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SSR MARKER LINKED TO RF1 FERTILITY RESTORER GENE OF COTTON (*GOSSYPIUM HIRSUTUM* L.)

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ABSTRACT

The cytoplasmic male sterility (CMS) system is convenient and efficient for hybrid seed production in Upland cotton (*Gossypium hirsutum* L.). However, it has not been widely used because of limited restorer lines carrying the restorer gene Rf1. The present study was carried out with the objective to identified restore fertility (Rf) (*Gossypium hirsutum* L.) line for CMS based male sterile line and specific gene for fertility restoration by using gene specific molecular marker. In present study, seven female lines, five male lines and 35 crosses were screened through SSR marker with primer UBC 607_{500}. Male lines and crosses had showed confirmatory results for fertility restoration. The restorer gene (Rf) sequences obtained was further submitted to Gene Bank (KX090570) and had been released in public domain. Thus, all male parent lines conformed as Rf1 restorer gene present in R-line and may be used in CMS-R based hybrid seed production programme.

INTRODUCTION

In agricultural advanced countries artificial emasculation and pollination method of exploitation of hybrid vigour in cotton has not been possible only due to labour paucity and cost. This is limiting factor for the poor/marginal farmers to grow hybrids. Therefore, an immediate need for evolving an alternative cross seed production technique which would require comparatively less human resources was felt. This situation prompted intensive search for investigation on male sterility in utilizable form in cotton. However, with the availability of male sterile line in *G.hirsutum* (Meyer, 1975), enough crossed seed can be produced with low cost and good purity. Thus, seed production through non conventional method (male sterility based) is profitable due to various reasons *viz*., accessibility in resources and reduced cost of hybrid seed production by eliminating the process of emasculation and commercial exploitation of hybrid vigour. Most of the cotton hybrids in India are produced manually by using conventional method of hand emasculation and pollination thus making hybrid seed production expensive. In the recent years, the phenomenon of male sterility being used for heterosis breeding which has helped to reduce the cost of hybrid seed production about 70 per cent (Meshram and Marawar, 1995). In cotton, different sources of male sterility are available. Out of which genetic male sterility (GMS) and cytoplasmic genetic male sterility (CGMS) are mainly used in both diploid and tetraploid species of cotton. Low productivity of male sterility based hybrids than their fertile counterpart may be minimized through modifying genes in genotype which will give high yields in suitable A and B line (Thomson, 1976). For this efforts were made to widen genetic base for cytoplasm which imparts sterility without much deterioration to economic traits of cotton with greater number of restorers (Meshram *et al*., 1995). Suitable and diversified cytoplasm with good number of restorer line would provide wider selection base, thus reducing selection pressure for sterility and more combinations can be worked out. In cotton (*G.hirsutum*), several different sources of CMS have been developed (Meyer, 1975; Stewart, 1992; Meshram *et al*., 1994; Yuan *et al*., 1996), which makes this material potentially useful for hybrid cotton production and for studying the interactions between CMS and restorer genes (Rf). With its male sterility conferred by the exotic cytoplasm from *G. harknessii* (with genome designation D2-2), CMS-D2 (Meyer, 1975) is the first system developed for cotton. Developed by Stewart (1992) from the cytoplasm of *G.trilobum* (with genome designation D8), CMS-D8 is the second publicly accepted CMS system. Its male fertility can be independently restored by two restorer genes, namely sporophytic Rf1 (designated D2 restorer gene hereafter) from the D2-2 genome and gametophytic Rf2 (designated D8 restorer gene hereafter) from the D8 genome (Weaver and Weaver, 1977; Stewart and Zhang, 1996; Stewart *et al*., 1996; Zhang and Stewart, 2001a, 2001b). At present the only stable and dependable CMS source under various environments is of *G. harknessii* which in interaction with

genome of *G.hirsutum* produces male sterility. The dependency of single source of cytoplasm for the development of hybrid cotton is likely to narrow down the genetic basis of hybrid development through the specific molecular marker base screening. Identification of molecular markers closely linked to the nuclear Rf genes could help breeders to distinguish restorer and non-restorer plants at early seedling stage without crossing them to CMS lines. The present investigation was, therefore, conducted to validate the SSR markers closely linked to the nuclear Rf gene under male sterile cytoplasm in commercial cotton (*Gossypium hirsutum* L).

