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PARENTAGE CONFIRMATION OF COTTON HYBRIDS USING MOLECULAR MARKERS

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Abstract

Parentage of F_1 hybrids of cotton was verified using random amplified polymorphic DNA (RAPD) and microsattelite (SSR) assays. Out of 500 primers surveyed, 3 random and 3 EST based SSR primers were found polymorphic between two cotton parents (FH-883 and FH-631S). These highly informative primers not only differentiated the parent genotypes but also confirmed the parentage of their true F_1 hybrids. Primer OPM-07 amplified two polymorphic loci designated as OPM07_800 and OPM07_925, while primers OPU-01 and OPV-01 amplified OPU01_850 and OPV01_650 alleles, respectively. Polymorphic SSRs were named as MGHES06_95, MGHES17_220 and MGHES24_230. DNA markers OPM07_925, OPU01_850, MGHES24_230 and one allele of MGHES06_95 were specific to FH-883, while the others markers were specific to FH-631S. Our findings revealed that RAPD and SSR procedures are excellent genomic tools for parentage confirmation and hybridity determination, and would also enhance efficiency of our breeding programmes through marker assisted selection.

Introduction

Cotton is the leading fiber crop worldwide with the production of 26.75 million metric tons in 2006-07 (Anon., 2007a) that makes possible world commerce of raw cotton of about $20 billion annually (Rong *et al*., 2005). Cotton is the main cash crop in Pakistan, the basis of the national textile industry and a major source of foreign exchange sharing 60% of the total export and hence contributes substantially to the national economy. During 2006-07 area under cotton cultivation was 3.08 million hectares and the production was 13 million bales in Pakistan (Anon., 2007b). The main goals of cotton breeding programs worldwide are the genetic enhancement of yield and more recently, fiber quality (Jiang *et al*., 2000).

For the improvement of agronomically and economically important traits, plant breeding generally recombines traits present in different parental lines of cultivated and wild species. Conventional breeding programmes reach this goal by generating an F_1 hybrid and F_2 segregating population and then screening the phenotypes of pooled or individual plants for presence of desirable traits, which is followed by a process of repeated backcrossing, selfing and testing. During this process breeder depends on accurate screening methods and availability of lines with clear-cut phenotypic characters, which is time consuming and difficult to achieve with classical methods (Beckmann & Soller, 1986). Use of molecular markers facilitate these breeding processes, since it can provide means of detecting and resolving complications and accelerate the generation of new varieties and allow association of phenotypic traits with genomic loci (Jiang *et al*., 2000). Ideal molecular markers are stable, abundant and detectable in plant tissues regardless of growth, differentiation and defense status. These properties make molecular markers indispensable for crop improvement.

A number of DNA fingerprinting techniques are available for detection of polymorphism (Semagn *et al*., 2006). Restriction fragment length polymorphisms (RFLPs) are very reliable markers in linkage analysis and crop breeding however, time consuming, expensive and require large amount of DNA for restriction and hybridization

analysis (Paterson *et al*., 1993). Most of the DNA marker assays use polymerase chain reaction (PCR), among them are random amplified polymorphic DNA (RAPD), microsatellites or simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphisms (SNPs). RAPD is much faster and cheaper than RFLP analysis and uses only minute amounts of DNA (Williams *et al*., 1990). Microsatellites are typically the repeat unit of 1-6 nucleotides and SSR analysis is performed by using pairs of specific primers flanking tandem arrays of microsatellite repeats. SSR markers are codominant and extremely polymorphic (Liu *et al*., 2002). AFLP is robust and reliable for DNA fingerprinting of different genomes because it combines the use of restriction enzymes and PCR amplification (Vos *et al*., 1995). The AFLP system is technically intricate and expensive to set up, but it detects a large number of loci (up to 100). SNPs are the single base substitutions or small insertions and deletions (Indel) in homologous genomic regions. SNPs are more frequent and codominant in nature (Lindblad *et al*., 2000).

