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Molecular characterization of genetic male sterile genotypes in diploid cotton (*Gossypium arboreum*) and development of male sterility specific SCAR marker

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Abstract

Genetic male sterility (GMS) in the *Gossypium arboreum* was found to be under the control of recessive gene. This GMS system has been used for diploid cotton hybrid breeding in India. In the present study, RAPD marker was used to characterize the Hisar GMS and SRT-1GMS lines which are derived from the repeated back crosses. Male sterility in these genotypes is governed by the single recessive gene *ams*$_1$. From the survey of 60 random decamer primers, 34 were found to be polymorphic generating 60.73 per cent polymorphism between male sterile and male fertile plant. Out of the polymorphic primers OPAB3, OPAB4, OPAB5, OPAB19, OPH20, OPI2, OPI3 and OPI7 showed a notable differences in the amplicon profile of male sterile and their fertile counterparts. Dendrogram revealed two distinct clusters in which all the male sterile and fertile plants made independent clusters indicating genetic differences between them for sterility. The primer OPI3 was found to be male sterile specific in repeated PCR by consistently producing a specific fragment of 486 bp only in the sterile plants which has been later converted into an locus specific Sequence Characterized Amplified Regions (SCAR) marker. The RAPD markers associated with male sterility and putative SCAR marker specific to male sterility may facilitate for the utilization of the GMS system in hybrid breeding in the Asiatic cotton.

Key words: Asiatic cotton, Genetic male sterility, Random Amplified Polymorphic DNA, Sequence Characterized Amplified Regions marker.

Introduction

Male sterility is the most important approach to exploit the heterosis in various crops. Male sterility found in cotton can be divided into Cytoplasmic male sterility (CMS) and genetic or genic male sterility (GMS). Although the CMS system, has been considered more attractive than the GMS system, it has its own limitations like instability of the sterility or partial restorability of the male sterility, limited availability of the restorers and possible undesirable cytoplasmic effects. On the contrary, GMS system can be more stable for sterility since it is governed by the nuclear genes, easier to use in diverse cytoplasmic sources and restorers and has a shorter breeding time than the CMS system. However, the major drawback of GMS system stems from that one has to remove 50 % of the male fertile plants in a commercial seed production plots.

In cotton perhaps the first case of genetic male sterility was reported by Justus and Leinweber [1] in upland cotton (*Gossypium hirsutum*), later on several cases of genetic male sterility were reported by several workers. A total of 18 loci have been identified in upland cotton, three in Egyptian cotton (*G. barbadense*) and two in Asiatic cotton (*G. arboreum*) for genetic male sterility. Male sterility in cotton was found to be governed by both recessive and dominant genes. However the cases of recessive male sterility are higher than the dominant male sterility. In the Asiatic cotton the two loci reported for genic male sterility so far are under the control of single recessive gene designated as "*ams*$_1$" [2] and "*arms*" [3 and 4] respectively. Several genic male sterile genotypes of diploid cotton were developed in India by transferring the *ams*$_1$ and *arms*$_1$ genes and the genotypes were classified into two major

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groups *viz*., Hisar Source GMS lines and Akola Source GMS lines. These lines have been successfully used in the heterosis breeding and it led to the release of the world's first GMS based diploid cotton hybrid AAH-1 (Hisar Source) in 1999 and several other hybrids *viz*., AKDH-7 (Akola source), G.cot MDH-11 (Hisar source) and CICR-2 (Hisar source) for commercial cultivation in different cotton zones of India.

The commercial hybrid seed production using GMS system in Asiatic cotton did not become popular and economically viable because of the sole drawback of the GMS system, the need of rouging out 50 % of fertile plants in each generation from seed production plots. Moreover the male sterile and male fertile plants can be distinguished only after the anthesis. Some possible solutions to this problem were proposed in the previous reports [5] *viz*., pyramiding several male sterile genes, introducing a recessive conditional lethal gene (l) , use of seed characters or seedling phenotype and using DNA markers [6] linked to the male sterility. However the molecular marker linked to the male sterility in diploid cotton GMS lines has not yet been reported.

In the present study, considering its rapidity and non-requirement of sequence information specific to the crop, the Random Amplified Polymorphic DNA (RAPD) system was adopted to characterize the diploid cotton GMS lines. This paper describes the identification RAPD markers associated with the genic male sterility in Asiatic cotton and conversion of RAPD marker specific to the male sterility into putative SCAR (Sequence Characterized Amplified Regions) marker.

