



# Fertile grounds: exploring male sterility in cotton and its marker development

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## Abstract

The high cost of producing conventional hybrid cotton seeds led to more research efforts on cotton male sterility systems. There is a lack of studies on cytology, histology, morphological variation, yield, and altered restorer backgrounds to identify and develop male sterility markers in cotton hybrids. Hybrid cotton can be efficiently produced by exploiting genetic male sterility. Among the 19 Genetic Male Sterility (GMS) genes discovered, the lines with *ms5ms6* genes are mostly utilised to establish successful hybrid cotton in India. Molecular markers closely associated with the MS alleles are identified to facilitate the efficient and rapid backcrossing of male-sterility genes into elite lines or cultivars by marker-assisted backcrossing. The majority of the markers which are random DNA markers (RDMs), are probably lost, when recombination occurs. In contradiction, molecular markers (functional markers, or FMs) within the genic region can be identified and employed in crops for diverse traits, if prospective characteristic genes are known. In this review, the mechanism of male sterility, its gene expression level, and the need for functional markers for the male sterility trait in cotton have been put forward.

**Keywords** GMS · CMS · CGMS · FM · Upland cotton

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## Introduction

### Economic importance of cotton

*Gossypium* sp., commonly known as cotton, is a principal cash crop used for fibre, as well as edible oil, and protein worldwide. Among the 50 species reported in the genus, four are predominantly cultivated, and *G. hirsutum* produces 90% of the total cotton fibre. Although genetic engineering technology has been standardised in cotton to a greater extent [1], the phenomenon of heterosis and exploitation of hybrid vigour is still gaining traction in China and India [3]. Strong heterosis has been reported by several researchers for fibre quality, number of bolls, boll weight and seed cotton yield in upland cotton [1].

### Role of male sterility in hybrid production

In cotton, intraspecific and interspecific hybrids are produced conventionally through manual emasculation and pollination. But the disadvantages of traditional hybrids are two-fold. Firstly, the emasculation process is tedious

and requires employing numerous skilled labourers, which drives up the cost of hybrid seeds. Secondly, the hand emasculation process damages the female reproductive system, which impairs hybrid seed production. Given the above facts, unearthing male sterility becomes vital in cotton.

It is an efficacious and effortless way of developing quality hybrid cotton seeds [2]. On the basis of genes responsible for inheritance of male sterility, it can be categorised into three types, namely, nuclear male sterility (also known as genic male sterility; GMS), cytoplasmic male sterility (CMS) and cytoplasmic genetic male sterility (CGMS). In upland cotton, the first line with heritable partial male sterility was discovered by Justus and Leinweber in 1960. Suguna (CPH2), the first GMS-based hybrid in history, was created in 1978 at the Central Institute for Cotton Research (CICR) Regional Station in Coimbatore, India. There are also the CGMS-based hybrids, which include PKV Hy 3, PKV Hy 4, and MECH 4.

### Male sterility genes

According to [3], anther development can be categorized into 14 typical stages based on genetic and cytological factors. Based on cell proliferation, development, differentiation, maturation and degradation, the anther and pollen formation process can be fit into four stages namely, pre-meiotic phase (specification of PMCs and tapetum), meiotic phase (differentiation of tapetal cells), post-meiotic phase (development of pollen wall and mature pollen) and dehiscence of anther and pollen germination phase. An anomaly in any of the stages brings about male sterility in flowering plants (Figure 2). Many transcriptomic, epigenetic and biochemical studies have been conducted to elucidate the

mechanism of MS at molecular level. Nevertheless, genes associated with MS have not yet been identified in many species [4–7]. In GMS cotton, gene identification, mechanism understanding and manipulation of heterosis have been decoded with continual research.

