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AUTHENTICATION OF SSR MOLECULAR MARKERS ASSOCIATED WITH GENETIC MALE STERILITY IN COTTON (*GOSSYPIUM HIRSUTUM* L.) PARENTAL LINES

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ABSTRACT

Genetic male sterility serves as a highly efficient approach for generating hybrid cotton. In India, successful hybrid cotton development has predominantly relied on the utilization of lines containing the ms_5ms_6 gene among various identified GMS gene-containing lines. In this current research, the validation of three GMS female lines, along with their fertile counterparts and the original non-GMS parental lines, was conducted using SSR markers with the primers BNL 1227 and NAU 2176.

At the Main Cotton Research Station, Athwa Farm, N.A.U, Surat, India, three GMS female genotypes, their corresponding fertile counterparts, and the original parental lines lacking GMS were employed for the validation of molecular markers associated with GMS in cotton. This validation process involved DNA extraction and subsequent PCR amplification.

Our investigations yielded conclusive evidence regarding the presence of the sterility gene in GMS lines. This demonstrates the genetic purity of the genotypes, and it was observed that the selected primers effectively validated both GMS and their respective original parental groups. Consequently, the primers BNL 1227 and NAU 2176 provide a rapid and dependable method for identifying the genetic male sterility system controlled by the ms5ms6 gene in cotton (*G. hirsutum* L.). Moreover, they serve as an efficient alternative to the time-consuming and labor-intensive grow-out test (GOT) for cotton.

Key words : Genetic male sterility, *G. hirsutum* L., SSR primers, ms_5ms_6 gene, Validation.

Introduction

Cotton, renowned as the leading fiber producer, holds a prominent position as a cash crop in our nation. India stands as a trailblazing nation in the large-scale cultivation of cotton hybrids. In recent times, there has been a stagnation in cotton productivity, making it imperative to explore methods like heterosis exploitation to enhance yields and fiber quality. This enhancement is crucial to ensure the competitiveness of Indian cotton against synthetic materials and foreign production. For the commercial harnessing of hybrid vigor, male sterility has emerged as a pivotal tool. Within the realm of cotton, heterosis plays a significant role in elevating both fiber yield and quality (Zhang and Pan, 1999). Male sterility, specifically genic male sterility (GMS), proves highly effective in generating hybrid cotton. It surpasses cytoplasmic genetic male sterility (CGMS) in terms of efficiency and cost-effectiveness, primarily because manual emasculation and pollination are time-intensive and less economical. In contrast to CGMS, GMS offers the advantage of facilitating random mating for hybrid development, as nearly all commercial cultivars can serve as restorer lines. However, a notable drawback of GMS lies in the fact that offspring resulting from GMS plants

pollinated by heterozygous pollinators theoretically yield a 1:1 segregation of male sterile and fertile progeny. Consequently, the fertile offspring must be identified and removed, a process that often proves uneconomical (Kaul, 1988).

Much effort has been made in genetic identification and manipulation of heterosis in GMS cotton. Since, Justus and Leinweber (1960) first found a heritable partial male-sterile line in upland cotton (*Gossypium hirsutum* L.), 19 GMS genes in tetraploid cotton have been identified, including seven single recessive genes (ms_1, ms_2, ms_3, ms_{13}, ms_{14}, ms_{15} and ms_{16}), four duplicate recessive genes (ms_5ms_6 and ms_8ms_9) and eight single dominant genes (Ms_4, Ms_7, Ms_{10}, Ms_{11}, Ms_{12}, Ms_{17}, Ms_{18} and Ms_{19}) (Justus and Leinweber, 1960; Richmond and Kohel, 1961; Justus *et al*., 1963; Allison and Fisher, 1964, Weaver, 1968; Weaver and Ashley, 1971; Bowman and Weaver, 1979; Turcotte and Feaster, 1985; Percy and Turcotte, 1991 and Zhang *et al*., 1992). Except for Ms_{11}, Ms_{12}, ms_{13}, Ms_{18} and Ms_{19} identified in *Gossypium barbadense*, all were found in *G. hirsutum*. Among these GMS lines, only ms_{14} and ms_5ms_6 lines have been utilized successfully in developing hybrid cotton in India. Our objectives in this research is to validate the available molecular markers linked to the GMS genes in tetraploid cotton (*G. hirsutum*).