MATERIALS AND METHODS

The parents includes seven CMS based lines GJHV-156, 4384, LRK-516, GSHV-97/443, GSHV-97/13, GSHV-4093, GSCMS-10 and five restorer lines AKH-03-8h, AKH-7859, DR-16, DR-8, AKH-076R. The seeds of these parental lines were obtained from the Research Scientist (Cotton), Main Cotton Research Station, NAU, Surat. These parents were crossed in Line x Tester (Kempthorne, 1957) mating design to get 35 different crosses (Table 1). The crossing programme was carried out at MCRS, NAU, Surat. First, identified and conformed restorer gene (Rf) among male parents and compared with non restorer line as female. These parents were crossed in Line x Tester mating design to get 35 crosses and molecular analysis was done. Further Parents and its crosses were analyzed for molecular identification and conformation of restorer gene.

DNA extraction

For DNA extraction, young and tender leaf sample were taken and homogenized in liquid nitrogen. After complete homogenization, 1 mL of extraction buffer (50 mM Tris, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 0.1 ml â-mercaptoethanol, 2% CTAB and 1% PVP) was added to the samples, which were then incubated for 1 hr at 65ºC. DNA was extracted and purified according to method described by Saghai Maroof *et al*. (1984) and Ayachit *et al*. (2013). RNA was removed by RNase treatment. DNA was quantified using Nano spectrophotometer and diluted to 50ng/ìL for PCR amplification.

PCR conditions and electrophoresis

PCR (Polymerase chain reaction) for specific band of DNA genome analysis was performed in 25 ìl reaction volume containing 50 ng genomic DNA, 2.5 ìl PCR buffer (MBI Fermentas, Hanover, USA), 200 ìM dNTPs (Merk, Bengaluru, India), 1.5 U Taq DNA polymerase (MBI Fermentas) and 0.4 ìM primer using a thermal cycler (Eppendorf, Germany). The reaction mixture was placed on DNA thermal cycler (Biometra). The program was performed as 1 cycle of 94ºC for 5 min and 35 cycles of 94ºC for 1min, 53ºC for 45sec and 72ºC for 2min, then, a final extension step 72ºC for 10min. The specific Forward primer as UBC607 (GGTGGGCAGGGTTGTTGT) and reverse primer as UBC607 (GGTAGCGCTTGAAAACGACAT) are designed from conserved part of DNA for restorer gene. PCR products were electrophoresed on 1.2% (w/v) agarose gels, in 0.5X TBE Buffer at 90 V for 1hr and then stained with ethidium bromide (0.4ìg/mL). Gels with amplified fragments were visualized and photographed under UV light (Kalaria *et al*., 2013).

Sequencing of amplified PCR product

Amplified PCR product was purified using BigDyeR Terminator v3.1 Cycle Sequencing clean up method described in Sambrook and Russel (2001) for sequencing. Purified product was used for Sequencing by Ready Reaction Kits (Applied Biosystem) using ABI Prism3130 automatic sequencer (Applied Biosystems, U.S.A) with same reverse and forward primers. The derived sequence was analyzed by performing online BLAST sequence homology test. (Kalaria *et al*., 2013, 2014)

