium bromide staining.

The area of plant biotechnology has witnessed significant developments in molecular biology techniques and genetic engineering advancements dealing with the study and manipulations of plant genetic resources at the molecular level. Isolation of good quality and quantity of genomic DNA from *Gossypium* species is challenging task due to the presence of high levels of phenolic, polysaccharides constituents. Until now, several protocols have been published for the purpose, but none of them found to be universally applicable. Most of the earlier reported cotton DNA isolation protocols (Dabo *et al.,* 1993; Paterson *et al.,* 1993; Permingeat *et al.,* 1998; Chaudhry *et al.,* 1999; Zhang and Stewart, 2000; Li et al., 2001; Abd-Elsalam *et al.,* 2007) are laborious, time consuming and moreover proved to be successful only for the young leaves.

In the present study, DNA was isolated by designing modified buffers to reduce problems associated with secondary metabolites. Use of

suspension buffer containing 0.8 M NaCl is useful for the removal of polysaccharides from DNA solutions by increasing their solubility in ethanol. 0.5 M osmoprotectant, such as sucrose and 2% Triton X-100 is used in suspension buffer to stabilize the nuclear membrane, thereby facilitating the isolation of intact nuclei by centrifugation. Non-ionic detergent, Triton X-100 helps to lyses chloroplasts and mitochondria, leaving the nuclei intact (Varma *et al.,* 2007). To avoid oxidation of phenolic compounds during cell lysis, β-mercaptoethanol is used instead of polyvinyl pyrollidone (PVP) as PVP sometimes interfere with nucleic acid (Puchooa *et al.,* 2004). In one of the experiment Permingeat *et al.,* (1998) used glucose as a reducing agent in cotton (*Gossypium hirsutum*) to avoid problems with phenolics. By using described protocol, the DNA yield ranged from 700-800 µg/g of leaf tissue (Table 1), which indeed is highest than the reported protocols of Dabo *et al.,* 1993; Paterson *et al.,* 1993; Permingeat *et al.,* 1998; Chaudhry *et al.,* 1999; Zhang and Stewart, 2000; Li *et al.,* 2001 and Abd-Elsalam *et al.,* 2007. The purity of the DNA samples were confirmed by its A_{260} spectrophotometer reading which showed 1.7 to 1.9 and $A_{260/280}$ ratio was less than 2, indicating DNA preparation were free from proteins, polyphenolics/polysaccharide and RNA (Table 1).

Further the quantity and quality of DNA isolated from cotton species were checked on 0.8% agarose gel (Figure 1a) compared with known molecular weight markers. Subsequently isolated DNA was restricted with restriction enzymes and separated on agarose gel with undigested genomic DNA (Figure 1b), which showed more than 98% of the DNA was digested. To check DNAs efficacy, it is used as a template in the PCR to amplify transgene (430 bp gene). The expected product was amplified at 430 bp (Figure 2). In Southern analysis, it is very difficult to locate transgene in the genome if the DNA preparation is not good, as it is entirely dependent on the concentration of genomic DNA and its purity. The DNA isolated from this protocol was used to identify transgene in the cotton genome by non radio labeled method, which showed hybridizable signals against non radio labeled probe (Figure 3). In addition to cotton, this protocol is helpful to the researchers who are working on plant samples containing high levels of phenolics and carbohydrates. The present optimized protocol yield good quality and quantity DNA even from the older-leaf samples of cotton and it is reliable, fast, simple and economical which is useful for southern hybridization and PCR based applications.

References

Abd-Elsalam, K.A., Asran-Amal, A., El-Samawaty, A.M.A. 2007. Isolation of high-quality DNA from cotton and its fungal pathogens. *Journal of Plant Diseases and Protection*. **114 (3):** 113–116.

Chaudhry, B., Yasmeen, A., Husnain, T., Riazuddin, S. 1999. Mini-scale Genomic DNA Extraction from Cotton. *Plant Molecular Biology Reporter*. **17:** 1–7.

Dabo, S.M., Mitchell, E.D. Jr., Melcher, U. 1993. A method for the isolation of nuclear DNA from cotton (*Gossypium*) leaves. *Anal. Biochem.* **210:** 34–38

Li, H., Luo, J.A., Hemphill, J.K., Wang, J.T., Gould, J.H. 2001. A Rapid and High Yielding DNA Miniprep for Cotton (*Gossypium* spp.). *Plant Molecular Biology Reporter*. **19:** 1–5.