In cotton, the availability of GMS sources have been documented in American, Egyptian, and *arboreum* species. Both recessive and dominant genes are accountable for governing male sterility in tetraploid cotton. About 19 GMS genes, seven single recessive (*ms1*, *ms2*, *ms3*, *ms13*, *ms14*, *ms15*, and *ms16*), eight single dominant (*Ms4*, *Ms7*, *Ms10*, *Ms11*, *Ms12*, *Ms17*, *Ms18*, and *Ms19*) and four double recessive (*ms5ms6* and *ms8ms9*), have been recorded in tetraploid cotton [8, 9]. All the above genes were discovered in *G. hirsutum* except *Ms11*, *Ms12*, *Ms13*, *Ms18*, and *Ms19*, which were reported in *G. barbadense* (Table 1). Among the above, only *ms14* and *ms5ms6* lines have been effectively used in China and India to generate hybrids [10]. Male partial genic sterility has been reported to be conferred by the single recessive genes *ms1* and *ms3* [8, 11]. Richmond and Kohel, 1961, observed that stable male sterility was imparted by paired recessive genes (*ms5* and *ms6*) and a single recessive gene (*ms2*) [12]. Justus et al., (1963), reported that, in a greenhouse condition, *ms3* plants produced more fertile flowers than in the field. However, recessive genetic mechanisms with advantages of stable and absolute male sterility, particularly by *ms5* and *ms6*, have been frequently exploited in cotton hybrid cultivation since fertile GMS maintainers are needed [13].

### Markers for male sterility trait

Male sterility becomes an essential trait in plant species for the complete success of hybridization with ease. To fulfil the requirements of breeding objectives, male sterility plays a major role in getting rid of the difficulties in manual emasculation, thereby solving the problems of the need for skilled labour, time-consuming efforts, labour wages etc. Marker-assisted selection (MAS) has been proven prominent in this cash crop owing to the accessibility of molecular markers and genetic linkage maps. Markers have the utmost importance in transferring the GMS allele to the elite genetic background by MAS. Despite the uses and significance of molecular markers for improvement of other important traits, its necessity in GMS is inevitable. This is because of the practical difficulty that, the screening of sterile plants is only possible during the reproductive stage of the crop. The molecular markers for GMS help in screening of plants in the seedling stage itself and in decreasing the cost and resources of hybrid seed production. This study aims to investigate the genetic basis of male sterility in cotton and develop reliable markers for its identification.

**Table 1** List of male sterility genes identified in cotton

Gene	Species	Identified By
<i>ms</i> <sub>1</sub>	<i>G. hirsutum</i>	Justus and Leinweber, 1960
<i>ms</i> <sub>2</sub>	<i>G. hirsutum</i>	Richmond and Kohel, 1961
<i>ms</i> <sub>3</sub>	<i>G. hirsutum</i>	Justus et al., 1963
<i>MS</i> <sub>4</sub>	<i>G. hirsutum</i>	Allison and Fisher, 1964
<i>ms5ms6</i>	<i>G. hirsutum</i>	Weaver, 1968
<i>MS</i> <sub>7</sub>	<i>G. hirsutum</i>	Weaver and Ashley, 1971
<i>ms8ms9</i>	<i>G. hirsutum</i>	Rhyne, 1971
<i>MS</i> <sub>10</sub>	<i>G. hirsutum</i>	Bowman and Weaver, 1979
<i>MS</i> <sub>11</sub>	<i>G. barbadense</i>	Turcotte and Feaster, 1979
<i>MS</i> <sub>12</sub>	<i>G. barbadense</i>	Turcotte and Feaster, 1985
<i>ms</i> <sub>13</sub>	<i>G. barbadense</i>	Percy and Turcotte, 1991
<i>ms</i> <sub>14</sub> (Dong A)	<i>G. hirsutum</i>	Tianzhen et al., 1994
<i>ms</i> <sub>15</sub> (Lang A)	<i>G. hirsutum</i>	Tianzhen et al., 1994
<i>ms</i> <sub>16</sub> (81 A)	<i>G. hirsutum</i>	Tianzhen et al., 1994
<i>ar.ms</i>	<i>G. arboreum</i>	Meshram et al., 1997
<i>ams</i> <sub>1</sub>	<i>G. arboreum</i>	Singh and Kumar, 1993

Source Technical Bulletin no: 24 from CICR Report - Male Sterility in Cotton

## Types of male sterility in cotton

### Genetic male sterility (GMS)

One of the best tools for accelerating hybrid breeding is GMS. It is the term used to describe pollen sterility resulting from nuclear genes. Environmental factors such as temperature (TGMS), photoperiod (PGMS), and humidity (HGMS) can have an impact on male gametogenesis in environment-sensitive GMS (EGMS) [14, 15]. In particular, intentional suppression of autogamy and subsequent promotion of allogamy is done through the transmission of advantageous male sterility genes. Thus, GMS has served the purpose of harnessing the yield linked with hybrid vigour [14, 16]. Numerous genes that regulate gene transcription, protein metabolism, and other physiological processes such as anther dehiscence and pollen germination, have been identified by cytological and biochemical investigations of several GMS mutants [17].