Materials and methods

Two pairs of near isogenic lines (Hisar GMS and SRT-1 GMS) of Hisar source derived by the repeated back crossing were used in the present investigation. These lines (NILs) were grown in the field at Main Agricultural Resarch Station, Dharwad during *kharif*, 2009. Male sterility was examined by visual observation based on the dichotonomous criteria (Fig. 1). Male fertile plants had normal pollen grains and long staminal column whereas the sterile plants had no pollen grains in mature anthers and produced short staminal column.

DNA isolation and RAPD analysis

Genomic DNA was isolated from the fresh leaves by following the modified CTAB extraction method (Seghai-Maroof *et al*., 1984). The concentration of DNA was quantified spectrophotometrically and also by gel electrophoresis using 0.8 per cent agarose with known concentrations of DNA. For the spectrophotometric

Fig. 1. Dihiscent and Indehiscent anthers in the male fertilele (F) and male sterile (S) plants of Diploid GMS lines

analysis, 5 ml of DNA sample was diluted to 300 ml of TE buffer. The spectrophotometer readings were recorded at 260/280 nm. A good DNA preparation generally exhibits the A260 / A_{280} > 1.80. DNA concentration was calculated using OD values at 260 nm using the formula, Concentration of DNA (ml/ml) = OD at 260 nm x 50 x dilution factor. To test the quality of DNA, samples were run on 0.8 per cent agarose gel in 1X TAE buffer and stained with ethidium bromide. DNA was evaluated by comparing it with a standard undigested DNA sample. The sample was treated with RNase enzyme, to remove RNA.

The RAPD analysis was performed with DNA of individual male sterile and fertile plants from two pairs of NILs. Commercial kits of random decamer primers obtained from Operon Technologies Inc. Alamedas, USA were used. Polymerase Chain Reaction (PCR) was performed in 25 µl volume containing primer, DNTPs mix, Kcl, Tris-Hcl (P^H 8.0), $Mgcl_2$ and *Taq* DNA polymerase. The amplification profile included a pre-incubation at 95°C for 4 minutes leading to 37 cycles of melting at 94°C for 1 minute, annealing at 37°C for 1.3 minutes and synthesis at 72°C for 2 minutes followed

by a final extension at 72°C for 10 minutes. The amplified products along with 2 ml of loading dye (bromophenol blue) were separated on 1.2 per cent agarose gel at 80 volts (45 volts/cm of gel) using 1X TAE buffer of pH 8.0 containing ethidium bromide (0.5 ml/10 ml of gel). Lambda DNA-EcoRI/Hind III double digest was used as DNA molecular weight marker. The amplified fragments were scored as "1" for the presence and "0" for absence of band generating "0" and "1" matrix and percent polymorphism was calculated. Pair-wise genetic similarities (Sij) between genotypes were estimated by DICE similarity coefficient. Clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on unweighted pair group arithmetic mean (UPGMA) using SAHN module of NTSYS-PC version 2.02.

SCAR conversion

The RAPD marker which was found to be specific to genic male sterility after repeated PCR has been chosen for the conversion into an locus specific and reliable SCAR (Sequence Characterized Amplified Regions) marker. The sterile specific DNA fragment generated by the RAPD marker was excised from the 1 % agarose gel with a scalpel and blade and purified using DNA gel extraction kit. The re-amplified DNA products were cloned to pTZ57R/T vector and used for transformation of *Escherichia coli* DH5α. The recombinant plasmids were screened using colony PCR method and the cloned DNA fragments were sequenced (Genie, Benguluru). Based on the sequence information pair of primers (Forward and Reverse) of SCAR marker were developed using PRIMER 3.0 software.

Results and discussion

Although, the GMS system is preferred to the CGMS, but the main drawback of this system is the offspring of GMS based hybrid F_2 will segregate for male sterility and produce the male sterile and male fertile plants. In order to maintain the pure-breeding male sterile lines the male fertile segregants (Ms/ms) are required to be rouged out from the seed production plots. Though, many researchers made an attempt to differentiate the male sterile and fertile plants at the early stages (seedling stage) through the morphological differences, it has not been successful because of lack of suitable morphological markers. Since, male sterile and fertile plants could be differentiated only at the flowering stage through the flower morphological trait differences maintenance of breeding true male sterile plants (lines) in the seed production plots is difficult.

