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Full Length Research Paper

Genetic purity testing of cotton F1 hybrid DHH-11 and parents revealed by molecular markers

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Cotton F1 Hybrid DHH-11 and its parents CPD-420 (male) and CPD-423 (female) was analyzed for the development of markers, identification of Hybrids and ascertaining genetic purity in *Gossypium hirsutum* L. through PCR based Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSRs) DNA Markers. Twenty RAPD primers and nineteen ISSR primers were used for analysis. RAPD primer, OPA 7 was found to be heteroallelic for parents. OPA 7 identified male specific amplicons of about 350bp in the Hybrid DHH-11. The other repeats of different base pairs were homoallelic for both the parents. ISSR primer, IS 15 was found to be heteroallelic for parents. IS 15 identified a female-specific amplicon of about 1000bp in the Hybrid DHH-11. Comparison of the RAPD and ISSR banding pattern of the parents with respective hybrid clearly identified genuine hybrid. Thus RAPD and ISSR markers individually have their own merits in the identification of parents and their hybrids. However, a combination of two PCR based markers 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. This study suggested that efficiency of RAPD and ISSR assay was evaluated successfully for hybridity determination, which would be a valuable genomic tool for the cotton breeders.

Keywords: Genetic Purity, *Gossypium*, RAPD, ISSR.

INTRODUCTION

Cotton is an important cash crop in India; it is commonly called as 'white gold' (Kumar et al., 2007). India was the first country in the world to domesticate cotton for the production of cotton fabrics, when members of the Indus Valley Civilization began to grow the fiber in 1750 BC for manufacturing textiles (Thomasson 2010). After China, India is the largest producer and consumer of cotton, the country accounting for a little over 21.83% of the global cotton production in 2010-11 (Cotton Incorporated, USA 2011). The country's output for the year 2010-11 cotton season is estimated at a record 31.2 million bales of 170 kilograms (kg) each, with cotton cultivated area totaling 11.1 million hectares and yielding 475 kg of cotton per hectare (The Cotton Corp. of India Ltd. Reports 2010-11). In case of organic cotton production, India is the world's leading producers in 2009-10 for the third straight year, growing over 80 percent of the organic cotton produced globally and increasing its production of the fiber by 37 percent over 2009-10 (Marquardt 2011). For the improvement of agronomically and economically important traits, plant breeding generally recombines traits present in different parental lines of cultivated and wild species.

Gossypium hirsutum varieties have been developed from crosses between closely related ancestors but only limited increases in productivity. Pressure for higher productivity in cotton farming has stimulated the search for more exotic germplasm, but although breeding methods have increased the efficiency of transferring alleles from exotic germplasm sources to cotton breeding gene pools many germplasm sources still remain underused. The genetic diversity ensures protection procedures against diseases and pests and thus provides a basis for future genetic gains (Esbroeck et al., 1998).

Molecular markers have been widely used in genetic

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analyses, breeding studies and investigations of genetic diversity and the relationship between cultivated species and their wild parents because they have several advantages as compared with morphological markers, including high polymorphism and independence from effects related to environmental conditions and the physiological stage of the plant (Bertini et al., 2006). For research involving cotton (*Gossypium hirsutum* L.) the most widely used molecular methods has been the Random Amplified Polymorphic DNA (RAPD) technique (Multani and Lyon 1995; Lu and Myers 2002) and Inter Simple Sequence Repeats (ISSRs) (Wu et al. 1994), though allozymes (Wendel et al., 1992), Restriction Fragment Length Polymorphism (RFLP) (Wendel and Brubaker 1993), Amplified Fragment Length Polymorphism (AFLP) (Pillay and Myers 1999; Abdalla et al., 2001) and Simple Sequence Repeats (SSRs) or Microsatellites (Reddy et al. 2002) have all been used successfully in genetic diversity analyses in many species including cotton but the major limitations of these methods are low reproducibility of RAPD, high cost of Amplified Fragment Length Polymorphism (AFLP) and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Belaj et al., 2003). ISSR-PCR is a technique that overcomes most of these limitations. It is rapidly being used by the research community in various fields of plant improvement (Reddy et al., 2002) as molecular studies of the genetic diversity of cultivated cotton have generally shown low genetic diversity (Brubaker and Wendel 1994; Tatineni et al. 1996; Iqbal et al., 1997) thus more than one marker, likely to be promising for genetic purity testing of cotton.