Palle, S.R., Campbell, L.M., Pandeya, D., Puckhaber, L., Tollack, L.K., et al, (2013) RNAi-mediated ultra-low gossypol cottonseed trait: performance of transgenic lines under field conditions. *Plant Biotechnol J*. **11:** 296–304.

Permingeat, H.R., Romagnoli, M.V., Vallejos, R.H. 1998. A Simple Method for Isolating High Yield and Quality DNA from Cotton (*Gossypium hirsutum* L.) Leaves. *Plant Molecular Biology Reporter*. **16:** 1–6.

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.

Rahman, M., Shaheen, T., Tabbasam, N., Iqbal, M.A., Ashraf, M., et al, 2012. Cotton genetic resources A review. *Agron. Sustain. Dev.* **32:** 419–432.

Varma, A., Padh, H., Shrivastava, N. 2007. Plant genomic DNA isolation: An art or a science. *Biotechnol J.* **2:** 386–392.

Zhang J, Stewart JM (2000) Economical and Rapid Method for Extracting Cotton Genomic DNA. The Journal of Cotton Science 4:193-201

Table.1. Estimation DNA concentration by spectrophotometer

Genotype	DNA concentration per gram of fresh leaf		DNA concentration per gram of old leaf	
	Quality at A_{260}	Average yield	Quality at A_{260}	Average yield
G.hirsutum	1.76±0.03	769± 55ug	1.85±0.04	742± 38ug
G.arboreum	1.87±0.05	782±43 ug	1.73±0.02	775± 62ug