But, after the discovery of the PCR several DNA markers have been developed and conveniently used in the marker assisted selection for different traits in different crops. A number of markers systems such as RAPD, AFLP, SSR, ISSR, CAPS, SCAR and STMS are available now to use in the marker assisted selction (MAS). Among the several molecular techniques, Random Amplified Polymorphic DNA (RAPD) markers [7] is simple, widely used, efficient and relatively inexpensive. The technical simplicity and speed of RAPD methodology is a principal advantage [8]. These markers have been successfully used to discriminate intra and interspecific genotypes in cotton [9-12].

However, studies on cultivated Indian cotton genotypes are limited. Similarly, studies on genetic male sterile genotypes of cotton are very rare. Thus, RAPD markers are the best to start to get an insight into molecular diversity and marker development. These genetic markers which are heritable entries associated with economically important traits can be used by plant breeders as selection tools [13, 14]. This present study focused on identification of the male sterile and fertile plants of GMS genotypes in diploid cotton through RAPD markers.

Hisar GMS and SRT-1 GMS genotypes are the genic male sterile lines in which the sterility is found to be governed by a recessive gene ams_1. In a sibling population derived from consecutive back crosses between male sterile and male fertile are theoretically isogenic. RAPD analysis was carried out to identify an molecular marker that can distinguish between the male sterile and male fertile plants in GMS in this genotypes.

Total of sixty random decamer primers belonging to AB, H, I, L, M series of Operon Technologies Inc. were used to screen the diploid near isogenic lines, in which seven primers did not show amplification even after repeated PCR, nineteen primers produced monomorphic bands. The remaining 34 primers generated a total of 159 fragments of which 93 (60.73%) were polymorphic. Out of 4.68 fragments per primer, 2.74 fragments per primer were found to be polymorphic. The polymorphism per primer ranged from 14.29 with OPAB3 to 100 per cent with OPI3 and OPL7. The band profile obtained with the 34 primers is summarized in Table 1.

The number of amplified fragments varied with different primers, which is ranged from two (OPH9, OPH19, OPI12 and OPL7) to ten fragments (OPAB5). Highest numbers of amplified fragments were obtained

Table 1. Summary of RAPD amplicons and polymorphism in diploid GMS lines

Sl. No.	Primer	Sequence	Total No. of bands	No. of polymorphic bands	Percent polymorphism
1	OPAB1	CCGTCGCTAG	4	3	75.00
2	OPAB2	GGAAACCCCT	6	4	66.67
3	OPAB3	TGGCGCACAC	7	1	14.29
4	OPAB4	GGCACGCGTT	7	4	57.14
5	OPAB5	CCCGAAGCGA	10	3	30.00
6	OPAB17	TCGCATCCAG	6	4	66.67
7	OPAB18	CTGGCGTGTC	4	3	75.00
8	OPAB19	ACACCGATGG	5	2	40.00
9	OPAB20	CTTCTCGGAC	5	3	60.00
10	OPH1	GGTCGGAGAA	5	4	80.00
11	OPH2	TCGGACGTGA	4	3	75.00
12	OPH3	AGACGTCCAC	3	2	66.67
13	OPH4	GGAAGTCGGC	3	1	33.33
14	OPH5	AGTCCTCCCC	7	3	42.86
15	OPH6	ACGCATCGCA	3	2	66.67
16	OPH7	CTGCATCGTG	5	4	80.00
17	OPH9	TGTAGCTGGG	2	1	50.00
18	OPH12	ACGCGCATGT	5	4	80.00
19	OPH13	GACGCCACAC	5	2	40.00
20	OPH14	ACCAGGTTGG	4	3	75.00
21	OPH16	TCTCAGCTGG	3	2	66.67
22	OPH17	CACTCTCCTC	7	4	57.14
23	OPH18	GAATCGGCCA	4	2	50.00
24	OPH19	CTGACCAGCC	2	1	50.00
25	OPH20	GGGAGACATC	5	4	80.00
26	OPI2	GGAGGAGAGG	5	3	60.00
27	OPI3	CAGAAGCCCA	4	4	100.00
28	OPI4	TCCGCCTAGT	5	4	80.00
29	OPI7	CAGCGACAAG	6	2	33.33
30	OPI11	ACATGCCGTG	6	3	50.00
31	OPI12	AGAGGGCACA	2	1	50.00
32	OPI13	CTGGGGCTGA	3	1	33.33
33	OPI14	TGACGGCGGT	5	4	80.00
34	OPL7	AGGCGGGAAC	2	2	100.00
	Total		159	93	
No. of bands per primer			4.68	2.74	60.73

with the OPH series primers compared to others. The average fragment per primer was found out to be 4.68. Ying *et al.* [15] identified four RAPD markers linked to genic male sterility in Chinese cabbage found to be