### Cytoplasmic genetic male sterility (CGMS)

The combination of sterile cytoplasm and nuclear gene(s) conditioning sterility results in CGMS. In contrast to CMS, this kind of male sterility allows for the restoration of fertility. The R gene (s) located in the nucleus is responsible for restoring fertility. Therefore, the fertility or sterility of such plants is determined by the interaction of both nuclear genes and cytoplasmic factors. The primary bottleneck of CGMS is the laborious process of incorporating R genes into cultivated species or genotypes.

When alien cytoplasm from diploid wild species is introduced into cultivated tetraploid cotton, CMS ensues. Cotton contains the comparable independent restorer genes *Rf1* and *Rf2*. Gametophytic *Rf2*, which is from the D8 genome, is designated as the D8 restorer gene. Sporophytic *Rf1*, which is from the D2–2 genome, is designated the D2 restorer gene. The two restorer gene loci are closely linked, with a genetic distance of 0.93 cM on the same chromosome [20].

Male sterility could be produced by interaction of three diploid species namely, *G. harknessii*, *G. arboreum*, and *G. anomalum*, with the *G. hirsutum* nuclear genes. However, the stability of cytoplasm of *G. arboreum* and *G. harknessii* are affected by heat stress. The *G. harknessii* cytoplasm and its interaction with *G. hirsutum* genome produces reliable and stable CMS in cotton across many environments. But this also has some linkage drag causing certain unfavourable effects on micronaire value, ginning outturn and susceptibility to bacterial blight [18].

### Chemical hybrid agent (CHA) / male gametocides

The labour-intensive nature of emasculation, currently limits the production of hybrid cotton. It impelled scientists and cotton breeders to employ innovative techniques like chemical hybrid agents (CHA), which have been already shown to be effective in crops such as wheat, rape and rice [19]. The effects of most common CHAs like ethrel and benzotriazole were studied in cotton. It was resulted in a range of 96.8 to 100% pollen sterility by both the agents. Nevertheless, a substantial decline in yield characteristics was associated with it. On the other hand, plants sprayed with lower ethrel concentrations could not exhibit a discernible decrease in yield. Thus, it is conceivable to use ethrel as a hybridizing agent with *G. hirsutum*. [20].

Furthermore, glyphosate, a significant herbicide, was considered a possible candidate for CHA after it was found to induce male sterility in cotton. [21]. The sterile phenotypes, that resulted in glyphosate application, were having the following typical characteristics: (1) shorter and fused filaments; (2) greater interspace between androecium and stigma; (3) indehiscence of anther; (4) tapetum degradation; (5) enhanced expression of *G6-EPSPS* (roughly eight times, in pistil) and (6) increase of ascorbate peroxidase (APX) activity in stamens, but decrease in other organs. Compared to manual emasculation, it is reliable in inducing male sterility because of its ability to cause complete pollen abortion and lack of negative effects on  $F_1$  yield traits and fibre quality [22]. However, the reproductive portions were observed to suffer significant harm from glyphosate. It has been reported to cause aberrant flowers, along with non-dehiscent anthers with less viable pollen grains having uneven shapes [23].

### Transgenic male sterility

Biotechnological approaches have been widely employed to develop transgenic plants in several crops, cotton (*G. hirsutum* L.), soybean (*Glycine max* (L.) Merr.), canola (*Brassica napus* L. and *B. rapa* L.) and maize (*Zea mays* L.), for exploitation in commercial scale. Agrobacterium-mediated genetic transformation and particle bombardment have been used to develop transgenic male sterility in crops like citrus, wheat, soybean, tobacco, rapeseed, rice, sesamum and mustard. Transgenic male sterility is the term used to describe male sterility caused by genetic engineering. The 'A' line incorporates the 'barnase' gene, which causes male infertility. Another gene, *barstar* can be utilized to restore fertility since it inhibits the activity of the male sterility gene *barnase*. It has been reported that up to 50% of the transgenic plants exhibit stable male sterility, and in a few instances, around 90% of transgenics exhibited stable male sterility in

few crops like mustard, rapeseed, and tobacco, but the same is not yet available in cotton. Therefore, in the near future, this approach can be used to produce commercial hybrid cotton [24].

