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Modified Cetyl Trimethyl Ammonium Bromide Method Improves DNA Quality and Quantity in Cotton: The Benchmark for Plant DNA Extraction

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Abstract

A wide range of plant genomic DNA extraction or isolation methods are available; however, many of those are limited in their application for a diversified range of plant species. With special emphasis on robustness and versatility, we have improved the cetyl trimethyl ammonium bromide method and isolated high-quality DNA from cotton leaf samples. The major modifications made to the protocol described here were a reduction of sample treatment steps and change in β-mercaptoethanol concentration (to 0.2%) resulting in a robust, rapid, and reproducible plant DNA extraction protocol that can be used for a broad range of plant species and tissue types.

Keywords: *Cotton, DNA, leaf samples, PCR, SSR markers*

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INTRODUCTION

Plants normally produces secondary metabolites that interfere not only with extraction of ultrapure quality genomic DNA but also with the subsequent reactions such as polymerase chain reaction (PCR) and related genetic analyses [1,2]. The widely used genomic DNA extraction procedures rely on lengthy protocols that use hazardous chemicals or expensive commercially available kits. Examples include the cetyl trimethyl ammonium bromide method and its modifications [3, 4], which use reagents like liquid nitrogen, hydrochloric acid, sodium hydroxide, 2-mercaptoethanol, phenol, and chloroform that are either toxic or caustic and, therefore, require use of a fume hood. These procedures are lengthy with a minimum of 5 to 6 hour per extraction [4] and are also expensive. Such methods are, therefore, not suitable for large-scale DNA extractions in laboratories with minimum resources [1, 5].

Using DNA markers in plant breeding with marker-assisted selection has greatly improved the precision and efficiency of selection, ultimately, leading to the accelerated development of new crop varieties.

In the early to mid-1990s, restriction fragment length polymorphism and random amplified polymorphic markers were commonly used for rice-breeding research. Later on, simple sequence repeats (SSRs) became the most widely used markers in cereals. The first SSRs were reported in 1980s in cotton and tomato, which were adequate for marker-assisted evaluation of germplasm and the construction of framework linkage maps.

SSR markers (microsatellites) are sequences of short tandem repeats of bi, tri, tetra nucleotide tandem repeats distributed across the genomes of plants. They have been successfully employed in many genetic diversity studies [6,7] and are useful for a variety of applications in plant genetics and breeding because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance, and good genome coverage [8].

Transgenic cotton plants expressing Cry1Ac and Cry2Ab, from the soil bacterium *Bacillus thuringiensis* (Bt), provide effective control of certain lepidopteran pests. The mode of action of Cry proteins is highly specific to species of insects. Bollgard® II contains two genes,

Cry1Ac and Cry2Ab, and their controlling sequences/promoters. The genes and their controlling sequences produce endotoxins in biotech varieties by expression in the cells of the cotton plant. The endotoxins are very specific in their mode of action. Toxins are effective against Lepidoptera and to some extent; Cry2Ab is effective against Diptera.

The amount of toxin expressed by plant tissues was first performed by Rochester using Enzyme-linked immunosorbent assay (ELISA) using green leaves collected from plants with different treatments. ELISA is used to quantify in vitro constitutive expression of Cry1Ac and Cry2Ab proteins.

Cotton (*Gossypium hirsutum L*) is an important fiber crop of global significance, which is, cultivated in tropical and subtropical regions of more than 70 countries the world over. The large-scale cultivation of Bt cotton is likely to usher in an era of eco-friendly cotton cultivation with reduction in the number of insecticidal applications (40% to 60% less), which in turn will enable better sustenance of parasites and predators in cotton crop.

Genetic purity analysis is carried out using SSR markers. SSR markers are codominant producing polymorphic markers from each of the parental line of the hybrid and hence it would be useful for hybridity and purity testing.

Cotton crop was mostly susceptible to insect pests, which particularly, suffered lot due to bollworm resistance problem to organophosphates and pyrethroids. Among the insect pests, bollworms were the most serious pests of cotton, which developed resistance against most of the recommended insecticides thus making cotton the cultivation unprofitable, which led the way for evolution of Bt era. Thus, the transgenic cotton plants with Cry1Ac and Cry2Ab genes were produced. ELISA is carried out to test the constitutive expression of Cry proteins in Bt Cotton.

MATERIALS AND METHODS

Plant Materials Used and Experimental Conditions

Two genotypes of *Gossypium hirsutum* (superior JKC725 and inferior JKC737 with respect to fiber quality) are used in this study. These cotton varieties are parents of two mapping population developed R&D Centre, JK Agri Genetics Ltd., Ravalkol, Hyderabad during 2013 to 2014. The intraspecific mapping population (recombinant inbred lines [RIL]) of cotton was also generated at the same location.

