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Identification of Molecular Markers Associated with Genic Male Sterility in Tetraploid Cotton (*Gossypium hirsutum* L.) through Bulk Segregant Analysis Using a Cotton SNP 63K Array

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Abstract

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Genic male sterility (GMS) is one of the most important economic traits for cotton (*Gossypium hirsutum* L.) hybrid seed production. The GMS trait conferred by two recessive alleles *ms5* and *ms6* in homozygous constitution is widely used for cotton hybrid seed production in India. Identification of molecular markers closely linked to the *ms5* and *ms6* alleles would be useful in effective transferring in a short time male-sterility genes into cultivars or elite lines using marker-assisted backcrossing. Here, we describe a quick method to identify markers for GMS genes using bulk segregant analysis (BSA) in the interspecific (*G. hirsutum* × *G. hirsutum*) biparental population. The parents and bulks were genotyped with a cotton single nucleotide polymorphism (SNP) 63K array that contains 63 058 SNP markers including 45 104 intraspecific and 17 954 interspecific SNP markers. Four SNP markers were found to be linked with the *Ms5* and *Ms6* genes. The markers i23493Gh and i46470Gh linked with the *Ms5* gene, and other two markers i08605Gh and i08573Gh linked with the *Ms6* gene are located on chromosome 12 and 26, respectively. A simple and cost effective tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS-PCR) assay was optimized for screening a large number of breeding samples with the identified SNP markers in a short time. The molecular markers developed in this study will facilitate the marker-assisted selection (MAS) and accelerate the development of new GMS lines to use in cotton hybrid seed production.

Keywords: *Gossypium hirsutum*; hybrid; male-fertility; polymorphism; test cross

Male sterility is an effective and economical pollination control system in the production of hybrid seeds. Plant male sterility systems such as three-line breeding using cytoplasmic nuclear-encoded male sterility (CMS), two-line breeding system using genic male sterility (GMS) and environmentally sensitive genic male sterility (EGMS) system have been widely used in various crops for production of commercial hybrids (Zhou *et al*. 2005). The CMS-based hybrid system has been successfully exploited in production of hybrids in various crops such as rice, maize, sorghum, onion and sunflower (Wang *et al*. 2010). However, the complicated breeding procedures, longer breeding period of CMS lines and costly maintenance of the parental system are limitations of the CMS system (Lakshmi Praba &

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Thangaraj 2005). Genic male sterility system has several advantages over the conventional CMS system as it does not require any maintainer and restorer lines for developing hybrids.

In cotton, GMS lines are being used for development of hybrids in both tetraploid and diploid cotton as it reduces the cost of hybrid seed production by eliminating the process of hand emasculation. In tetraploid cotton, GMS genes are well characterized and classified based on the nature of inheritance of genes. So far 19 GMS genes have been reported in tetraploid cotton species; four single genes with a dominant allele for sterility (*Ms4*, *Ms7*, *Ms10* and *Ms17*), six single genes with a recessive allele for sterility (*ms1*, *ms2*, *ms3*, *ms14*, *ms15* and *ms16*), four duplicate genes with recessive alleles (*ms5* and *ms6*; *ms8* and *ms9*) have been identified in *G. hirsutum* and the genes *Ms1*, *Ms11*, *Ms12*, *Ms18* and *Ms19* have been identified in *G. barbadense* (Chen *et al*. 2009). Although several genes have been identified, a recessive genic male sterility system conditioned by the *ms5* and *ms6* alleles attracts great attention to cotton breeders due to its prominent advantages in stable and complete male sterility in hybrid seed production (Paroda & Basu 1993).

Conversion of elite genotypes into GMS recessive homozygous lines needs selfing after each backcross to isolate recessive alleles; hence a higher number of generations is required as it is a relatively complicated genetic control system. With the advent of new technologies such as next-generation sequencing (NGS) and the availability of SNP (single nucleotide polymorphism) marker information provides an accurate approach to ease the conversion of male sterility traits to the elite cotton lines. Infinium SNP array is the latest SNP assay technology that has been widely used for genome mapping in humans, animals and plants as this technique enjoyed the advantages of high specificity, reproducibility and high call rate (Oliphant *et al*. 2002; Steemers & Gunderson 2007). Recently, Texas A&M University (TAMU) and Illumina, USA, jointly formed a cotton consortium and developed a cotton SNP 63K array to use in intraspecific and interspecific crosses for mapping the genes/QTLs for agronomically important traits within the cultivated cotton species, *Gossypium hirsutum* L. and other cotton species (Hulse-Kemp *et al*.2015).

