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Short Research Article

Identification of Suitable SSR Marker for Testing of Genetic Purity of Cotton Hybrids

Menka[1], Amarendra Kumar[2*] and S. K. Yadav[1]

[1]Division of Seed Science and Technology, Indian Agricultural Research Institute, New Delhi (110 012), India
[2]Dept. of Plant Pathology, Bihar Agricultural University, Sabour, Bhagalpur, Bihar (813 210), India

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Correspondence to

E-mail: kumaramar05@gmail.com

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Abstract

India has the largest area in the World under cotton cultivation but the productivity is quite low due to inadequate supply of inputs including genetically pure seed to the farmers. Only high quality seeds of assured genetic purity can be expected to respond fully to all other inputs. Among the different quality aspect genetic purity is of utmost importance and enables farmers to exploit the full potential of hybrids. Grow Out Test (GOT) is mandatory to test the genetic purity of cotton hybrids as per the Indian minimum seed certification standards. But, it is labour intensive, tedious, time consuming and the expression of morphological characters may be influenced by environmental conditions. Alternatively, molecular markers could be used to assess the genetic purity to obtain quick, reliable and reproducible results. The genetic purity of two inters specific cotton hybrids namely, DCH 32 and RAHB 87 were assessed by microsatellite markers. Out of the 40 primers studied eight markers viz., (BNL 840, BNL 2590, BNL 3103, BNL 169, BNL 2544, BNL 2895, BNL 2572 and BNL 3441) were found polymorphic among the parents of both the hybrids. All the two hybrids were clearly distinguished from their selfed females and off types using these SSRs markers. Hence, these SSR markers can be used for the genetic purity testing of the cotton hybrids as it is accurate, rapid and without any influence of environment.

1. Introduction

Cotton is the world's most important source of natural textile fibre and a significant oilseed crop. Cotton production provides income to approximately 100 m families, across approximately 150 countries that are involved in cotton import and export. Cotton and Textile exports account for nearly one-third of total foreign exchange earnings of India, each year at an estimate of ₹ 750 billion (Anonymous, 2011). At present, in India more than 90% area under cotton cultivation is covered by hybrid cotton alone (Anonymous, 2012).

The increased production and productivity is credited to the release of high yielding hybrids for commercial cultivation which occupy around 22% of the total area under cotton. The yield of these hybrids in many places is about 50% higher than the pure line varieties (Rana et al., 2006). Hybrid cotton seed production is a labour intensive affair as it is produced by hand emasculation and pollination thereby making the hybrid cotton seed costly. Cotton being an often cross pollinated crop, there are chances of contamination with the foreign pollen. Also, due to manual error there may be selfing of female parent. Maintaining genetic purity is of utmost importance that helps to exploit the full potential of hybrids. When a seed lot is passed from one generation to another, some form of genetic contamination is likely to occur, which cannot be detected morphologically and may go on accumulating unnoticed in the population finally leading to deterioration of genetic worth of that variety. Therefore, a reliable method to discriminate between cultivars as well as to assess genetic purity of seed samples will enable seed producers to monitor and maintain adequate levels of genetic purity at each generation of seed production and multiplication. This will ultimately ensure high quality seeds.

Therefore, to ensure the genetic purity of hybrid cotton seeds Grow Out Test (GOT) is mandatory for certification as per Indian Minimum Seed Certification Standards. In this technique the morphological characters are scored at various stages of plant growth up to maturity (Selvakumar et al., 2010). The main limitations of this method are time consuming, labour intensive and require more space which now-a-days has become scarce. Moreover, expression of certain morphological characters is influenced by the environmental factors leading to false positive and false

negative results. Conventional GOT requires one full season thus exclude the possibility of immediate use of hybrid seed for cultivation. This results in extra expenditure incurred on the safe storage which ultimately increases the hybrid seed cost (Nandakumar et al., 2004). If the storage condition is not proper there may be reduction in seed vigour and viability. The biochemical markers such as isozymes and seed storage proteins have been suggested for genetic purity determination (Dadlani et al., 1997; Mehetre and Dahat, 2001; Borle et al., 2007; Rakshit et al., 2008) but the main weaknesses of biochemical marker are its low abundance and sensitivity to environmental and experimental conditions. These limitations can be overcome by the application of molecular markers i.e. a rapid, reliable and reproducible technique for testing of genetic purity of cotton hybrids.