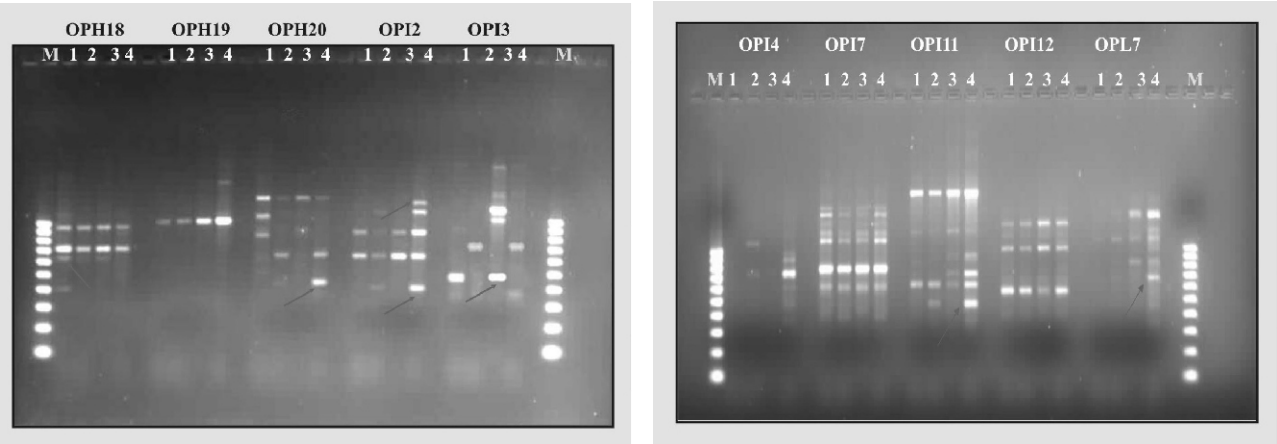

Fig. 2. RAPD banding pattern of diploid cotton GMS lines. (M: 1000 bp ladder, 1: SRT-1 sterile, 2: SRT-1 fertile, 3: Hisar sterile, 4: Hisar fertile)

inherited in a Mendelian manner. Since the lines used in the present study are near isogenic lines, any polymorphic fragment present in either of the male sterile and fertile line is a putative marker useful to screen the mapping population for distinguishing between the male sterile and fertile plants.

Pair-wise similarity coefficient value for four near isogenic lines was calculated. Overall similarity indices ranged from 0.77 to 0.85. Least similarity (0.77) was observed between fertile and sterile plant of genotype SRT-1 and between sterile plants of genotype HISAR and fertile plant of SRT-1 genotype. Whereas, highest similarity (0.85) was observed between fertile plants of genotypes HISAR and fertile plant of genotype SRT-1(Table 2). The dendrogram (Fig. 3) revealed two distinct clusters. All fertile plants made independent cluster (II), similarly, all sterile plants made another independent cluster (I). Together, they formed a single cluster at similarity coefficient of 0.78 [20].

To differentiate between fertile and sterile plants of each GMS genotype, out of the 60 RAPD primers used, the primers OPAB3, OPAB4, OPAB5, OPAB19,

Table 2. Similarity matrix based on RAPD profile analysis in near isogenic diploid GMS lines

		SRT-1		HISAR	
		Sterile	Fertile	Sterile	Fertile
SRT-1	Sterile	1.00	0.77		
	Fertile	0.77	1.00		
HISAR	Sterile	0.82	0.77	1.00	0.78
	Fertile	0.78	0.85	0.78	1.00

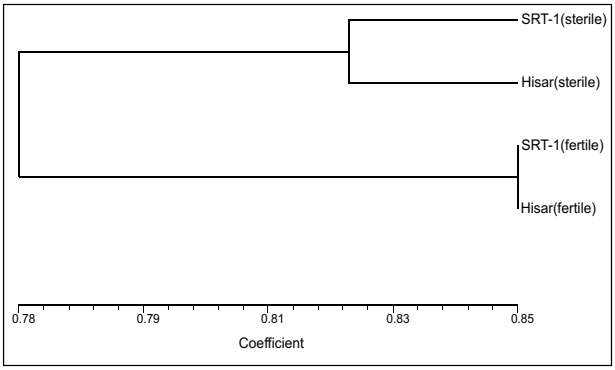

Fig. 3. Dendrogram generated from pooled data of RAPD profile using UPGMA analysis in the diploid near isogenic GMS lines