Bulked segregant analysis (BSA) is an efficient method for rapid identification of molecular markers linked to any specific genes or genomic regions (Michelmore *et al*. 1991). BSA was successfully employed for mapping the fertility-restorer genes using 6K array (Yu *et al*. 2014) and salt tolerance traits using 50K chip in rice crop (Tiwari *et al*. 2016). In cotton, earlier *Ms11* and *ms8*, *ms3*, *ms9* GMS alleles have been mapped to chromosome 12, 16 and 26, respectively (Mandaliya & Thaker 2016). Chen *et al*. (2009) reported mapping of *ms5*, *ms15* on chromosome 12 and *ms6* on chromosome 26 with the SSR markers using an interspecific population in tetraploid cotton. However, for employing the genes for male sterility in MAS and cloning, further fine mapping is necessary. In this study, we employed the BSA technique using cotton SNP 63K array to develop a set of SNP markers that are linked with GMS alleles (*ms5*, *ms6*) which will be helpful in MAS-based introgression breeding programs in tetraploid cotton (*G. hirsutum*).

MATERIAL AND METHODS

Plant material. Intraspecific crosses were made between two homozygous parents, *G. hirsutum* var. RC403, a male-sterile donor line (*ms5ms5 ms6ms6*) and *G. hirsutum* var. RC370, a male-fertile recurrent line (*Ms5Ms5 Ms6Ms6*). The F_1 plants (*Ms5ms5 Ms6ms6*) were crossed with male-sterile donor parent (RC403) and produced BC_1 progeny for fine mapping. The part of F_1 progenies were allowed to self-pollinate the produced F_2 population for evaluation of pollen fertility. Pollen fertility and sterility of each plant were checked during anthesis and confirmed through the iodine in potassium iodide (IKI) staining; also the colour of pollen grains was observed with a phase contrast stereomicroscope (Nikon Eclipse.50i, Nikon, Japan). The genotype of each BC_1 fertile plant (*Ms5ms5 Ms6ms6*/*Ms5ms5 ms6ms6* or *ms5ms5 Ms6ms6*) was determined in a test-cross population by crossing the BC_1 fertile plant with the male-sterile donor parent (RC403). Chi-square (χ^2) tests were performed for segregation of phenotypic data from expected Mendelian ratios. The BC_1 fertile plants which showed 3:1 segregation (*Ms5ms5 Ms6ms6*) in the test-cross population were combined with the male-sterile (*ms5ms5 ms6ms6*) lines for mapping the recessive *ms5* and *ms6* alleles. Of these, 20 male-sterile and 20 male-fertile plants were selected and four bulks were made for genotyping with the SNP markers using cotton SNP 63K chip.

SNP assay. Genomic DNA was isolated from male-sterile and male-fertile plants as described by

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Paterson *et al*. (1993). DNA was quantified using a NanoDrop spectrophotometer and stored at –20°C until further use. Two male-sterile bulks (SB1 and SB2) and two male-fertile bulks (FB-1 and FB-2) were made by pooling 10 samples of DNA (20 ng/ul) in equal amount of male-sterile plants (*ms5ms5 ms6ms6*) and male-fertile plants with genotype *Ms-5ms5 Ms6ms6* separately. Cotton SNP 63K array that contains 63 058 SNPs represented from *G. hirsutum* (45 014 SNPs), *G. barbadense* (9579 SNPs), *G. tomentosum* (2397 SNPs), *G. mustelinum* (2226 SNPs), *G. armorianum* (1890 SNPs) and *G. longicalyx* (1 862 SNPs) was used for high throughput genotyping of sterile and fertile bulks. Illumina assay protocol was followed for DNA fragmentation, chip hybridization, single-base extension through DNA ligation and signal amplification. Data for the markers were clustered using the 18 Genome Studio Genotyping Modules (Ver. 1.9.4, Illumina, Inc., USA) (Hulse-Kemp *et al*. 2015). An ABH scoring system was followed for scoring the SNP alleles as described by Hulse-Kemp *et al*. (2015).