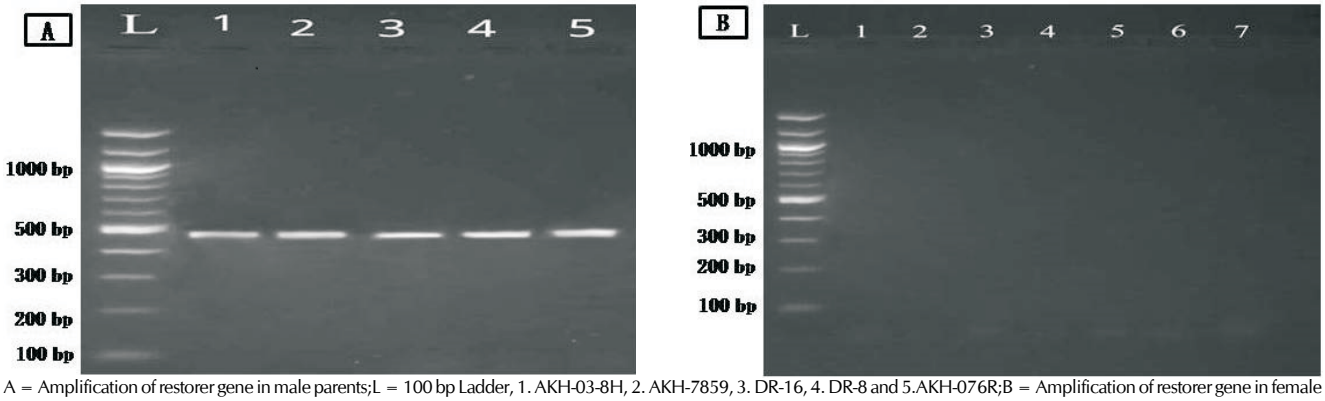
Sequence analysis

Database searches for partial restorer gene sequences, Basic local alignments and similarity index analysis were carried out using NCBI-BLASTN program (http: //blast.ncbi.nlm.nih.gov). Multiple nucleotide (nt) sequence alignments were performed for part of DNA isolate with other restorer gene reported from India and worldwide by using CLUSTALW (2.1) programme (http://www.ebi.ac. uk/clustalw) (Kalaria *et al*., 2013, 2014).

RESULTS AND DISCUSSION

Table1: Present/Absent of specific band for restorer fertility in hybrids.

Sr. No.	Hybrids	Present / Absent of specific band for restorer fertility Rf_1
1.	GJHV-156 x AKH-03-8h	Present
2.	GJHV-156 x AKH-7859	Present
3.	GJHV-156 x DR-16	Present
4.	GJHV-156 x DR-8	Present
5.	GJHV-156 x AKH-076R	Present
6.	4384 x AKH-03-8h	Present
7.	4384 x AKH-7859	Present
8.	4384 x DR-16	Present
9.	4384 x DR-8	Present
10.	4384 x AKH-076R	Present
11.	LRK-516 x AKH-03-8h	Present
12.	LRK-516 x AKH-7859	Present
13.	LRK-516 x DR-16	Present
14.	LRK-516 x DR-8	Present
15.	LRK-516 x AKH-076R	Present
16.	GSCMS-10 x AKH-03-8h	Present
17.	GSCMS-10 x AKH-7859	Present
18.	GSCMS-10 x DR-16	Present
19.	GSCMS-10 x DR-8	Present
20.	GSCMS-10 x AKH-076R	Present
21.	GSHV-97/443 x AKH-03-8h	Present
22.	GSHV-97/443 x AKH-7859	Present
23.	GSHV-97/443 x DR-16	Present
24.	GSHV-97/443 x DR-8	Present
25.	GSHV-97/443 x AKH-076R	Present
26.	GSHV-4093 x AKH-03-8h	Present
27.	GSHV-4093 x AKH-7859	Present
28.	GSHV-4093 x DR-16	Present
29.	GSHV-4093 xDR-8	Present
30.	GSHV-4093 x AKH-076R	Present
31.	GSHV-97/13 x AKH-03-8h	Present
32.	GSHV-97/13 x AKH-7859	Present
33.	GSHV-97/13 x DR-16	Present
34.	GSHV-97/13 x DR-8	Present
35.	GSHV-97/13 x AKH-076R	Present



A = Amplification of restorer gene in male parents;L = 100 bp Ladder, 1. AKH-03-8H, 2. AKH-7859, 3. DR-16, 4. DR-8 and 5.AKH-076R;B = Amplification of restorer gene in female parents;L = 100 bp Ladder, 1. GJHV-156, 2. 4384, 3. LRK-516, 4. GSCMS-10, 5.GSHV-97/443, 6.GSHV-4093 and 7. GSHV-97/13

Figure 1: Electrophoresis analysis of restorer gene using PCR amplification parental lines