### GMS - atop the CGMS

The drawback of GMS is that, in theory, heterozygous pollinators will result in segregation as 1:1 male sterile and fertile plants in the progenies. Accordingly, it is sometimes not economically feasible to score and eliminate the fertile offsprings [25]. Despite that, in comparison, the GMS line is efficient and inexpensive to a greater extent in commercial hybrid cotton seed production. Practically, since every commercial cultivar can act as its own restorer line in GMS, it can promote random mating to generate hybrids, in contrast to CMS. One among the breeding advantages of GMS, contrasted with CMS, is the effortless transfer of the MS trait to an array of genetic backgrounds. It is achieved by consecutive backcrossing and inheritance with complete penetrance in subsequent generations [26].

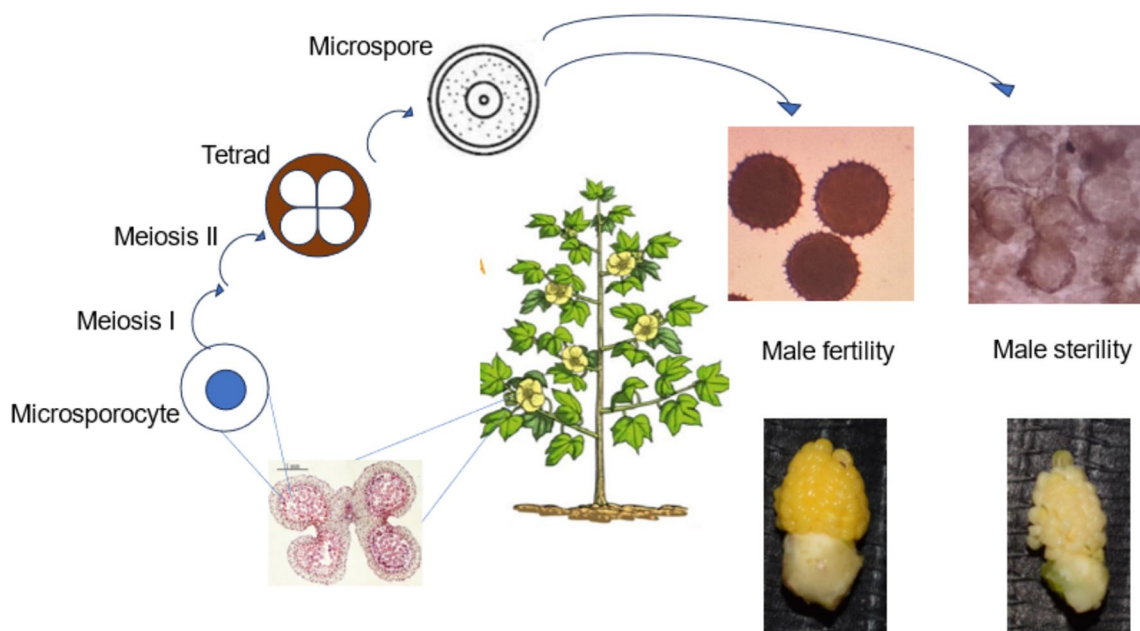
There also exists evidence regarding the undesirable consequences of the sterile cytoplasm in the case of CGMS. The yield of hybrid cotton is negatively impacted by sterile cytoplasm, which was evident in the study of [27]. The yield reduction of the hybrids produced by employing restorers has been reported to be 4.76 to 15.63%, which could be due to the sterile cytoplasm from *G. harknessii*. Nonetheless, no adverse impact was detected regarding ginning out turn and further characteristics of fibre quality. Even though, CMS-based hybrids showed an enhanced span length, the hybrid

combinations showed the largest reduction in micronaire value, up to 30%. In a similar vein, CMS-based hybrids showed an increase in tenacity value, which was mirrored in the strength/length ratio. To identify the right hybrids with higher yield potential and better fibre quality utilizing CMS system, a larger number of cross combinations with suitable restorer and stable CMS lines are employed. In this manner, the negative yield-reducing effect of sterile cytoplasm could be mitigated [27].