Tetra-primer ARMS-PCR amplification. The tetra-primer ARMS-PCR assay was developed to validate the candidate SNP markers in the individual samples of sterile and fertile bulks as it is simple, rapid, inexpensive, and it can distinguish homozygote from heterozygote SNPs (Ye *et al.* 2001). Primers were designed for the SNP by the software on the website: http://cedar.genetics.soton.ac.uk (Collins & Ke 2012). We designed two outer primers and two inner primers (forward and reverse) for each selected SNP locus and gradient PCR was carried out on a thermal cycler (Eppendorf Vapo Protect, Hamburg, Germany) to determine the optimal annealing temperature for each primer set (Table 1). The PCR assay was conducted in a 20 µl reaction containing 50 ng of template DNA, 0.4 µl of each inner primer and outer primer (10 µM), 200 µM dNTPs, 2.5 mM $MgCl_2$, 2 µl of 10× buffer and 0.5 U of *Taq* DNA polymerase. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C to 68°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were separated by running on 3% agarose gel with ethidium bromide and the bands were scored as homozygous and heterozygous based on the presence of SNP allele in each sample.

RESULTS

F_2 and BC_1 populations. All F_1 progeny was fertile. The male sterility of RC403 was controlled by

Table 1. Sequence of primers for single nucleotide polymorphism genotyping for *ms5* and *ms6* alleles and characteristics for tetra-primer amplification refractory mutation system PCR assay

Marker	Primer sequence (5'–3')	Product size (bp)	Annealing temperature (°C)
i23493Gh	FIP: TTGATGTTCAATTTATTACCCAGATCACAT RIP: TCTAATATTCATTGGGCCACATAAGAATG FOP: CTGAGTTTGGCTTATCTTCAATTCTGGT ROP: GCTATTTGATACCTGGGTTTGCTTTTAA	T allele : 236 C allele: 214 common: 391	66
i46470Gh	FIP: AATACTAAGCTTACCTTATTTTCATTACCG RIP: AAAGGGTTAGGTAAGTGTAAAATTCCTT FOP: TAAGACACATCAAGCAACTCTTAATTACA ROP: ATTTGTAATTACGAATAAGACCCTGTGT	G allele :236 A allele : 292 common: 470	66
i08573Gh	FIP: AGACACTGAATGATGTTGCCGAACCG RIP: GCATTCTATTGGGGTGGAACTCGAATCTT FOP: CGAACATGCCGAGAGTTTGTCAGCTTAG ROP: GCTGGTAAATAACCGGGGGCTTTTTCTT	G allele : 201 A allele: 157 common: 303	66
i08605Gh	FIP: GGAAGCAAACTCTGTTAAGGGTACAC RIP:ACTTGTAAAAATCACATTTTGAGCTTATAA FOP: ACTAGGTTCAACAGTCAACTGAACTGTT ROP: ATAGAGAAGTGCATGGATTGAAGTTTAC	C allele: 199 T allele: 218 common: 361	62

FIP – forward inner primer; RIP – reverse inner primer; FOP – forward outer primer; ROP – reverse outer primer

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Table 2. BC_1 and F_2 segregation patterns for the cross of male-sterile parent RC403 × male-fertile recurrent parent RC370 of cotton

Generation	Total No. of plants	No. of male-fertile plants	No. of male-sterile plants	Expected ratio	χ^2	*P*–value
BC_1	297	219	78	3:1	0.13	$0.50 < P < 0.75$
F_2	284	268	19	15:1	0.34	$0.50 < P < 0.75$

two recessive alleles (*ms5ms5 ms6ms6*). This was confirmed in the F_2 segregation with 268 fertile and 19 sterile plants which fitted with 15:1 segregation ratio ($\chi^2 = 0.34$, $P = 0.72$) shown in Table 2. The segregation ratio of BC_1 population with 219 male-fertile to 78 sterile plants is not significantly different from a 3:1 ratio ($\chi^2 = 0.13$, $P = 0.72$). Among 219 male-fertile BC_1 plants, 66 plants segregated at a 3:1 ratio being heterozygotes for both genes while the remaining 153 plants which showed a 1:1 segregation ratio being either *Ms5ms5 ms6ms6 or ms5ms5 Ms6ms6* genotype in their test-cross.