Significant efforts have been dedicated to the genetic identification and manipulation of heterosis in GMS cotton. The journey began with the discovery by Justus and Leinweber in 1960 of a partially heritable male-sterile line in upland cotton (*Gossypium hirsutum* L.). Subsequently, 19 GMS genes in tetraploid cotton have been pinpointed. These genes encompass seven single recessive genes (ms_1, ms_2, ms_3, ms_{13}, ms_{14}, ms_{15} and ms_{16}), four duplicate recessive genes (ms_5ms_6 and ms_8ms_9) and eight single dominant genes (Ms_4, Ms_7, Ms_{10}, Ms_{11}, Ms_{12}, Ms_{17}, Ms_{18} and Ms_{19}). Notably, all but Ms_{11}, Ms_{12}, ms_{13}, Ms_{18}, *and* Ms_{19} were identified in *Gossypium hirsutum*, with the remainder found in *G. barbadense*. Among these GMS lines, only ms_{14} and ms_5ms_6 lines have been successfully employed in the development of hybrid cotton in India. In this research, our primary objectives revolve around validating the existing molecular markers associated with GMS genes in tetraploid cotton (*G. hirsutum*).

Materials and Methods

Plant materials

During the 2017-2018 period, we utilized three female GMS genotypes, along with their fertile counterparts and the original parental lines without GMS. These plant materials were cultivated at the Main Cotton Research Station, Athwa Farm, N.A.U, Surat, India and were employed in the validation of molecular markers associated with GMS in cotton.

Following are the genotypes used in these experiment:

Name of the genotypes

GMS lines		Non GMS original parental lines
Sterile	Fertile counterpart	
GMS Gregg	GMS Gregg	Gregg
G(T)-84	G(T)-84	GT-84
S-D-2	S-D-2	SD-2

DNA extraction

We employed fresh young leaf tissues, less than one week old, to extract genomic DNA from both fertile and sterile plants in GMS cotton, as well as from non-GMS cotton. This extraction was carried out using the CTAB method described by Paterson *et al.* (1993). In summary, we ground 4 g of leaf tissue into a fine powder in liquid nitrogen, which was then transferred to a 50 ml centrifuge tube. To this, we added 20 ml of ice-cold DNA extraction buffer containing 2% (w/v) CTAB, 1.4 M NaCl, 0.005 mM EDTA, 2% (w/v) polyvinylpyrrolidone (PVP), 0.2% mercaptoethanol, and 0.1 M TrisHCl at pH 8.0 and incubated the mixture for 1 hour with intermittent shaking.

Subsequently, the homogenate was allowed to cool to room temperature and 10 ml of Chloroform: Isoamyl alcohol (24:1 v/v) was added. The contents were gently mixed by inverting the tube 25-30 times, followed by centrifugation at 2700 rpm for 5 minutes. The clear aqueous phase was carefully transferred to a fresh centrifuge tube and the steps were repeated if the aqueous phase appeared cloudy. The supernatant from the final Chloroform-Isoamyl alcohol extraction was transferred to 15 ml Falcon tubes.

To each tube, approximately 0.6 volume (about 5.4 ml) of ice-cold isopropanol was added, and the tubes were inverted 20-30 times. The DNA was then resuspended in a 65 °C water bath for 10-30 minutes. After resuspension, the samples were centrifuged at 10,000 rpm for 5 minutes and the supernatant (containing DNA) was transferred to fresh tubes, discarding the pellet. The DNA could be stored in frozen TE at 4°C or indefinitely at -20°C in 70% ethanol. The DNA concentration was determined spectrophotometrically at 260 nm and its quality was confirmed by electrophoresis in a 0.8% (w/v) agarose gel.