The objective of the present study was to test genetic purity and identify the markers for cotton F1 hybrid DHH-11 and it's parents revealed by using combination of RAPD and ISSR markers.

MATERIALS AND METHODS

Plant material

The material for the present investigation comprised of intraspecific cotton F1 hybrid DHH-11 and its parents CPD-420 (male) and CPD-423 (female) were obtained from Agriculture Research Station, Dharwad Farm, University of Agricultural Sciences, Dharwad. The seed were germinated in the pot house and the authenticity of plants was confirmed phenotypically in flowering stage. DHH-11, CPD-420 and CPD-423 are all tall plants having 171, 148 and 164 cm height and 50% flowering at appx. 68, 77 and 71 days respectively with palmate (normal) leaf shape, shallow leaf incision and hairy leaf pubescence, cream petals without spot, cream colour anther and normal bract type. DHH-11 and CPD-420 have green leaf colour and exerted position of stigma whereas CPD-423 have light green leaf colour and embedded position of stigma. DHH-11 and CPD-423 have medium leaf size and ovate boll shape while CPD-420 have small leaf size and rounded boll shape. CPD -420 and CPD-423 showed less numbers of serration on bract while DHH-11 showed more (Reddy et al., 2007).

Sample Collection

Four to five fresh, young, healthy leaves were collected from pot house and kept at 4ºC in the sterilized cooling box to avoid drying and minimize the phenolics compound level.

DNA Extraction

About 4-5 gram collected leaves were taken for DNA isolation. A modified procedure of Edward et al., (1991) was used, in which 2% polyvinyl pyrrolidone (PVP) was added in DNA extraction buffer to avoid co-isolation of phenolics and polysaccharides with 5-10ug of RNAase. Quality and quantity of DNA were estimated by measuring O. D. at 260/280nm in UV Spectrophotometer. Intactness of genomic DNA was checked by gel electrophoresis.

RAPD and ISSR analysis

A total of 20 RAPD primers (Almeda CA, USA) and 19 ISSR primers (4 primers were with 3bp repeats of (AGC) 4 anchored at 5'end, 12 primers were 2 bp repeats (CA)6 and (GT)6 anchored at 5'end and 3 primers were 2 bp repeats (GA)5 anchored at 3' end), selected from 55 ISSR primers that were designed and screened for PCR amplification were synthesized from Bangalore Genei Pvt. Ltd. (Table 1) A 25ul PCR reaction mixture contained 40 ng of genomic DNA, 1x PCR buffer, 200 ∝M of each dNTP (Q-Biogene, USA), 1U *Taq* polymerase (Q-Biogene, USA) and 15 ng Primer (Q-Biogene, USA). An initial denaturing step of 6 min. at 94°C was followed by 35 PCR cycles (denaturing at 94°C for 45 sec., primer annealing at 36°C for 1 min. and primer extension at 72°C for 2 min.) in RAPD. An initial denaturing step of 10 min. at 94°C was followed by 35 PCR cycles (denaturing at 94°C for 1 min., primer annealing at 49°C for 1 min. and primer extension at 72°C for 2 min.) in ISSR. A final step of 10min at 72°C was carried out for polishing the ends of PCR products in RAPD and ISSR. The DNA amplification was carried out in Biometra UNO thermoblock thermo cycler.

Table 1- 1 RAPD and ISSR primers with sequences (5'-3') used in the study.