Bulk segregant analysis. Genotyping of the male-sterile and male-fertile bulks along with their parents was done using high density cotton SNP 63K array that contains 63 058 SNP markers. Of these, 46 297 markers were homozygous for the male-sterile parent and 46 409 markers were homozygous for the male-fertile parent with the average call rate of 98%. Heterozygous markers were eliminated for the parental polymorphic analysis. The intraspecific markers showed a higher polymorphic rate (5.89%) than the interspecific markers (1.10%) between the parental lines. Overall, 2855 SNP (4.52%) markers showed polymorphism between the parental lines (Table 3). In further analysis, 24 markers showed polymorphism between the male-sterile bulks and the male-fertile bulks. Among them, a set of four SNP markers such as i23493Gh, i46470Gh i08573Gh and i08605Gh was found to be at the homozygous condition in male-sterile bulks similar to the sterile parent and heterozygous in male-fertile bulks (Table 4). It was also found that the markers i23493Gh and i46470Gh are located on chromosome 12 and the other markers i08573Gh and i08605Gh are located on chromosome 26 in the existing high density SNP linkage map constructed by using the intraspecific cross population developed using Phytogen 672 and Stoneville 474 (Hulse-Kemp

Table 3. Single nucleotide polymorphism (SNP) origin for 63K array and SNP between the male-sterile parent RC403 and male-fertile recurrent parent RC370

SNP origin	No. of SNPs in 63K array	No. of polymorphic markers	Polymorphism (%)
G. hirsutum (*Gh*)	45 104	2 657	5.89
G. barbadense (*Gb*)	9 579	146	1.52
G. tomentosum (*Gt*)	2 397	40	1.67
G. mustelinum (*Gm*)	2 226	08	0.36
G. armorianum (*Ga*)	1 890	02	0.11
G. longicalyx (*Gl*)	1 862	02	0.11
Total	63 058	2 855	4.53

Table 4. Evaluation of polymorphism for single nucleotide polymorphism (SNP) markers associated with the male-sterility alleles

SNP ID	Chr.	Polymorphic SNP alleles	RC403	RC370	SB-1	SB-2	FB-1	FB-2
i23493Gh	12	T/C	CC	TT	CC	CC	TC	TC
i46470Gh	12	A/G	GG	AA	GG	GG	AG	AG
i08605Gh	26	T/C	CC	TT	CC	CC	TC	TC
i23493Gh	26	A/G	AA	GG	AA	AA	AG	AG

RC403 – male-sterile donor parent; RC370 – male-fertile recurrent parent; SB-1, SB-2 – male-sterile bulks; FB-1, FB-2 – male-fertile bulks

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et al. 2015). This result was similar to the earlier report that ms5ms6 genes were located on chromosome 12 and 26, respectively (Rhyne 1991; Chen *et al*. 2009). Based on this analysis, the markers i23493Gh and i46470Gh were linked with ms5ms5 gene and the markers i08573Gh and i08605Gh were linked with ms6ms6 gene in our male-sterile and male-fertile bulks.

Validation of SNPs for male-sterility genes. For the validation of BSA approach, the tetra-primer ARMS-PCR protocol was optimized for the linked SNP markers to genotype the individual samples of sterile and fertile bulks. Annealing temperature was fixed to be 62°C for i08605Gh and 66°C for the other three SNPs in the gradient PCR. The sizes of DNA fragments amplified with those primers are shown in Figure 1. A comparison of genotype and phenotype data revealed perfect co-segregation among them. It was evident that these markers are closely associated with the genic male sterility trait governed by paired recessive genes and can be used for marker-assisted selection-based GMS genes introgression breeding program.