Table 1 : Primer sequences used in PCR analysis and molecular marker test.

Primer name	Primer sequences 5' - 3'			
	Forward primer	Tm (^{0}C)	Reverse primer	Tm (^{0}C)
BNL 1227	CATCAAGATCTATCTCTCTCTATACCG	61.90	TTTACCCTCCGATCTCAACG	57.30
NAU 2176	TGGCACTTGCTAAACCATAA	53.20	GGTTTGAGCAGAAGGGTATG	57.30

Table 2 : PCR reaction mixture for amplification of SSR Primer.

S. no.	Stocks	Quantity
1.	Sterilized Millipore water	16 µl
2.	Taq Buffer (10x)	2.5 µl
3.	dNTPs (2.5 mg each)	2.0 µl
4.	Taq DNA polymerase (3U/ µl)	0.5 µl
5.	Forward primer (10 pmoles/ µl)	1.0 µl
6.	Reverse primer (10 pmoles/ µl)	1.0 µl
7.	DNA template (50 ng/ µl)	2.0 µl

Table 3 : Programme of the thermocycler for the amplification of SSR primer.

Stages	Time	Temperature	Cycles
Denaturation	5 min	94°C	1
Denaturation	1 min	94°C	

PCR amplification

PCR amplifications for SSR markers were carried out following the protocols outlined in Zhang *et al.* (2000) and *Zhang et al.* (2002). The specific primer sequences employed in this experiment are provided in Table 1 and a concise outline of the reaction mixtures is presented in Table 2. The thermocycler program utilized for SSR primer amplification is detailed in Table 3. The resulting polymerase chain reaction products (25 µl) were combined with 1X gel loading buffer (3 µl) and subjected to electrophoresis at 70 V for a duration of 65-70 minutes. Subsequently, the gel was immersed in an ethidium bromide solution (2 µl/100 ml 1X TAE buffer) for 40 minutes, and the bands were visualized using UV illumination in a Gel Doc system (UVITEC). The size of the amplified fragments was estimated by reference to a 100 bp DNA size standards marker.

Results and Discussion

PCR amplifications employing SSR markers were conducted on female genetic male sterile lines, their corresponding fertile counterparts and the original parental lines without genetic male sterility (non-GMS lines). The results, as illustrated in Fig. 1, revealed the presence of a specific ~170 bp DNA band amplified by the BNL 1227 primer pair. This observation indicated the presence of the sterility gene ms_5ms_6 in all genetic male sterile lines and their fertile counterparts, while it was conspicuously absent in all non-GMS original parental lines (as summarized in Table 4).

Similarly, Fig. 2 displayed the presence of a specific ~90 bp DNA band amplified by the NAU 2176 primer pair. This finding also confirmed the presence of the sterility gene ms_5ms_6 in all genetic male sterile lines and their fertile counterparts, with its absence in all non-GMS original parental lines, further corroborated in Table 4. These results collectively provide robust evidence of the genetic makeup of the tested cotton lines, highlighting the presence or absence of the ms_5ms_6 sterility gene and underscore the utility of these markers in rapidly and accurately identifying the genetic male sterility system in cotton. In our current investigation, we employed primers previously described by Chen *et al.* (2009) to conduct genetic analyses on cotton lines with male sterility traits (GMS lines), their corresponding fertile counterparts and the original parental lines without genetic male sterility (non-GMS lines). The objective was to validate the presence of the genetic male sterility system in cotton using molecular markers. Among the various cotton genotypes that underwent testing, we observed that GMS Gregg (fertile counterpart), GMS Gregg (sterile), GMS G(T)-84 (fertile counterpart), G(T)-84 (sterile), GMS S-

Table 4 : Present or absent of specific band for sterility in parents.

