Excised radicle tips as a source of genomic DNA for PCR-based genotyping and melting curve analysis in cotton

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Genomic DNA isolation in cotton is complicated because of the presence of secondary metabolites that are inhibitory to PCR amplification. We report here that radicle tips, but not other parts of cotton seedlings, yield high-quality DNA that is readily amenable for PCR. The radicle-tip-excised seedlings retain viability because of the formation of adventitious roots. We demonstrate the utility of this method in distinguishing homozygotes from heterozygotes in a cotton breeding population and in hybrid seed purity testing.

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1. Introduction

In cotton (*Gossypium* spp.), the isolation of good quality DNA is complicated by the presence of phenolic compounds and alkaloids such as gossypol that have an inhibitory effect on polymerase chain reaction (PCR) amplification (Dabo *et al*. 1993; Porebski *et al*. 1997; Horn *et al*. 2004). The incorporation of several steps for removal of phenolics and alkaloids makes the process of cotton genomic DNA isolation a tedious process (Loomis 1974; Paterson *et al.* 1993).

We have tried to overcome this problem by identifying tissue(s) of cotton seedlings from which DNA can be isolated without the need for elaborate procedures to remove inhibitory substances. We report here that the tips of the radicles of 2-day-old seedlings meet this criterion as DNA isolated from this tissue is highly amenable for PCR and restriction enzyme digestion. The utility of this approach for the cotton research community is demonstrated by (i) performing heterozygosity analysis for a *cry1Ac* transgene in a segregating breeding population and (ii) distinguishing interspecific *Gossypium* hybrids from the two parental lines using high-resolution melting curve analysis (HRMA), a recently introduced technique that is an efficient, accurate and inexpensive method for identifying DNA polymorphisms.

2. Materials and methods

2.1 *Germination of cotton seedlings and isolation of root tip*

Cotton seeds were germinated in moistened filter paper. After 48 h, radicle tips (~1.5 cm length; figure 1) were collected and genomic DNA was isolated. The cotton seeds used were of the following genotypes: Narasimha (courtesy: Regional Agricultural Research Station, Acharya NG Ranga Agricultural University, Lam Farm, Guntur, India); a population segregating for a *Bacillus thuringiensis* (Bt) *cry1Ac* gene (from a breeding program of M/s Nuziveedu Seeds Ltd); G. *barbadense* (NC105) and *G. hirsutum* (NC1108Bt) and the interspecific hybrid (NCHB-990Bt) from Nuziveedu Seeds.

Keywords. Cotton; *cry1Ac*; genomic DNA isolation; high-resolution melting curve analysis; radicle tip; seed purity testing

Abbreviations used: Bt, *Bacillus thuringiensis*; HRMA, high-resolution melting curve analysis

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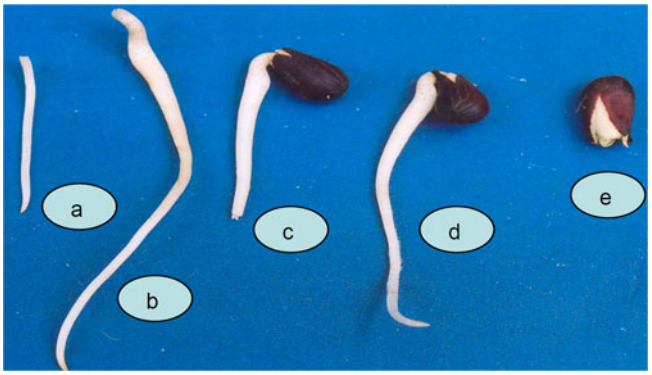

Figure 1. Different parts of cotton seedlings from which genomic DNA was isolated. 48-h-old cotton seedlings were grown in petri dishes and the different parts of the seedlings were excised as described in Materials and Methods. These parts are: a, terminal part of radicle (1st ~1.5 cm from the tip); b, complete radicle; c, cotyledon with proximal part of radicle; d, 48 hr old seedling; and e, cotyledon after radicle excision.