OPH20, OPI2, OPI3 and OPI7 showed polymorphism in both the pairs of near isogenic diploid GMS lines. The RAPD banding pattern in diploid GMS lines using different primers is depicted in Figure 2. When the diploid NILs were repeatedly screened with primers identifying polymorphic bands for confirmation and further studies, only primers like OPAB19, OPH20, OPI2, OPI3 and OPI7 could show consistent polymorphic bands in the plants. This kind of observation is common in RAPD based analysis of polymorphism. Similarly, Mane [16] failed to reproduce the polymorphism in 46 of 47 random decamer primer pairs in rice. The observations in other plant species are similar [17-19]. Bharati [20] identified RAPD primers OPY15 showing consistency in polymorphism in cotton GMS genotypes.

In the present investigation, primers *viz.*, OPAB19, OPH20, OPI2 and OPI3 showed genetic differences between fertile and sterile plants of both the diploid GMS

genotypes. So, they can be considered as putative markers to use them for linkage studies

Conversion of RAPD marker into SCAR marker

Lack of reproducibility is inherent in the RAPD technique itself. Non-specific primer binding for various reasons is a main concern. RAPD markers, therefore, are used for analysis of variability in the absence of better markers. Once identified, consistent RAPD markers are converted to sequence defined specific markers such as Alleles Specific Associated Primers (ASAPs) developed in chickpea [21] as well as sequence characterized amplified regions (SCARs) in rice [22], tomato [23], and chickpea [21]. The AFLP markers ware used for conversion into SCAR marker for genetic male sterility in *Brassica napus* [24 and 25] and *Capsicum annum* [26].

Out of the 34 polymorphic RAPD primers, one primer OPI3 was found sterile-specific by producing a reproducible specific fragment at 486 bp only in the male sterile plants of the Hisar GMS and SRT-1 GMS lines (Fig. 4). The male sterile specific fragment was eluted, gel purified, re-amplified, cloned and sequenced. Based on the sequence information SCAR marker was developed using the PRIMER 3.0 software. The male sterile specific Putative SCAR marker was then designated as DMS1 (Dharwad male sterile 1). The sequence information of the Putative SCAR marker is given in Table 3. This putative SCAR marker may be useful in future as a male sterile specific marker in marker assisted selection for commercial exploitation of heterosis using the genetic male sterility system in diploid cottons.

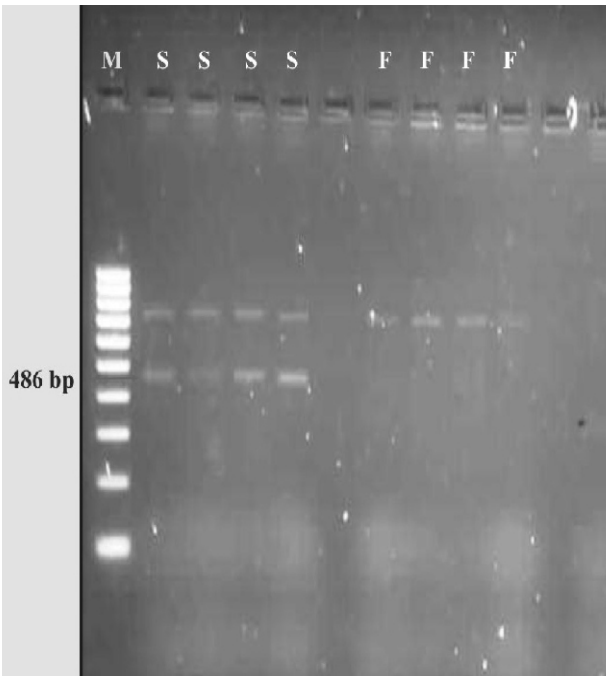

Fig. 4. Reproducible banding pattern in diploid cotton GMS lines using RAPD marker OPI-3 (M: 1000 bp ladder, S-male sterile, F-male fertile)

Table 3. Sequence and PCR parameters of SCAR marker

Marker designation	Direction	Primer sequence	Annealing temperature (^{0}C)
DMS1	Forward	5'AAAGAAATCTTG TAAAGAAATACTC CA 3'	57
	Reverse	5'CCTCATGATAAAAT GATGTTGC 3'	

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