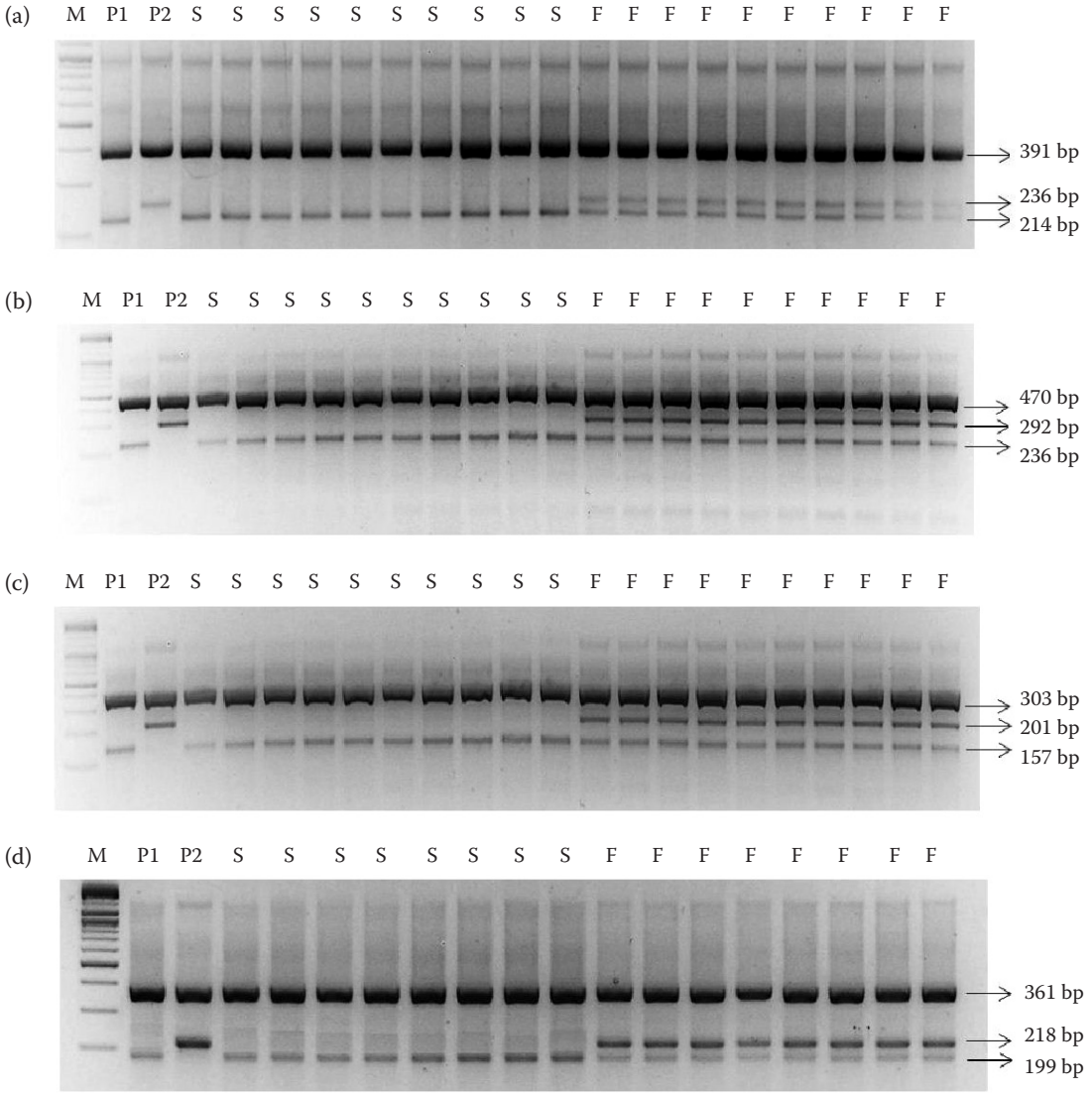

Figure 1. Tetra-primer amplification refractory mutation system PCR fragments of four single nucleotide polymorphism (SNP) markers linked to the *ms5* and *ms6* alleles in BC_1 plants of male-sterile and male-fertile bulks: i23493Gh marker associated with *ms5* allele (a); i46470Gh marker associated with *ms5* allele (b); i08573Gh marker associated with *ms6* allele (c); i08605Gh marker associated with *ms6* allele (d)
M – marker (3 Kb DNA ladder); P1 – homozygous male-sterile parent (RC403); P2 – homozygous male-fertile recurrent parent (RC370); S-male – sterile BC_1 progenies; F-male –fertile BC_1 heterozygous progenies