2.2 *Isolation of genomic DNA*

A 1.5 cm region beginning from the tip of the radicle of a 2-day-old cotton seedling (figure 1) was excised using sterile scissors and pulverized in liquid nitrogen using an Eppendorf (1.5 mL) tube and a pestle. 200 µL of grind buffer [4 parts of homogenization buffer (0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA and 0.03 M Tris- HCl, pH 8.0) and 1 part lysis buffer (0.25 M EDTA, 2.5% SDS, 0.5 M Tris-HCl, pH 9.2)] was added and held at 65°C for 10 min. 3 µL of Dnase-free Rnase solution (10 mg/mL) was added and incubated for 20 min after brief homogenization. 133 µL of 3M K-acetate (pH 4.7) was added and the sample was incubated on ice for 30 minutes. The sample was centrifuged in a microfuge for 10 min at 13 K and the sample was transferred to a new Eppendorf tube. The DNA was precipitated using 700 µL of ice cold 100% ethanol. The sample was left at room temperature for 2 min and subsequently spun in a microfuge for 10 min at 13 K. The pellet was washed in cold 70% ethanol and spun in a microfuge for 2 min at 13 K. The ethanol wash was repeated and the pellet was air dried and dissolved in 20 µL nuclease-free water. DNA purity was judged by the ratios of absorbance at A260/280 (assessing protein contamination) and A260/230 (assessing contamination with carbohydrates/polyphenolics).

2.3 *PCR analysis and restriction enzyme digestion*

PCR amplification with cotton ubiquitin gene (DQ 1164410) primers (forward primer, 5′CCTAGAGGTCGAGTCTTCG GAC3′ and reverse primer, 5′ CCGAAGGTAGAAGCG GACTCTACTC3′) was performed as follows: an initial denaturation at 94°C for 2 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. Restriction enzyme digestion of this PCR-amplified fragment was performed with *Hae*III restriction enzyme as per manufacturer's (New England Biolabs, Ipswich, USA) instructions. PCR amplification with *cry1Ac*-gene-specific primers and primers for the endogenous locus at which the *cry1Ac* gene is inserted were performed using 50 ng of genomic DNA as template, 2 pmol of each primer, 0.1 mm dNTP and 1 unit of *Taq* polymerase. A hot start was used with 7 min denaturation at 95°C followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min. PCR was performed in a PTC-100 TM Programmable Thermal Controller, MJ Research, Inc. PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. The sequences of primers for amplification of *cry1Ac* and endogenous locus are proprietory information of Nuziveedu Seeds and Monsanto India Ltd.

2.4 *Melting curve analysis for establishing hybridity*

50 ng genomic DNA each of the parental lines (NC105 and NC1108Bt) and their hybrid (NCHB-990Bt) were used as templates for PCR amplification in a 20 µL volume of Light-Cycler 480 Probes Master mix (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 mM $MgCl_2$ and 200 nM primer.

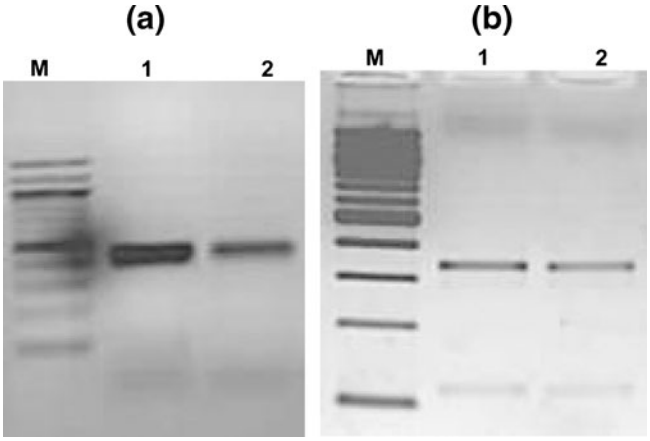

Figure 2. PCR Amplification and restriction digestion of cotton ubiquitin gene fragment. Genomic DNA was isolated from radicle tips of 48-h-old cotton seedlings. (**a**) Cotton ubiquitin-gene-specific primers were used to amplify a 453 bp fragment. (**b**) Restriction digestion of this PCR-amplified fragment with *Hae*III yields the expected 336 bp and 117 bp fragments. Lanes 1 and 2 illustrate results from two representative seedlings and M, the 100 bp DNA size marker.

The forward (5′ CCAGTTAGCACCAATTTAGG 3′) and reverse (5′ CCACAATAACACACTGGAATC 3′) primers of JESPR 56 cotton microsatellite marker were used. PCR cycling and HRMA was performed on an LC480 machine (Roche, Mannheim, Germany). The PCR program consisted of an initial preheating at 95°C for 10 min (ramp rate 4.4°C/s), followed by 45 amplification cycles. Each cycle comprised an annealing step at 58 °C for 10 s, an elongation step at 72°C for 10 s and denaturation at 95°C for 10 s.