S. no.	Genotypes	Present / Absent of specific band for sterility
GMS lines (Sterile)		
1.	GMS Gregg	Present
2.	G(T)-84	Present
3.	S-D-2	Present
GMS lines (Fertile counterpart)		
4.	GMS Gregg	Present
5.	G(T)-84	Present
6.	S-D-2	Present
Non GMS original parental lines		
7.	Gregg	Absent
8.	GT-84	Absent
9.	SD-2	Absent

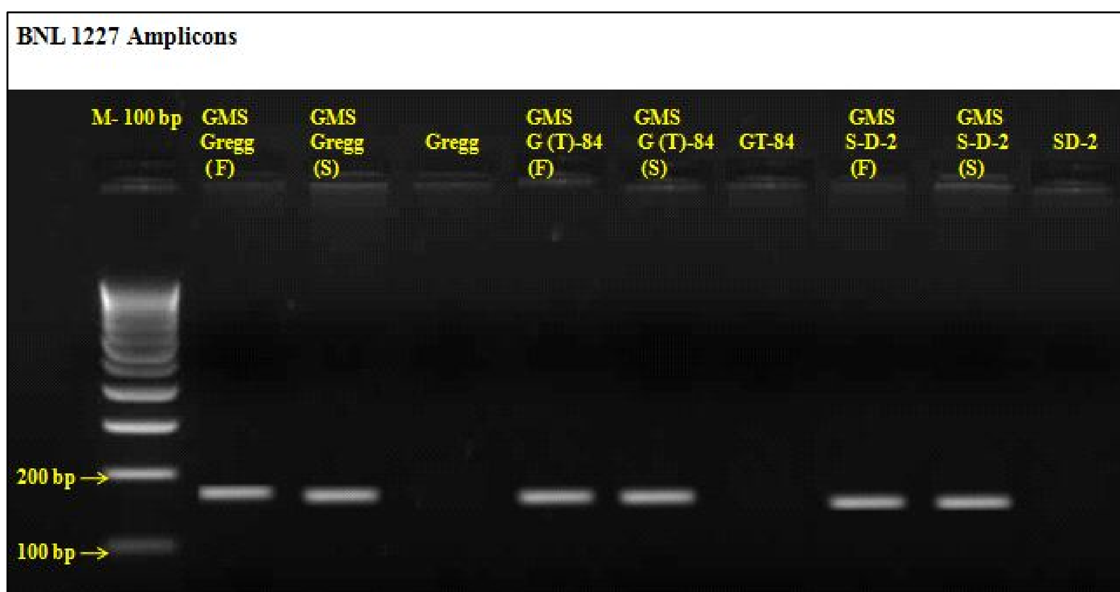

Fig. 1 : Amplified fragments of BNL 1227 primer in cotton genotype. Where, F = fertile counterpart, S = sterile and bp = base pairs.