Briefly, for the generation of intraspecific RIL, two *Gossypium* genotypes contrasting for fiber quality traits JKC737 and JKC725 were crossed and the resulting F_1 plant was self-pollinated to obtain the F_2 offspring that were further self-pollinated and advanced to F_6 generation using single-seed descent method to obtain RILs. An intraspecific mapping population comprising 180 RILs was used for construction of linkage map and further for QTL analysis.

Fresh or frozen tissues of plants were used for DNA extraction. Fresh tissue, quickly frozen in liquid nitrogen, gave satisfactory yields after up to 3 months of storage. The protocol was standardized for 2 g samples that can be handled in a 50 ml disposable screw capped tube, while similar conditions on mini scale using 0.2 g tissues can be scaled down and easily fit into an Eppendorf tube.

Equipment

- A refrigerated tabletop or floor centrifuge capable of 2,700 g or higher with swinging tube rotor and adapter for 50 ml tubes.
- A water bath at 65°C.
- A microcentrifuge that can reach to 10,000 rpm or higher speed.

Reagents

A. Cotton nuclei extraction buffer: (this buffer is modified from [9])
- 0.35 M glucose,
- 0.1M Tris-Cl (pH 8.00),
- 0.005 M Na2-EDTA (pH 8.00),
- 2% PVP-10,
- 0.1% (w/v) ascorbic acid,
- 0.2% (w/v) 2-mercaptoethanol.

B. Cotton nuclei lysis buffer: (this buffer is modified from [10])
- 0.14 M sorbitol,
- 0.22 M Tris-Cl (pH 8.00),
- 0.8 M NaCl,
- 0.22 M Na-EDTA (pH 8.00),

- 0.8% (w/v) CTAB,
- 1% (w/v) PVP-10,
- 1% Sarkosyl,
- 5 mg/ml proteinase-K,
- 0.1% ascorbic acid,
- 0.2% 2-mercaptoethanol.

C. DNA resuspension buffer:

- 100 mM Tris-Cl,
- 10 mM EDTA.

D. PCR reaction buffer: (Boehringer Mannheim).

E. Primers.

F. Nucleotide: (Pharmacia).

Protocol for Cotton Genomic DNA Extraction

Grind about 5 g leaf tissue with mortar and pestle in liquid N_2, transfer the powder into an 50 ml polypropylene tube (50 ml) and store at 20°C.

↓

Add 25 ml of ice-cold extraction buffer, mix well, and put on ice until centrifugation at 3750 rpm for 20 minutes (4°C); remove supernatant (use swinging bucket rotor).

↓

Add 15 ml lysis buffer to the pellet and vortex to resuspend; incubate in at 65°C water bath for 30 minutes. Mix the tubes periodically by stirring or rocking the tubes every 10 minutes.

↓

Add 15 ml of chloroform: Isoamyl alcohol (24:1) and mix gently, inverting tube, until an emulsion forms.

↓

Centrifuge at 3750 rpm for 20 minutes (15°C). Transfer upper phase to a new 50 ml tube. Repeat CIA extraction once.

↓

Add equal volume of cold isopropanol, mix gently, and let set at room temperature for more than 1 hour.

↓

Spool/scoop out the DNA on a 9-inch glass-hook pipet (new and unused) and let it air-dry under the hood or vacuum. If DNA cannot be spooled, centrifuge at 2500 rpm for 10 minutes to form DNA into pellet. Rinse the pellet with cold 70% EtOH. Let tubes dry upside down on paper towels (keep track of tube labels!).

↓

Once pellet is dry, place pellet into 3 ml TE buffer for 30 min at 65°C. Add RNase to a concentration of 20 μg/ml, mix gently, and incubate at 37°C for 15 minutes.

↓

Add 0.1 volume of 3M Na Acetate and two volumes of 95% ethanol for DNA precipitation. Place at −20°C for 1 to 12 hours (overnight).

↓

Centrifuge at 3750 rpm for 10 minutes (15°C). Wash with 70% ethanol.

↓

Dry the pellet and dissolve in 0.5 ml TE Buffer.

↓

Determine both concentration and quality of the DNA with spectrophotometer with a 40μl/800μl dilution (1/20), and by running undigested DNA in a 1% agarose gel.

Estimation of Purity and Concentration of DNA

DNA purity was judged by the absorbency at A260/280 and A260/230 by measuring contamination from proteins and carbohydrates/polyphenolics. RNA contamination was assessed by electrophoresis.

Polymerase Chain Reaction and Primers

Direct PCR is used to perform PCR directly DNA extracted from plant leaves without prior DNA purification. In this method, DNA was placed directly into the PCR reaction mix (Table 1).