The high-resolution melting was carried out at 95°C for 1 min (ramp rate 4.4°C/s), 40°C for 1 min (ramp rate 2.2°C/s) and 75°C for 1 min (ramp rate 4.4°C/s), followed by continuous acquisition for 25 cycles. The sample was later cooled to 40°C. Fluorescence data for melting curves were acquired by integrating the signal during a linear temperature transition to 95°C at 0.1–10°C/s. Fluorescence data were converted into melting peaks by software that plotted the effect of temperature on fluorescence as a negative derivative of fluorescence relative to temperature versus the temperature i.e. −dF/dT vs T (Ririe *et al.* 1997; Bernard *et al.* 1998 and Wittwer *et al.* 2003; Vossen *et al.* 2009; Wu *et al* 2010). The software also plotted difference curves wherein one of the parents was taken as a baseline (*G. hirsutum*) and subtracted the fluorescence of each of the other two samples with regard to this baseline.

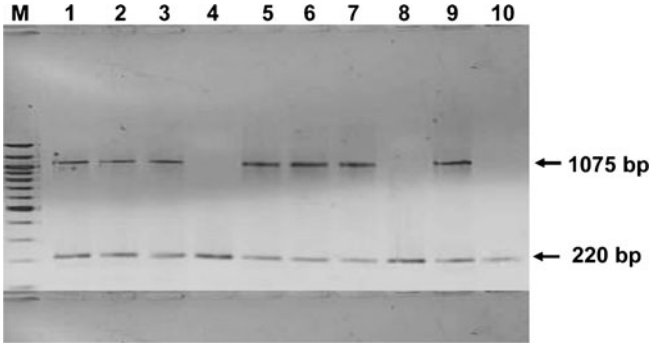

Figure 4. Heterozygosity analysis for a *cry1Ac* gene in a segregating population. Genomic DNA was isolated from radicle tips of 48-h-old cotton seedlings. PCR was performed and genotypes were assessed as described in materials and methods. The 220 bp fragment is indicative of the presence of the *cry1Ac* gene. The 1075 bp fragment represents the endogenous locus. Plants that are homozygous for the *cry1Ac* gene are in lanes 4, 8 and 10. The remaining plants are all heterozygous for *cry1Ac*. M, 100 bp DNA size marker.

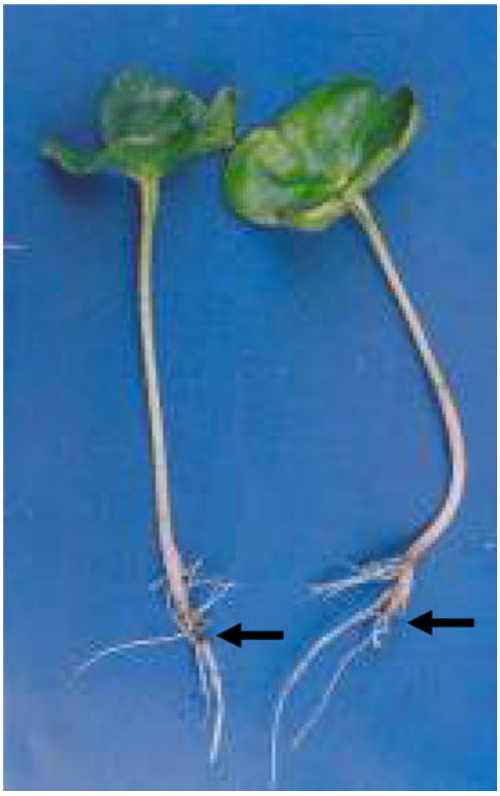

Figure 3. Adventitious root formation in radicle tip excised cotton seedlings. The radicle tips were excised from 48-h-old cotton seedlings. After excision, seedlings were transferred to sterile soil. 10 days later, adventitious roots were found in excised (seedling on the right; see arrow) and unexcised seedlings (seedling on the left).