Sr. No.	Primer code	Primer Sequence (5'-3')
RAPD Primers		
1.	OPA 1	CAGGCCCTTC
2.	OPA 2	TGCCGAGCTG
3.	OPA 3	AGTCAGCCAC
4.	OPA 4	AATCGGGCTG
5.	OPA 5	AGGGGTCTTG
6.	OPA 6	GGTCCCTGAC
7.	OPA 7	GAAACGGGTG
8.	OPA 8	GTGACGTAGG
9.	OPA 9	GGGTAACGCC
10.	OPA 10	GTGATCGCAG
11.	OPA 11	CAATCGCCGT
12.	OPA 12	TCGGCGATAG
13.	OPA 13	CAGCACCCAC
14.	OPA 14	TCTGTGCTGG
15.	OPA 15	TTCCGAACCC
16.	OPA 16	AGCCAGCGAA
17.	OPA 17	GACCGCTTGT
18.	OPA 18	AGGTGACCGT
19.	OPA 19	CAAACGTCGG
20.	OPA 20	GTTGCGATCC
ISSR Primers		
1.	IS 1	(AGC)4 GA
2.	IS 2	(AGC)4 GG
3.	IS 3	(AGC)4 GT
4.	IS 4	(AGC)4 GC
5.	IS 5	(CA)6 AT
6.	IS 6	(CA)6 AC
7.	IS 7	(CA)6 GT
8.	IS 8	(CA)6 GC
9.	IS 9	(CA)6 GA
10.	IS 10	(CA)6 AA
11.	IS 11	(GT)6 TA
12.	IS 12	(GT)6 TG
13.	IS 13	(GT)6 CA
14.	IS 14	(GT)6 CT
15.	IS 15	(GT)6 AT
16.	IS 16	(GT)6 AC
17.	IS 17	CAG (GA)5
18.	IS 18	GCT (GA)5
19.	IS 19	GCA (GA)5

Resolution of PCR products

Amplified PCR products of RAPD and ISSR were separated on 1.6% agarose gel. 3 kb DNA ladder (Bangalore Genei Pvt. Ltd.) was used as a molecular size standard.

The gel were stained with Ethidium bromide (10mg/ml) and photographed under UV light in ALPHA.

RESULTS AND DISCUSSION

Genetic purity of cotton F1 Hybrid DHH-11 and its parents CPD-420 (male) and CPD-423 (female) were carried out

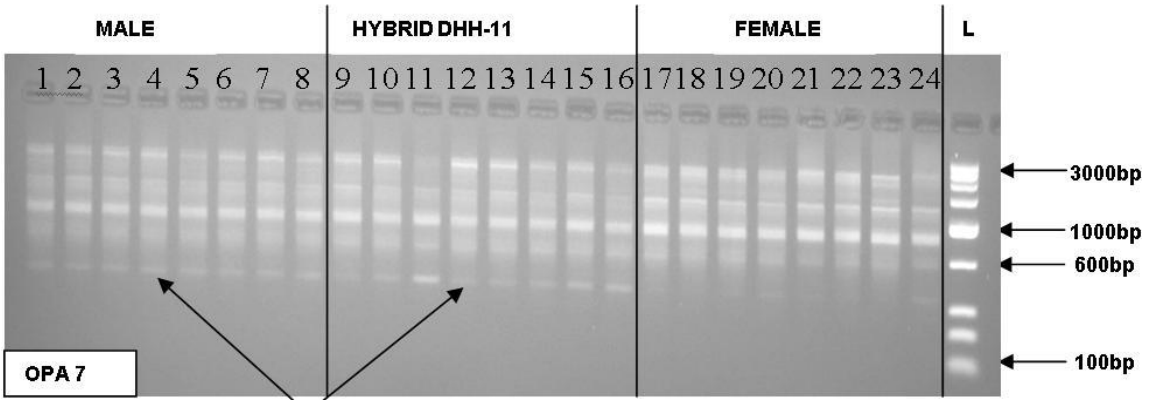

Fig 1. RAPD profile of OPA 7, Lane 1-8, CPD-420 (male); Lane 9-16, DHH-11 (F1 hybrid); Lane 17-24, CPD-423 (female); Lane L-3kb ladder. Primer, OPA 7 led to the conformation of hybridity of DHH-11 in which 1500 bp band present in Hybrid and Male but absent in Female.