Table 1: *Polymerase Chain Reaction Mix Composition.*

Composition (per reaction)	Concentration (per reaction)	Volume (in µl)
SIGMA water	—	4.3
2x phire plant buffer (2.5mM $Mgcl_2$ and 200µM dNTPs)	2x	7.5
Phire hot start II DNA polymerase enzyme	1 unit	0.2
forward primer	10 pm	1
Forward primer	10 pm	1
DNA solution	50 nano grams (approximately)	1
Total volume		15

Enzyme Linked Immunosorbent Assay (Sandwich ELISA)

Antibody coated plates of Cry1Ac and Cry2Ab (design) were loaded with leaf or seed samples prepared using 1X extraction buffer, conjugated antibody was added, and incubated for 45 minutes at room temperature. Then, plates were washed with 1X wash buffer, substrate was added to the plates which gives a blue colored complex (enzyme-substrate reaction) for the positive samples on incubation for 15 to 20 minutes. The blue colored complex is turned yellow on addition of stop solution and the absorbance was measured at 450 nm and the readings were observed based on the cutoff value.

CUT OFF = O.D OF NEGATIVE CONTROL + 0.1

RESULTS AND DISCUSSION

The percentage of purity of the filial generations was calculated by using the formulae:

Genetic purity analysis = total number hybrids-off types/total number of hybrids∗100

If the hybrid lot has a purity of above 98% will be passed into the market (Figure 1).

Table 2: *Markers Used for Screening Two Cotton Genotypes (JKC737 × JKC725): JESPR-178.*

S. No	Primer	Initial denaturation	Final denaturation	Annealing	Initial extension	Final extension
1	JESPR178	98°C–5 Minutes	98°C–10 Seconds	55°C–15 Seconds	72°C–20 Seconds	72°C–5 Minutes
		1 Cycle	39 Cycles			1 Cycle

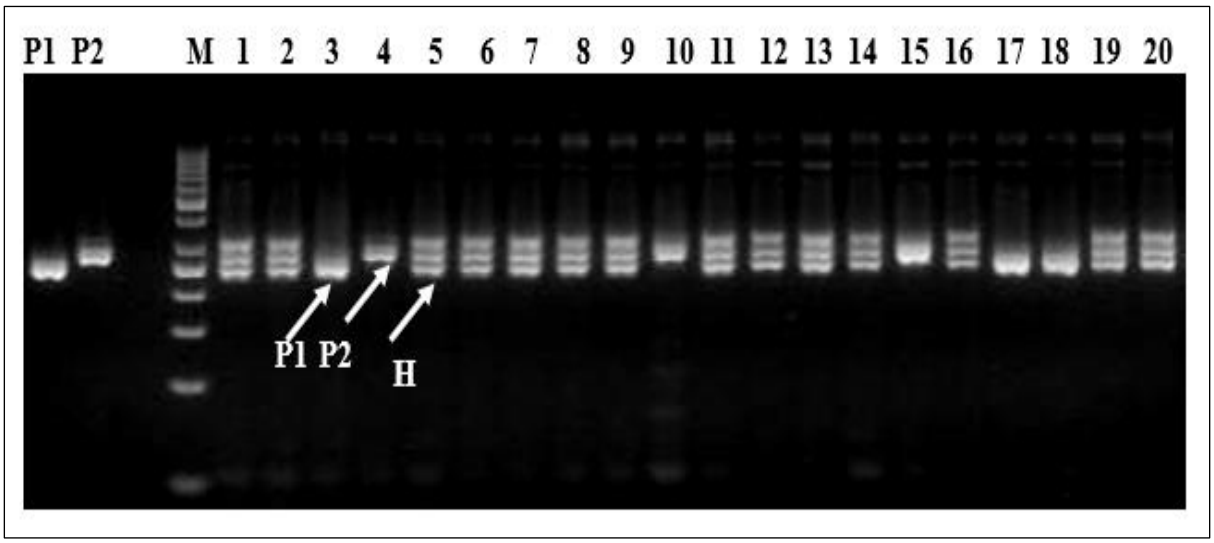

Fig 1: *Schematic Agarose Gel Electrophoresis Representation of Cotton Genotypes (i.e., JKC737 X JKC725).*

P1 and P2, male and female parent controls; M, 50bp ladder; Lanes, 1 to 20 samples; H, Hybrid

GPA% = 75% with both male and female contamination.

The aim of our study was to develop a rapid and cost efficient method for extraction of genomic DNA from fresh leaves and dry leaves of *Gossypium hirsutum*. The quality of DNA produced from this method needed to be high enough for downstream PCR-based genetic analysis.

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