### Genesis of MS

Male sterility arises due to varied reasons that differ on the basis of genetic background and type. Numerous loci regulate a series of essential steps involved in microsporogenesis genetically, and a loss-of-function mutation in any one of them could lead to the generation of underdeveloped microspores or non-functioning pollen grains (Fig. 1) and thus, male sterility [28]. The genes responsible for male sterility have been annotated in various species and are still not yet discovered in many species. As far as cotton is concerned, the genes and molecular mechanism of GMS and CMS have been exploited better enough at the transcriptional, biochemical and epigenetic levels. A majority of the research were executed on cytoplasmic and recessive genic male sterility, whereas only a few on dominant genic male sterility, particularly in cotton. This is owing to the unavailability of such mutants [29].

On grounds of the apprehension in the complex mechanism of male sterility and its responsible genes in the model crops, the germplasms were exploited to discover *ms*-related



**Fig. 1** Microsporogenesis in cotton

genes in other crops [30]. The genes, *ms5* and *ms6*, were hypothesized to have originated either from *G. tomentosum* or from the progeny of an interspecific hybrid with *G. hirsutum* [26]. While the precise molecular and biochemical reasons for male sterility produced by *ms5ms5* and *ms6ms6* remain ambiguous, research on similar species may be able to identify the mechanisms that account for pollen development failure in cotton. Four scenarios were identified as the root causes of male gamete development failure in Arabidopsis: Absence of tryphine, the substance that coats pollen; non-dehiscent anthers; non-functioning stamen that results in malformed anthers; and failure in microsporogenesis [31]. Each of the four cases could be responsible for male sterility in cotton. In view of the above fact, male sterility in cotton is ascribable to obstruction in the post-meiotic phase in GMS and abnormalities in the pre-meiotic phase in CGMS systems [32] (Fig. 2).

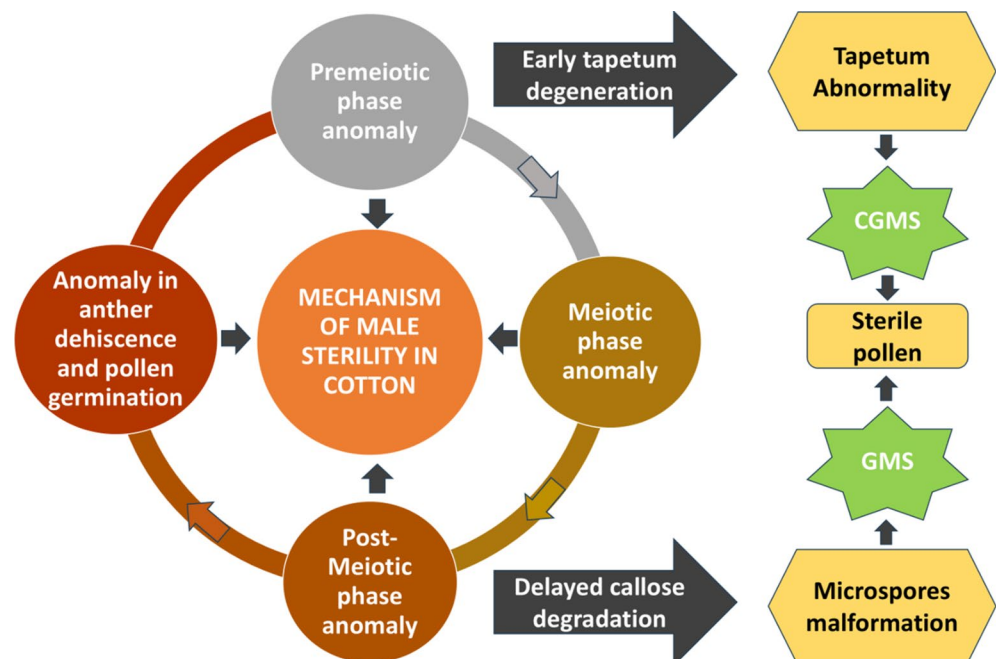
The phenomenon of GMS is a consequence of loss of function in the nuclear genes, accountable for male reproductive development. The microspores were vacuolated and nuclei were convoluted resulting in shrinkage of cytoplasm, and finally, disintegration of deformed developing microspores, forming the mechanism of GMS [32] (Fig. 2). Genetic studies of GMS in cotton revealed that non-dehiscent anthers and unviable pollens are the common forms of occurrence, resulting in failure of reproductive development. Furthermore, the metabolism of sugars has a significant role in the male reproduction of flowering plants. Any shortcomings in this metabolic pathway in the time of male reproductive development results in GMS [33]. According to physiological and biochemical studies carried out, male sterile plants have lower starch levels during the entire