Recent developments of molecular techniques and application of molecular markers have brought a new dimension into the traditional area of plant breeding. Molecular markers not only allow the easy and reliable identification of breeding lines, hybrids and cultivars (Bastia *et al*., 2001; Asif *et al*., 2005, 2006; Tabbasam *et al*., 2006) but also facilitate the monitoring of introgression, mapping of QTLs (Jiang *et al*., 2000), marker assisted selection (MAS) (Ribaut & Hoisington, 1998; Zhang *et al*., 2003) and estimation of genetic diversity (Mukhtar *et al*., 2002; Rahman *et al*., 2002, 2007). High-density genetic linkage maps (Guo *et al*., 2007; He *et al*., 2007) established using molecular markers, for economically important crops provide a basis for MAS of agronomically useful traits, for pyramiding of resistance genes and the isolation of important genes by map-based cloning strategies (Ribaut & Hoisington, 1998). The proposed research work was conducted to confirm the parentage of cotton hybrids with DNA markers. Efficiency of RAPD and SSR assay was evaluated successfully for hybridity determination, which would be a valuable genomic tool for the cotton breeders.

Materials and Methods

Plant material and DNA exraction: Two cotton genotypes (FH-883 and FH-631S) contrasting for fiber traits and their F_{1s} were used for the research work. DNA was extracted from these two selected cotton parents and their F_{1s} by CTAB method proposed by Murray & Thompson (1980). Young leaves were ground to a very fine powder in liquid nitrogen and transferred to a 50 ml Falcon tube. The 15 ml of hot 2x CTAB was added and incubated for 30-45 minutes at 65°C with occasional swirling. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed, then spun at 4000 rpm for 10 min. The supernatant was collected into a new 50 ml tube. Nucleic acid was precipitated with 0.6 volume of chilled isopropanol. Nucleic acid was pelleted at 4000 rpm for 5 minutes and supernatant was discarded. Pellet was washed with 70% ethanol and air dried before resuspending in 0.5 ml 0.1x TE buffer. The suspension was transferred to 1.5 ml eppendorf tube, added 7 µl of RNase and incubated at 37°C for 1 hour. Equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed, centrifuged for 10 min., at 13000 rpm and then supernatant was transferred to a new eppendorf. $1/10^{th}$ 3M NaCl was added, mixed gently and chilled ethanol (2 volumes) was added. After a spin at 13000 rpm for 10 minutes, discarded the supernatant, washed the pellet with 70% ethanol, air dried and resuspended it in 0.1x TE buffer. DNA concentration was measured with DyNAQuant 200 Fluorometer and also comparing with Quantification Standards, Phage λ DNA (GibcoBRL).

PCR amplification: Random decamers and SSR primers (including EST-SSRs) were surveyed for screening two cotton parents (FH-883 and FH-631S) and subsequently screening their F_{1s} with polymorphic RAPDs and SSRs. RAPD primers belonged to Operon series (A to Z) while SSRs belonged to the series of BNL, CM, JESPR and MGHES (EST based SSR primers). The sequences of these SSRs were obtained from publicly available cotton microsatellite database (CMD) (Blenda *et al*., 2006) and synthesized from GeneLink, USA. PCR was run on eppendorf mastercycler gradient, Germany. For RAPD assay PCR amplification was performed in 25 µl reaction volumes containing 15 ng/µl cotton DNA, 10x PCR buffer, 25 mM $MgCl_2$, 0.025% gelatin, 2.5 mM dNTPs (each of dATP, dGTP, dCTP and dTTP), 15 ng/µl random primer and 1.0 unit of *Taq* DNA polymerase. *Taq* DNA polymerase, 10x PCR buffer, $MgCl_2$ and dNTPs were obtained from Fermentas, while gelatin was obtained from Sigma. PCR profile was 94°C for 5 min., then 40 cycles of 94°C for 30 sec, 36°C for 30 sec, 72°C for 1 min and finally 72°C for 4 min. SSR amplification was performed in 20 µl reaction volumes containing similar reagents used for RAPD assay except for gelatin and primers. PCR profile for SSR amplification was similar to RAPD profile but cycles were 35 instead of 40 and annealing temperature ranged from 55 to 65°C.

Gel electrophoresis: RAPD products were electrophoresed on 1.2% agarose gels, while SSR loci were resolved on Metaphor agarose gels (2% standard agarose and 2% Metaphor agarose). Horizontal electrophoresis system HU-13 (Scie-Plas, UK) was used and after electrophoresis, finely resolved PCR products were visualized under UV light and photographed. The polymorphic primers between the two cotton parents were then used to survey their F_1 hybrids for parentage confirmation.