Molecular markers test the genetic purity of the seeds at nucleotide level. It is fast, reliable and require minimal amount of tissue for investigation (Rana et al., 2006). DNA markers such as RFLP (Pendse et al., 2001; Dongre and Parkhi, 2005) RAPD (Geng et al., 1995; Venu, 2001; Rao et al., 2002; Mehetre et al., 2007), AFLP (Rana and Bhat, 2004), SSR (Dongre and Parkhi, 2005; Saravanan et al., 2007; Selvakumar et al., 2010) and ISSR (Dongre and Parkhi, 2005; Rana et al., 2006) have been used to rapidly screen genetic purity of hybrid seed lots. It screens through the whole genome and produces enough polymorphism in closely related parental lines which can be exploited for ensuring the seed purity of parental lines and hybrids. The DNA markers have several advantages like, rapid, reliable and independence from the environmental influence. Dongare and Parkhi (2005) studied genetic purity of cotton F1 hybrid H6 and its parental lines G.Cot-10 (Male) and G.Cot-100 (Female) by using a combination of RAPD, ISSR and SSR markers. Rana et al. (2006) assessed genetic purity in eight commercial cotton hybrids and their parental lines using RAPD, ISSR and STMS markers.

Keeping in view the wide cultivation of cotton hybrids and the associated problem of availability of genetically pure seeds, the present research work was undertaken to identify the suitable molecular marker/s for genetic purity of two popular cotton hybrids of public sector.

2. Materials and Methods

2.1. Seed materials

Seeds of cotton hybrids namely DCH 32 and RAHB 87 and their parents procured from University of Agricultural Sciences, Dharwad were used for genetic purity testing.

2.2. DNA extraction

Seedlings were raised in sterlized sand under glasshouse conditions at 25 °C. Leaf samples from the seeds of parental lines and hybrids were collected from 15 days old seedlings. Leaf samples were used for DNA extraction through CTAB method following a modified procedure of Edwards et al. (1991). Purification of DNA was done to remove RNA, protein and polysaccharides which are the major contamination in DNA precipitate. Addition of CTAB in the DNA extraction buffer helps in the elimination of polysaccharides. RNA was removed by RNAse treatment and proteins were removed by phenol chloroform extraction.

2.3. DNA markers

Among the DNA marker techniques used for variety identification and verification, STMS is technically most robust, reproducible, co-dominant and rapid. Therefore, STMS markers were used for genetic purity testing of the intraspecific cotton hybrids. A total of 40 micro satellite primers belonging to BNL and CML series reported from Brookhaven National Laboratories (BNL) were randomly chosen across the cotton genome. They were custom synthesized by SAF Labs Private limited, Mumbai. These primers were screened on template DNA from parental lines and the hybrids. Details regarding the sequences of the primers are provided in Table 1.

2.4. Molecular analysis by SSR markers

The Polymerase Chain Reaction (PCR) was carried out in a final volume of 10 µl containing 1 µl DNA template (25 ng μl^{-1}), 0.4 µl dNTPs (10 mm, 2.5 mm each), 1 µl 10×PCR buffer containing $MgCl_2$ (25 mm), 0.2 µl *Taq* polymerase (5 U μl^{-1}), 0.4 µl of each of the two primers (forward and reverse), 6.6 µl nuclease-free water. The thermocycler (Eppendorf Company) was programmed as follows: 5 min at 94 °C; 40 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 30 sec at 72 °C and a final 7 min extension at 72 °C, followed by cooling down to 4 °C. The polymerase chain reaction products were separated in 3.5% (w/v) metaphor agarose stained with ethidium bromide solution. The electrophoresis was performed at 100 V for 180 min. The size of the amplified product was determined using 50 base pair ladder (NEX-GEN DNA ladder, PUREGENE) and the bands were visualized under UV in a Gel Doc Image Analysis System (Alpha Imager HP, Cell Biosciences).

3. Results and Discussion

Forty SSR markers were screened from the cotton marker database (www.cottonmarker.org) to ensure the genetic purity of the two cotton hybrids and their parental lines. Out of above mentioned 40 primers, eight primers were found to be polymorphic (BNL 169, BNL 2590, BNL 2895, BNL 840, BNL 3103, BNL 2544, BNL 2572 and BNL 3441) among their parental lines. These highly informative primers not only differentiated the two hybrids but also confirmed the

parentage of the F_1 hybrid. BNL 1440 showed polymorphism between parents of DCH 32 but showed female specific nature in RAHB 87. Primer BNL 3649 and CML 63 produced no amplification under different PCR conditions tried while in BNL 686 (2) no specific pattern was found. Remaining of the 28 markers were found to be monomorphic in the two hybrids and their parental lines. In Table 1 details of approximate amplified product size has been mentioned.