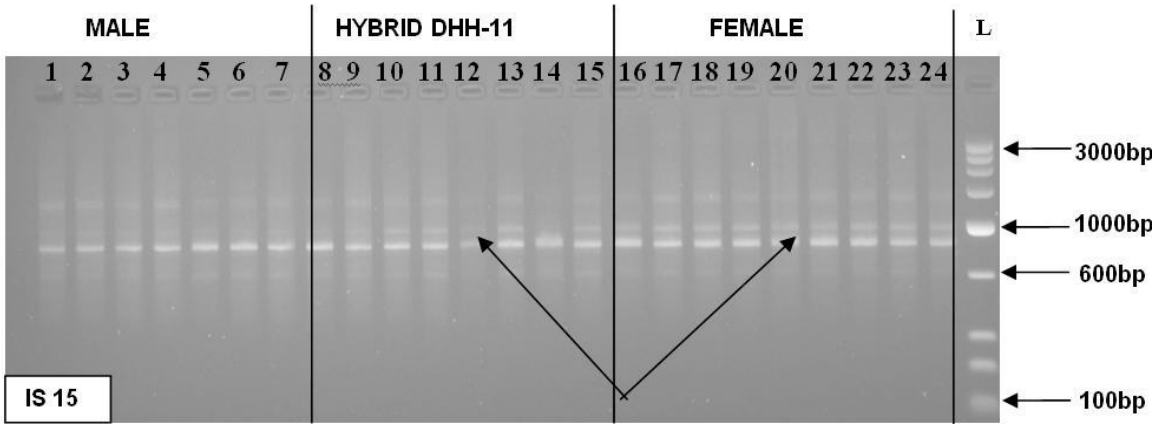

Fig 2. ISSR profile of IS 15 Lane 1-8, CPD-420 (male); Lane 9-16, DHH-11 (F1 hybrid); Lane 17-24, CPD-423 (female); Lane L-3kb ladder. Primer IS 15 led to the conformation of hybridity of DHH-11, in which 1000 bp band present in Female and Hybrid but absent in Male.

DigDoc 1201 gel documentation system Fig. 1 and Fig. 2. by using a combination of RAPD and ISSR markers. Out of 20 RAPD primers assessed only five primers-OPA 6, 7, 10, 12, 17 showed polymorphic amplification pattern between the parents. Further only one primer, OPA 7 [5'GAAACGGGTG3'] was found to be heteroallelic for parents. OPA 7 generated one male specific amplicon of 350bp was amplified in the Hybrid 'DHH-11'. The other repeats of different base pairs were homoallelic for both the parents (Fig. 1).

Out of 19 ISSR primers, three primers-IS 5, 7, 15 showed polymorphism in parents. Out of which only one primer IS 15 [5'(GT)6 AT3'] was found to be heteroallelic for parents. Primer IS 15 generated one marker of about 1000bp specific to female parent was amplified in the hybrid 'DHH-11'. The other repeats of different base pairs were homoallelic for both the parents (Fig. 2).

Use of RAPD technique, it is easier to identify true hybrids at early stages. This technique can be adopted for large scale screening of hybrids in cotton (Mehetre et al. 2004; Dongre and Parkhi 2005). RAPD analysis has also been utilized for hybrid identification and assessment of genetic diversity in wheat (Awan et al., 2008), rice (Haiyuan et al., 1998), maize (Iva et al., 2005), leucadendron (Lui et al., 2007), muskmelon (Park and rosby 2004), cyrtandra (Gesneriaceae) (Smith et al., 1996) and theobroma (Wilde et al., 1992). In the present study, OPA 7 showed specific polymorphism among the parents and the hybrid. It may be noted that ISSR-PCR markers have previously been used as a legal evidence for setting proprietary claim of disputed chili sample (Kumar et al., 2001). 14bp ISSR primer IS 15 of GT repeat anchored with AT expressed dominant markers of female parent in Hybrid 'DHH-11'.

CONCLUSION

The present study indicates that RAPD and ISSR

banding pattern of the parents compared with their respective hybrid clearly recognize true hybrid and it's profile showed that combination of PCR based molecular markers, likely to be promising for identification, registration and protection of commercial sample and will gain more and more influence on plant breeding in future and will speed up breeding processes considerably.

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