Results and Discussion

Out of 500 primers surveyed, 3 decamer random primers (OPM-07, OPU-01 and OPV-01) and 3 EST based SSR primers (MGHES-06, MGHES-17 and MGHES-24) were found extremely polymorphic between two cotton parents (Fig. 1 & 2). These highly informative primers not only differentiated FH-883 and FH-631S but also confirmed the parentage of their F_1 hybrids. The 3 random primers amplified collectively 22 fragments, while the number of resolvable loci were 8, 8 and 6 for the primers OPM-07, OPU-01 and OPV-01, respectively, however, each of the 3 informative and polymorphic SSR primers amplified two loci (Table 1). These informative primers amplified polymorphic as well as monomorphic loci and detection of more number of monomorphic loci illustrated the already reported narrow genetic base of cotton (Rahman *et al*., 2005).

OPM-07 and MGHES-06 independently verified the parentage of cotton hybrid, while OPU-01 along with OPV-01 and MGHES-17 in combination with MGHES-24 confirmed the parentage of F_1s (Fig. 1 & 2). OPM-07 amplified two polymorphic loci of 800 and 925 bp size, which was designated as OPM07_800 and OPM07_925, while primers OPU-01 and OPV-01 amplified polymorphic fragments of 850 and 650 bp length respectively, which were called as OPU01_850 and OPV01_650 respectively. SSR primer MGHES-06, produced one polymorphic locus with alleles of 95 and 100 bp size, which was named as MGHES06_95, while primers MGHES-17 and MGHES-24 produced polymorphic loci of 220 and 230 bp length respectively, which were labeled as MGHES17_220 and MGHES24_230 respectively. DNA markers OPM07_925, OPU01_850, MGHES24_230 and one allele of MGHES06_95 were specific for cotton parent FH-883, while others were specific for FH-631S.

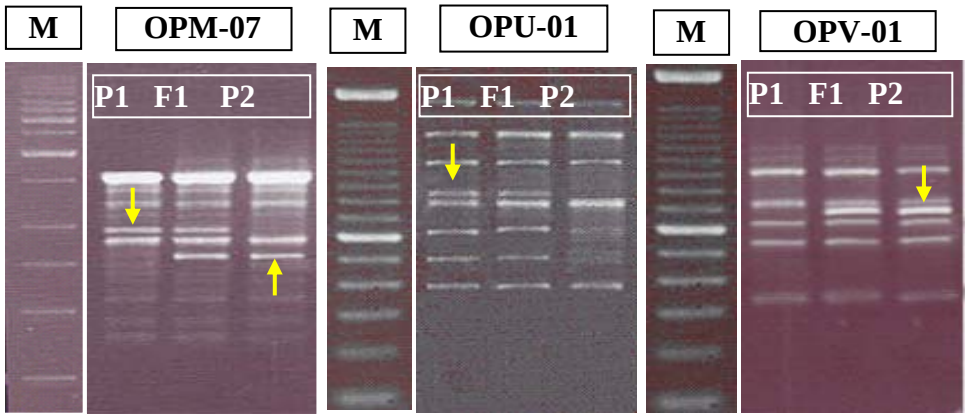

Fig. 1. Amplification profile of cotton parents and their F_1 with polymorphic RAPD primers (OPM-07, OPU-01 and OPV-01) resolved on agarose gels. M: marker (DNA ladder), P1: parent one (FH-883), P2: parent two (FH-631S), F1: first generation hybrid.