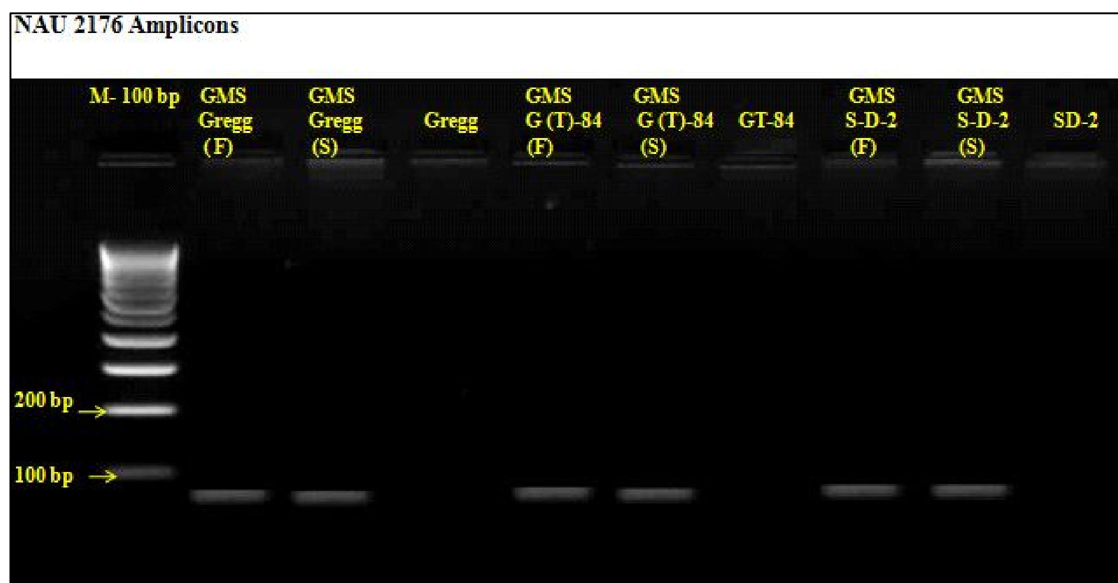

Fig. 2 : Amplified fragments of NAU 2176 primer in cotton genotype. Where, F = fertile counterpart, S = sterile and bp = base pairs.

D-2 (fertile counterpart), and GMS S-D-2 (sterile) all exhibited amplification with both primers, namely BNL 1227 and NAU 2176. This amplification indicated the presence of the male sterility genes in these lines, reaffirming their status as male sterile or fertile, as expected. Conversely, the non-GMS original parental lines, namely Gregg, GT-84 and SD-2, did not display any amplification with the gene-specific primers BNL 1227 and NAU 2176. This absence of amplification underscored the fertile nature of these non-GMS lines, as they lacked the male sterile duplicate recessive gene ms_5ms_6, which is responsible for the male sterility trait. Our findings provide conclusive evidence of the genetic makeup of these cotton lines.

The results we obtained in this study corroborate and validate the earlier work by Chen *et al.* (2009), confirming the reliability of the primer pair they proposed. In summary, both primers, BNL 1227 and NAU 2176, serve as efficient tools for swiftly and accurately identifying the presence of the genetic male sterility system, governed by the ms_5ms_6 gene, in cotton (*Gossypium hirsutum* L.). This research contributes to the field of cotton breeding and seed production, offering a valuable resource for cotton growers and breeders to ensure the purity and quality of hybrid cotton varieties.

Conclusion

The findings of our current research hold significant implications for the field of cotton breeding and hybrid seed production. This study has successfully validated the effectiveness of a specific molecular marker in the crucial task of assessing and preserving seed purity. The marker's primary function lies in its ability to detect and eliminate any contamination present in hybrid seed stocks at the seedling stage. This capability is of paramount importance because ensuring seed purity is a fundamental prerequisite for achieving consistent and reliable crop yields in cotton farming. The current research has confirmed the utility of this marker in assessing and maintaining seed purity by identifying and eliminating contamination in hybrid seed stocks at the seedling stage. This marker provides an efficient alternative to the time-consuming and labor-intensive grow-out test (GOT) for verifying hybrid purity. Consequently, both primers, BNL 1227 and NAU 2176, offer a rapid and dependable means of identifying the genetic male sterility system controlled by the ms_5ms_6 gene in cotton (*G. hirsutum* L.).

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Authors' contributions

Himalay R. Patel contributed to the research and technical writing under the guidance of D. H. Patel, while V. S. Ranpariya reviewed the research article for the final submission.

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Availability of data and materials

The data sets employed and/or scrutinized in the present study can be obtained from the corresponding author upon a reasonable request.

Declarations

Ethics approval and consent to participate : Not applicable.

Consent for publication : The manuscript has been reviewed by all authors, and they have provided their approval for its publication.

Competing interests : Not applicable.

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