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DISCUSSION

India is the pioneer country for commercial cultivation of cotton hybrids which covers more than 95% of the total cotton cultivation area. The majority of cotton hybrid seeds are produced by a conventional method which requires several trained labourers for hand emasculation and pollination. Thus, the cost of cotton hybrid seed production and the seeds of such hybrid become expensive. To circumvent the tedious hand emasculation and reduce the cost of cotton hybrid seed production, the development of hybrids using male sterility lines has to be accelerated as it eliminates hand emasculation since the pollen is sterile in female parent. Among the several GMS genes, duplicate recessive genes *ms5ms5 ms6ms6* conferred stable male sterility. However, it is difficult to develop GMS lines in a short time through conventional approaches as it requires alternate selfing after each backcross and a minimum of 10 to 13 generations. Molecular markers could play a vital role to avoid the progeny testing and alternate selfing, and in reducing the breeding cycle in a backcrossing method.

In cotton, four SSR markers have been reported for *ms5* and *ms6* alleles using the interspecific population of *G. hirsutum* × *G. barbadense* (Chen *et al*. 2009). But these markers are not amenable for high-throughput genotyping as their PCR amplicons resolved only through PAGE gel which is a time-consuming, cumbersome and laborious process. Recently, a haplotype SNP marker set has been reported for *ms5ms6* gene (Feng *et al*. 2015) which is based on an expensive TaqMan® assay method to genotype the breeding samples.

In this study, BSA was coupled with the cotton SNP 63K array genotyping method for rapid data generation and marker identification with less time and cost was adopted. Even though 63K array has a higher potential for mapping the gene in an intraspecific population, the moderately low level of polymorphism (4.53%) was obtained between the parents due to narrow genetic variation within the *G. hirsutum* germplasm. So, the number of bulks has been increased from a single bulk to two bulks for mapping the genes so as to increase the sensitivity of the assay.

A high-throughput genotyping method is preferred for the MAS-based breeding program as a large number of samples needs to be screened in a short time. Several fluorescence primer-based marker systems are available for high-throughput genotyping which needs expensive primers, chemicals and costly instruments to resolve a single nucleotide difference in the amplicon length (Wang *et al*. 2007), which may not be practical and affordable for most of the laboratories in developing countries. Among the several SNP genotyping methods, tetra-primer ARMS- PCR has been reported to be a rapid, reliable, simple and economical assay for SNP genotyping (Ye *et al*. 2001; Okayama *et al*. 2004). In our study, a simple tetra-primer ARMS-PCR assay has also been developed and validated with the genotyping data of BSA. This result revealed that the newly developed SNP markers are accurate and reliable to identify the plants with *Ms5ms5 Ms6ms6* gene combination in backcrossed samples which reduce the breeding cycle of GMS line development by eliminating the selfing after each backcross. It will also be useful to identify the male-fertile plants with either *Ms5ms5 ms6ms6* or *ms5ms5 Ms6ms6* gene combination for sib-mating with the male-sterile plant to produce the 1:1 ratio of sterile and fertile plants for hybrid seed production.

In summary, four SNP markers have been identified for *ms5ms5 ms6ms6* genic male-sterile genes in the intraspecific *G. hirsutum* population through BSA using cotton SNP 63K array. A cost effective, reliable tetra-primer ARMS-PCR assay has been optimized and validated for the identified SNPs to use in the molecular breeding program. The molecular markers identified in this study were able to identify the zygosity of the male-sterile and fertile alleles in backcrossed and selfed progenies for both the *ms5* and *ms6* alleles in the breeding program. Thus, these markers offer a great scope for improving the efficiency of conventional breeding program and accelerate the development of new GMS lines for providing cotton hybrids in a short period of time, thereby reducing the breeding program cycle.

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