Polymorphic markers produced unique banding pattern, it helped in not only distinguishing the parents, but also identified their true hybrids. Polymorphism between the individuals arises through nucleotide/s substitution and insertions or deletions (Williams et al., 1990). The polymorphism of SSR markers are based on the differences in the number of repeats in the amplified region.

Table 1: List of cotton markers that have amplified the fragment of the genome across the genome the hybrids and parental lines studied

Sl. No.	Primer	Repeat motifs	Approx. amplified product size (bp)	Sl. no.	Primer	Repeat motifs	Approx. amplified product size (bp)
1.	BNL 3442	$(CA)_{14}$, $(TA)_5$	110–150	21.	BNL 3449	$(CA)_{12}$, $(CT)_6$, $TA(CA)_{12}$	120–160
2.	BNL 2572	$(GA)_{23}$	200–250	22.	BNL 3441	$(AC)_{18}$, $(AT)_2$, $(AC)_{18}$, $(AT)_4$	200–220
3.	BNL 3556	$(AC)_{12}$, $(AT)_4$	150	23.	BNL 3627	$(TC)_{17}$	150–200
4.	BNL 3084	$(GA)_{12}$	150–170	24.	CML 60	$(AG)_8$	250
5.	BNL 3034	$(AG)_{12}$	140–160	25.	CML 66 (2)	$(AG)_{12}$	200–250
6.	BNL 3563	$(CA)_{13}$, $(TA)_4$	200–250	26.	BNL 3090	$(AG)_{31}$	200–250
7.	BNL 1350	$(CA)_8$, $(GA)_{16}$	150–200	27.	BNL 256	$(GA)_{17}$	200–300
8.	BNL 3065	$(AG)_{21}$	150–200	28.	BNL 2544	$(AG)_{11}$	200–250
9.	BNL 1672	$(AG)_{14}$	100–150	29.	BNL 3479	$(AC)_{15}$,$(TC)_6$,$T(AC)_{15}$,$G(CA)_2$	200–250
10.	BNL 1317(2)	$(AG)_{14}$	150–200	30.	BNL 1440	$(AG)_{15}$	200–260
11.	BNL 1679	$(AG)_{17}$	150–200	31.	BNL 2590	$(AG)_{11}$	150–200
12.	BNL 1053	$(AC)_{16}$	150–200	32.	CML 43	$(TC)_{20}$	200–300
13.	BNL 1064	$(CA)_{15}$, $(GT)_{13}$	130–140	33.	BNL 2895	$(GA)_{10}$	200–250
14.	BNL 3279	$(AG)_{15}$	200	34.	BNL 3103	$(GA)_{13}$, $(TC)_{14}$	150–200
15.	BNL 2960	$(GA)_{10}$	150–200	35.	BNL 2544	$(AG)_{11}$	200–250
16.	BNL 3599	$(TC)_{15}$	150–200	36.	BNL 2634	$(AG)_{11}$	200–250
17.	BNL 169	$(GA)_{15}$	200–250	37.	BNL 352	$(GA)_{13}$	150–200
18.	BNL 840	$(CA)_{19}$	150–200	38.	BNL 2544	$(AG)_{11}$	200–250
19.	BNL 3816	$(TG)_{15}$, $(TG)_5$, $TA(TG)_{15}$	150–200	39.	BNL 3649	$(TC)_{20}$	No amplification
20.	BNL 3255	$(GC)_6$, $AT(AC)_{14}$	220–240	40.	CML 63	$(TC)_{23}$	No amplification

The polymorphisms observed between the parents were used as markers for identification of hybrids. The SSR marker BNL 840, BNL 2590, BNL 3103 (Figure 1) produced 2 alleles in which allele 1 was specific to the female, allele 2 was specific to the male parent and both the allele were present in the hybrid. These primers amplified the polymorphic fragments of 150-200 bp size. The SSR markers BNL 2544, BNL 2895, BNL 2572, BNL 169 and BNL 3441 (Figure 2) also amplified two alleles of 200-250 bp amplicon size. Selvakumar et al. (2010) reported that six SSR markers (BNL 686, BNL 1679, BNL 3971, BNL 3955, CIR 407 and CIR 413) were useful in determination of the genetic purity of 3 cotton (*Gossypium* spp.) hybrids using SSR. These markers were clearly distinguished hybrids from their selfed females and off types.