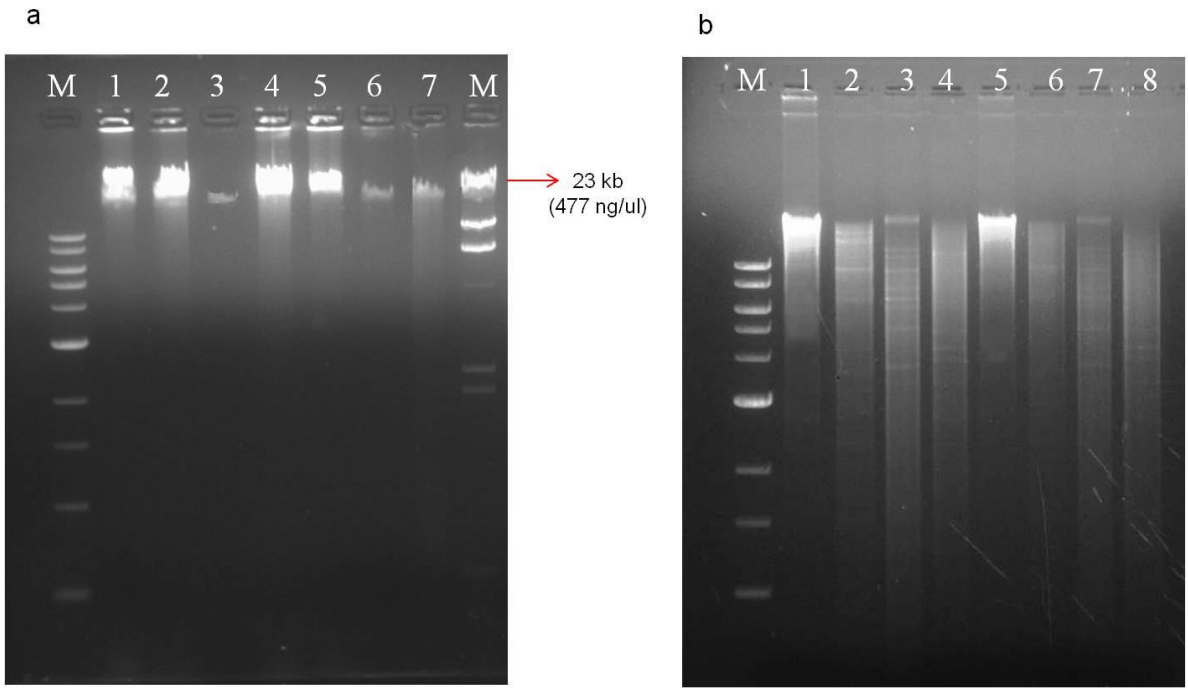

Figure 1. (a) Relative yields of genomic DNA from cotton leaves (1 µl DNA was loaded into each lane); *G.hirsutum* and *G. arboreum* DNA from young leaves (lane 1-2) isolated by described our procedure, DNA isolated from Macherey-Nagel kit (lane 3), *G.hirsutum* and *G. arboreum* DNA from old leaves (lane 4-5), *G.hirsutum* and G. *arboreum* DNA from Paterson et al. protocol (lane 6-7), DNA markers 1kb and lambda DNA *Hind* III digest. (b) Uncut genomic DNA from *G.hirsutum* (lane 1), restriction digestion of 1 µg genomic DNA from *G.hirsutum* using *Kpn* I, *Xba* I and *Hind* III (lane 2-4), Uncut genomic DNA from G. *arboreum* (lane 5), restriction digestion of 1 µg genomic DNA from *G. arboreum* using *Kpn* I, *Xba* I and *Hind* III (lane 6-8).

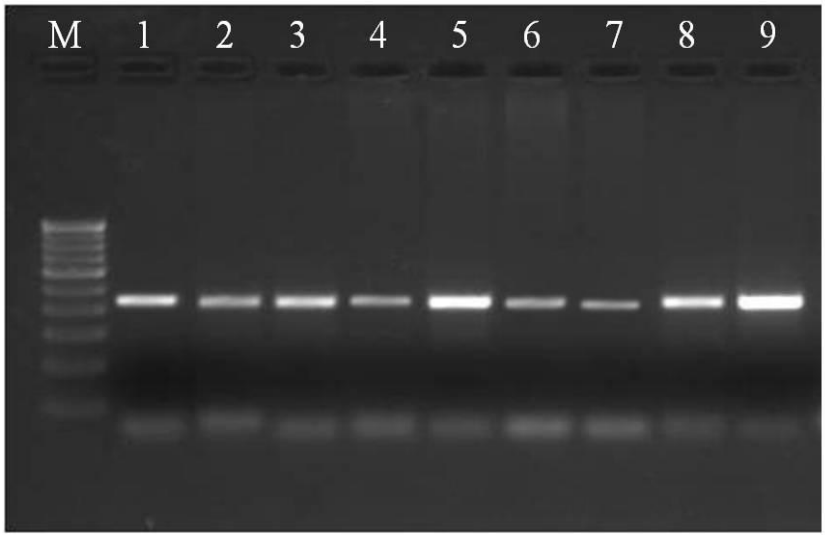

Figure 2. PCR analysis of transgenic plants; Agarose gel of PCR amplified 430 bp fragment (lane 1-9), 100 bp marker (M)

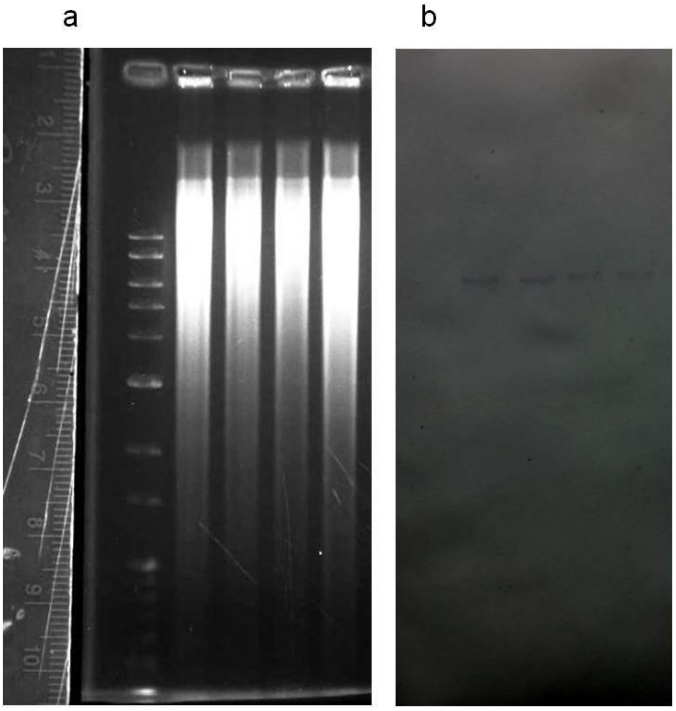

Figure 3. Southern analyses of transgenic tobacco plants (a) 10 ug of genomic DNA from one of the cotton transgenic line was digested with *Xba* I (b) Restricted fragments was hybridized with transgene non radio labeled DIG-11-dUTP probe