3. Results and discussion

3.1 *Isolation of genomic DNA from radicle tips of cotton seedlings*

The radicle tip (figure 1a) from each seedling was excised and collected in a 1.5 mL Eppendorf tube and genomic DNA was isolated as described in the section on materials and methods. To simplify the protocol, neither chloroform-isoamyl alcohol nor phenol-chloroform extraction was used. The protocol described here provided us with high-quality genomic DNA from small samples with the DNA yield ranging from 50 to 80 µg/100 mg of tissue. The absorbance ratios (A 260/280) for the DNA preparations were between 1.8 and 2.0, which is consistent with the recommended ratios for pure preparations. As per our estimate, considering the time taken for grinding the sample and centrifugation, a single person can extract DNA from 300 samples/day. The number of samples that can be analysed per day can be greatly increased by introducing automation. Genomic DNA isolated from any other part of the radicle or cotyledon other than the radicle tip (figure 1b–e) had an absorbance ratio that falls below 1.80.

3.2 *Genomic DNA isolated from radicle tips is amenable for PCR*

The genomic DNA isolated by this method using radicle root tips was used as template in a PCR assay to amplify a 45 3 bp internal fragment of the cotton ubiquitin gene (figure 2). PCR-amplified fragments were obtained reproducibly and upon staining with ethidium bromide, they appeared as clean and sharp bands. Restriction enzyme digestion of the PCR-amplified fragment with *Hae*III resulted in the expected 336 bp and 117 bp fragments. PCR products were not obtained when genomic DNA isolated from any other part of the radicle or cotyledon was used as a template. This points to the possibility that the terminal part of the developing radicle is either free from polyphenolics or their content is very much reduced in comparison to other parts of the radicle and cotyledons. The survival rate of the seedlings whose radicle tips were excised is close to 100% as adventitious roots are formed (figure 3). Therefore, this method is nondestructive.

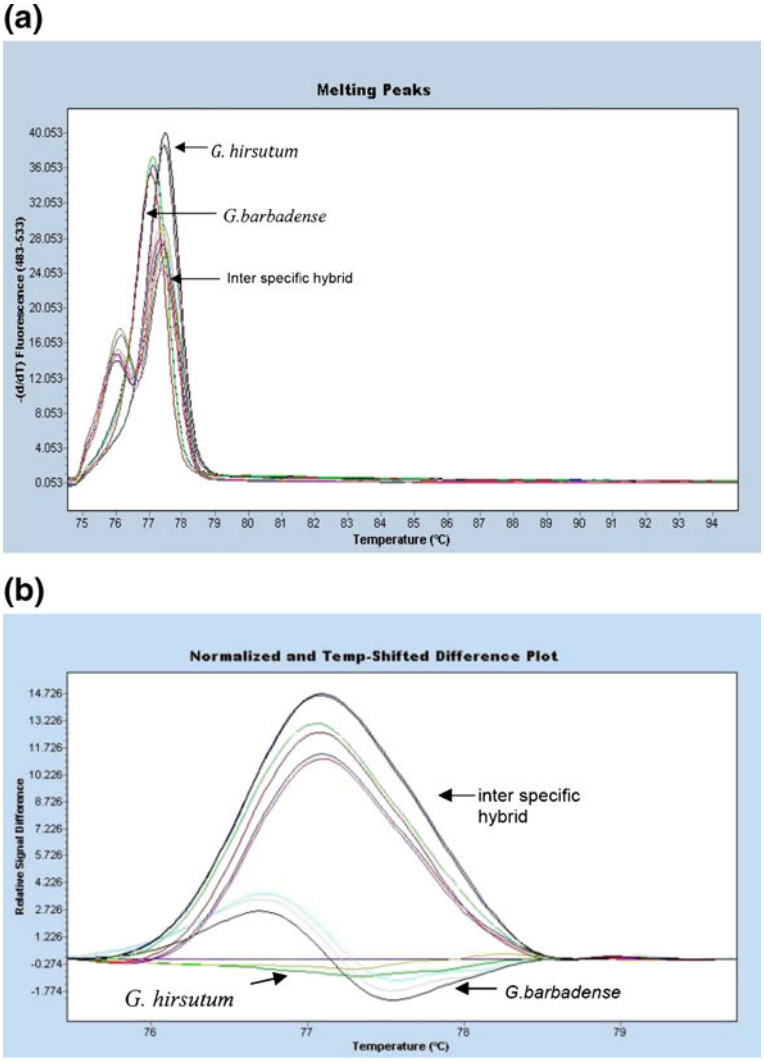

Figure 5. Determining hybridity using high resolution melting curve analysis of PCR amplified products. Genomic DNA was isolated from radicle tips of 48-h-old seedlings from the parental lines *G. barbadense* NC105 and *G. hirsutum* NC1108Bt as well as their interspecific hybrid, NCHB-990Bt. PCR was performed using JESPR 56 microsatellite marker. (**a**) Negative first derivative plots (−dF/dT vs T) of the parents and the hybrid. (**b**) Difference curves showing the variation in fluorescence with respect to temperature between *G. hirsutum* (baseline) and either *G. barbadense* or the hybrid. The difference curves show a clear difference between the genotypes. Each line corresponds to a single plant.