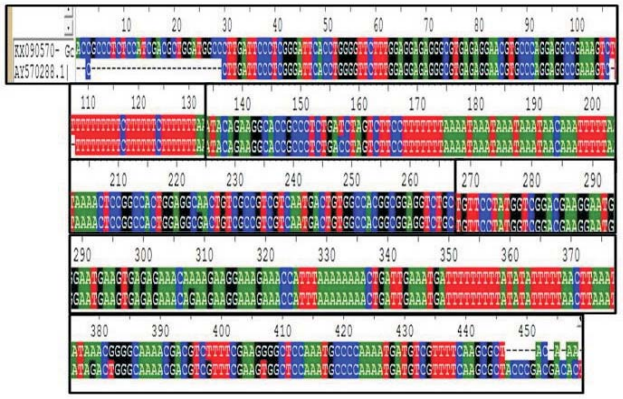
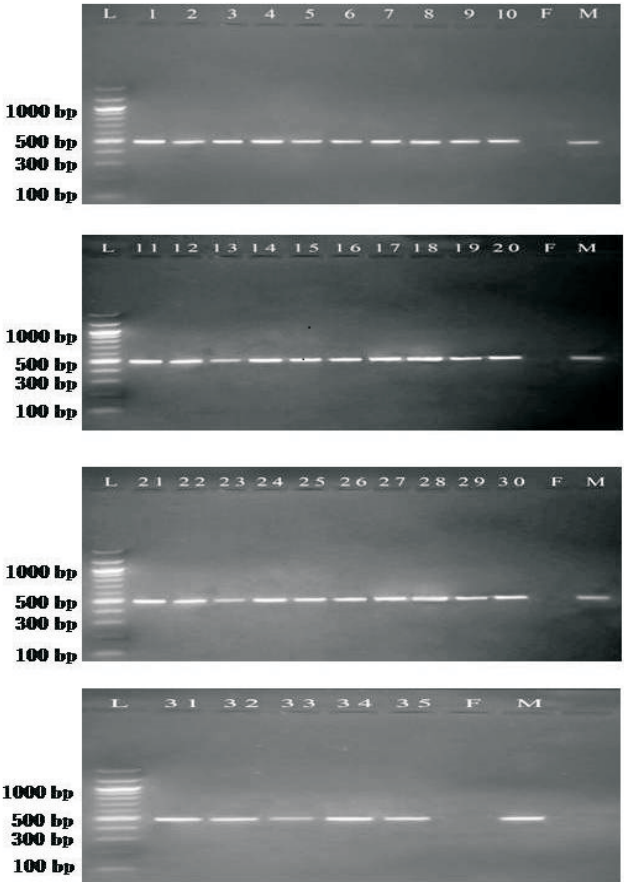


Figure 2: Multiple sequence alignment of amplified fragment with other restorer gene sequence of *Gossypium* spp. in NCBI

Identification and conformation analysis of SSR markers associated with Rf in Parental line

For identification of germplasm line as restorer, all parental line genomic DNA were isolated and run on 0.8% agarose gel. Further parental lines were screened out by using SSR primer (UBC 607). PCR amplification using SSR was carried out in both male and female parental line. DNA molecule as earlier used by Feng *et al*. (2005) and Ayachit *et al*.(2013). Result showed presence of specific ~500 bp DNA band fragment in all male parental lines while absent in all non restorer female parental lines (Fig 1). It indicated that restorer gene was present among all male parental lines while absent among all non restorer female parental lines. To generate and identified best fertile heterotic combination among the series of crosses, conformed five restorer line (R line) crossed with seven cytoplasmic genetic male sterile (CGMS) lines using line x tester method(Kempthorne, 1957). The amplified fragments was taken from one of the male parental lines (AKH-7859) and send for the sequencing. The sequences obtained were submitted to Gene Bank (KX090570) and had been released in public domain (www.ncbi.nlm.nih.gov/). The sequence showed highest percentage identity (98%), Query coverage (96%) and E value 0.0 with *Gossypium hirsutum* clone (Fig 2) UBC607-500 restorer (Rf1) gene, partial sequence, [AY570288.1].



L= 100 bp Laddder, F= Female parent, M= Male parent and 1 to 35= Hybrids name in Table 1.

Figure 3: Electrophoresis pattern of SSR marker in hybrids

Validation of restorer gene in F_1 generation.

Randomly selected ten plants from each plot were used to screened through SSR marker in F_1 generation. Result (Fig 3) showed presence of specific ~500 bp DNA band in all crosses samples which exhibited presence of restorer gene among F_1 generation. Similar findings were observed by Weaver and Weaver (1977) and Davis (1978).

The present studies, concluded that line x tester analysis in

cotton was undertaken with a view to elucidate the molecular marker linked to RF gene of restorer lines in CMS-R based hybrids in cotton. Total five male parent lines AKH-03-8h, AKH-7859, DR-16, DR-8 and AKH-076R and its segregating population showed the present of SSR marker of ~ 500bp linked to Rf gene. Thus, these five lines conformed as R-line and may be used as male lines in CMS-R based hybrid seed production programme.

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