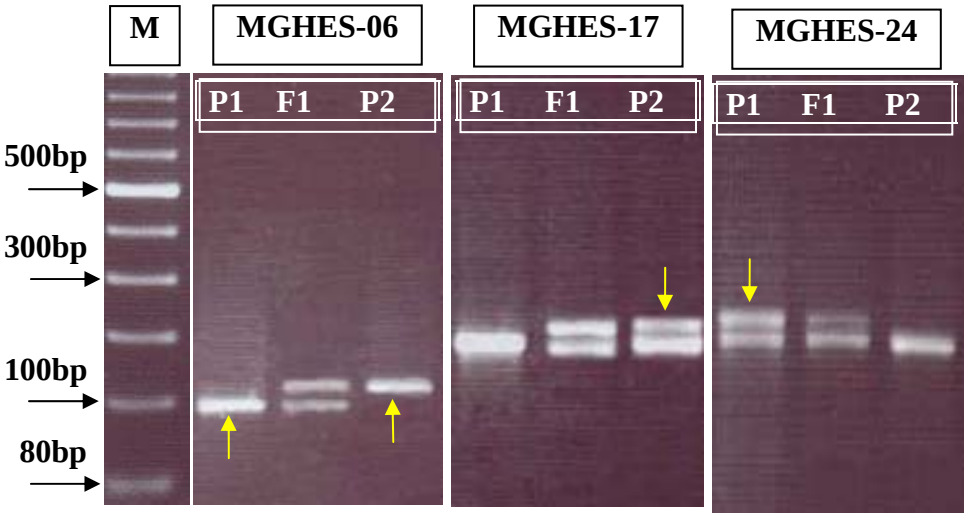

Fig. 2. Amplification profile of cotton parents and their F_1 with polymorphic SSR primers (MGHES-06, MGHES-17 and MGHES-24) resolved on Metaphor agarose gels. M: marker (DNA ladder), P1: parent one (FH-883), P2: parent two (FH-631S), F1: first generation hybrid.

Polymorphic molecular markers produced unique banding and not only discriminated the two cotton parents, but also identified their true hybrids. Polymorphism revealed by RAPDs is based on the position and orientation of primers annealing sites and the interval they span. Polymorphism between individuals can arise through nucleotide substitutions and insertions or deletions (Williams *et al*., 1990). SSRs are more informative and highly polymorphic and their polymorphism is based on differences in number of repeats in amplified regions. Many of the variations are due to the mutations within primer binding regions that may yield null alleles, whereas a mutation between the primer regions may result in new alleles.

Table 1. Informative RAPD and SSR primers.

Primers	Sequences	Total bands	Poly† bands	Ann temp‡
RAPD				(ºC)
OPM-07	CCGTGACTCA	8	2	36
OPU-01	ACGGACGTCA	8	1	36
OPV-01	TGACGCATGG	6	1	36
SSR				
MGHES-06	F=TCGCTTGACTTTCCATTTCC R=AACCCTCGGGATTATCGTCT	2	2	55
MGHES-17	F=AACCCTTCTTTTCCCCCTTT R=TCTTCACCGATGCCATTGTA	2	1	55
MGHES-24	F=CGCAACAACTGATGCAACTC R=AACCGATACCTCCGCTTCTT	2	1	55

† polymorphic, ‡ annealing temperature

RAPD and microsatellite analysis have been successfully employed for parentage verification, hybrid identification, cultivars characterization and purity testing in other crop plants (Asif *et al*., 2006; Bertini *et al*., 2006; Tabbasam *et al*., 2006). Yamagishi (1995) developed RAPD markers for *Lilium* species characterization and hybrid identification. In cherokee rose, RAPD analysis identified the erroneous classification of the hybrid 'Silver Moon' (Walker & Werner, 1997). Zhang *et al*., (2005) characterized and evaluated commercial cotton cultivars with microsatellites and found some specific SSR alleles for discriminating cotton germplasm. RAPDs and SSRs were also surveyed for verification of interspecific hybridization (Benedetti *et al*., 2000; Mei *et al*., 2004). Our results confirmed the efficacy of RAPD and SSR assay for the verification of hybridity and parentage identification. Moreover, it exemplified the importance of increased level of homogeneity and purity at intra-varietal level for better implementation of plant breeder's rights (PBRs).

Molecular markers linked to a gene of interest are the milestones and these tags are useful starters for identification of genes. Once the molecular markers closely linked to desirable traits are detected, MAS can be performed in early segregating populations and at early stages of plant development (Zhang *et al*., 2003; Francia *et al*., 2005). Therefore, it is safe to say that molecular markers will gain more and more influence on plant breeding in future and will speed up breeding processes considerably. In view of potential development of new strategies, the future for improvement of polygenic traits through DNA markers appears bright. Moreover, by adopting new and novel marker systems like EST-SSRs, SNPs, DNA chips and microarrays, indeed, some day it may be possible to select best lines for breeding based on RNA expression profiles as much as marker genotypes.

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