Another SSR marker BNL 1440 clearly distinguished DCH-32 from its parental lines but RAHB-87 hybrid exhibited female specific banding pattern. The female parent (DS-28) and the male parent SB(YF)-425 produced the amplicon of size of about 190 bp and 200 bp respectively and hybrid generated both the amplicons. On the other hand RAHB-87

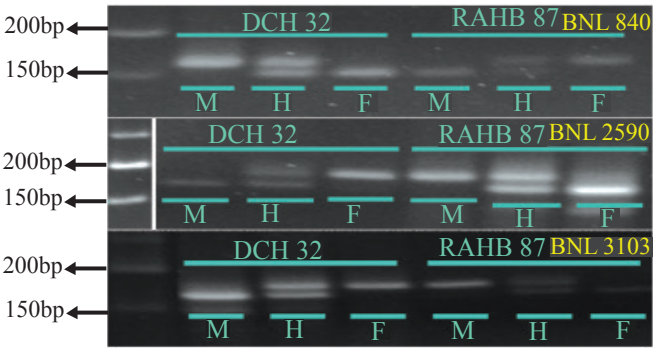

Figure 1: SSR profile of cotton hybrids (H) and their parental lines (M and F) obtained from BNL 840, BNL 2590, BNL 3103 and Ladder=50 bp DNA ruler from PUREGENE

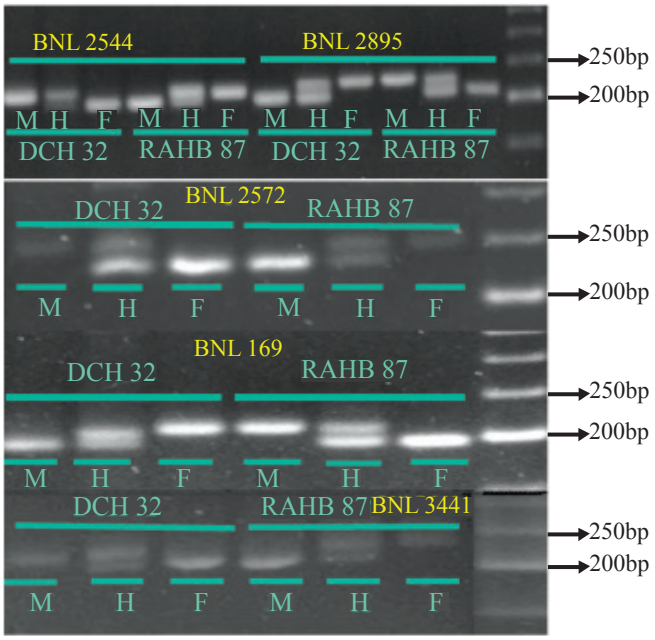

Figure 2: SSR profile of cotton hybrids (H) and their parental lines (M and F) obtained from BNL 2544, BNL 2895, BNL 2572, BNL 169, BNL 3441 and Ladder=50 bp DNA ruler from PUREGENE

hybrid and RAH (female parent) amplified 2 alleles of size 190 bp and 200 bp, while RAB (male parent) amplified allele of size 190 bp. Macha (2010) working on the genetic purity assessment in hybrid seed lots of DCH-32 and its parental lines observed that seven RAPD primers produced male parent specific marker. The marker CML 66(2) produced 2 distinguishable allele, allele 1 of amplicon size 190 bp was specific to the female parent in DCH-32 but in RHAB 87 it was specific to the male parent. Two allele of size 190 bp and 210 bp were present in both the hybrids.

RAPD and STMS markers have been successfully employed for confirmation of parentage, identification of hybrids, characterization of cultivars and testing genetic purity in other crops (Asif et al., 2006; Bertini et al., 2006, Tabbasam et al., 2006). Dongre et al. (2012) observed that a combination of two PCR based markers i.e. RAPD and ISSR can be used for testing the genetic purity of cotton seeds which will be more reliable substitute for GOT and a tool for seed certification. These results confirmed that identified SSR markers could be used for testing the genetic purity of cotton hybrids in a short span of time.

4. Conclusion

Eight primers were identified which could be effectively used to assess the genetic purity of the cotton hybrids, namely DCH 32 and RAHB 87. This study has shown that SSR markers are powerful biotechnological tool capable of detecting genetic purity status of cotton hybrids. It can be concluded that the inclusion of DNA analysis using SSR markers to determine genetic purity of cotton seed is recommended.

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