3.3 *Zygosity analysis for the cryIAc gene*

In a breeding program in which the *cryIAc* gene was introgressed into new genetic backgrounds, individual plants were genotyped to assess whether they are homozygous or heterozygous for the *cry1Ac* gene. Primers that are specific for the *cry1Ac* insertion event and a pair of primers that amplify the endogenous locus were used in a PCR assay to genotype individual plants as described in the section on materials and methods. The *cry1Ac*-gene-specific product (220 bp fragment) was amplified from all 10 plants that were tested (figure 4). However, the amplification of the endogenous locus (1075 bp fragment) occured in 7 plants. These plants were considered to be heterozygous for the *cry1Ac* gene. The absence of the 1075 bp allele indicates that the plant is homozygous for the *cry1Ac* gene. As per this criterion, 7 out of the 10 plants were heterozygous and the remaining 3 plants were homozygous for the transgene.

3.4 *Assessing hybridity using fluorescent melting analysis of PCR products*

We performed HRMA (excitation 485 nm and emission 500 nm) of PCR-amplified fragments obtained using primers for the cotton JESPR56 locus with template DNA from the parental lines NC105 and NC1108Bt as well as their hybrid (NCHB-990Bt). The JESPR56 marker was chosen as it was found to be polymorphic (as judged by PCR and gel electrophoresis followed by ethidium bromide staining) between NC105 and NC1108Bt in a screen of 20 cotton microsatellite markers (data not shown). The three genotypes were resolved based on their melting curves. The heterozygotes are distinguishable from the parents in the negative first derivative plots (figure 5a) as well as in the difference plots (figure 5b). Hybridity was also confirmed by agarose gel electrophoresis and ethidium bromide staining (data not shown).

Hybrid cotton seed production forms the mainstay of the flourishing seed industry in India. Assessing cotton hybrid seed purity is an essential component of quality control programs. The use of microsatellite markers for seed purity testing using PCR and gel electrophoresis has been described earlier (Yashitola *et al.* 2002). However, detection of polymorphism using electrophoresis is cumbersome and not easily amenable for automation. HRMA has been used extensively in human diagnostic applications (Vossen *et al.* 2009) and also in plants for detecting SNPs and microsatellite marker polymorphsims (Chagné *et al.* 2008; Croxford *et al.* 2008; Lehmensiek *et al.* 2008; Mader *et al.* 2008; Wu *et al.* 2010). In this study, we show that HRMA using microsatellite markers can be used for distinguishing parental lines from hybrids in cotton. The detection of microsatellite marker polymorphism using HRMA has the advantage that it can be fully automated. This automation will facilitate the handling of thousands of seed lots for purity testing in a timely manner. Time is of the essence in commercial hybrid seed production programmes in India as the gap between the hybrid seed production season and the growing season is limited and large numbers of seed lots have to be analysed. Although the present day costs of a HRMA instrument and the dyes used are higher than those used in conventional PCR and gel electrophoresis, we anticipate that costs will come down, and because of the savings accrued from automation, this method will be routinely used for hybrid cotton seed purity testing purposes in the future. HRMA also permits the application of SNPs in hybrid seed purity testing. This is likely to be an added advantage in crops like cotton wherein it is sometimes difficult to identify microsatellite markers that are polymorphic between parental lines of hybrids.

4. Conclusion

This work reports a simple and nondestructive method of genomic DNA isolation in cotton and demonstrates amenability for PCR amplification. The major novelty of this work is the identification of the radicle tips of seedlings as the tissue of choice for isolation of high-quality genomic DNA in cotton. Leaf is widely used as tissue for isolation of cotton genomic DNA. However, the DNA isolated from leaves requires elaborate cleaning procedures to match the quality of that isolated from radicle tips. Interestingly, no other parts of cotton seedlings other than radicle tips yielded DNA of the desired quality. This suggests that secondary metabolites that are inhibitory to PCR are absent from radicle tips but are present in other parts of cotton seedlings/plants. We anticipate that the methodology described here will find a lot of application in cotton breeding and seed purity testing programs.

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