period of pollen development and lower soluble sugar levels in young stamens [34].

In Photosensitive Genetic Male Sterility (PGMS) system, day length and photoperiod modulate the pollen fertility due to premature tapetum degeneration [35]. In expression profiling studies of sterile mutant and fertile wildtype (WT) anthers, under long-day conditions, induction of ubiquitin-proteasome system mutant uninucleate pollen (UNP) were observed to cause degradation of pollen proteins, that formed the basis of male sterility [36]. Both the reproductive and vegetative sections of plants are impacted by high temperature, although the former is more sensitive [37]. According to [38], the male reproductive system is more susceptible to High Temperature (HT) stress than the female reproductive system. As the anther development is exceptionally organized in comparison to other male reproductive components, it is more sensitive to HT stress [39]. Numerous genes, whose expression and regulation were impacted by HT, are involved in the intricate process of anther and pollen growth [38]. Flower developmental stages, the structure of anther and the cellular morphology of microspore are impacted, even by a single day of high-temperature stress [40].

Being a maternally inherited trait, CMS eventuate due to energy deficiency in mitochondria, CMS protein cytotoxicity and programmed cell death (PCD) of premature tapetum [35]. The transfer of diploid genome (A2) of *G. arboreum* to *G. anomalum* cytoplasm, having B1 diploid genome, created the first cytoplasmic male sterility. When *G. arboreum* pollen was used to pollinate the CMS inducer, male sterile plants were created and when *G. anomalum* pollen was used to pollinate the sterile lines, fertility was regained [41,

**Fig. 2** Mechanism of male sterility in cotton



42]. As these CMS lines had undesirable traits and the resultant male sterility was incomplete and unstable, a different native CMS system known as CMS-D2 was developed by transferring the commercial cotton genome (*G. hirsutum*) to *G. harknessii* cytoplasm [43, 44]. Tapetum plays a crucial role in case of pollen nourishment, development and maturation. However, premature degeneration of the tapetum in the later stages of pollen mother cell arrested the normal course of meiosis in sterile lines. These pre-meiotic abnormalities seemed to be the root cause of CMS in *Gossypium* sp [34] (Fig. 2).

A restorative gene,  $Rf_1$ , from *G. harknessii* was transferred to *G. hirsutum* genome to form the CMS-D2 restorer system [44]. Another restorer,  $Rf_2$  was also discovered in genome D8 of *G. trilobum* [45] and its cytoplasm was utilized to build CMS-D8 system [46]. A nonallelic relationship and tight linkage were discovered between  $Rf_1$  (sporophytically functioning) and  $Rf_2$  (gametophytically functioning) in chromosome LGD08 by [47, 48]. The fertility of both CMS-D2 and CMS-D8 systems could be restored by  $Rf_1$ , while  $Rf_2$  could restore the fertility of CMS-D8 sterile lines only [49].

In CGMS, the flower traits were consequentially different, whereas all other morphological traits were akin in fertile and sterile counterparts. The early stages of microspore development were indistinguishable in fertile and sterile plants. Some of the floral traits like the size of ovary, style, staminal column, anther number and anther filament length were decreased in CGMS lines, whereas their size differences were obscure in GMS lines [32]. Despite the fact that CMS produces nearly 100% sterility, a wider use of CMS has not been practicable because of the cytoplasmic effect on CMS stability and the presence of potential yield drag and limited genetic combinations of CMS and restorer lines [50].

## Gene expression in MS

It is essential to comprehend the complex mechanism, expression, regulation, and activity of several genes as well as the quantitative nature of male sterility. [28]. In prior studies, an array-based technique was implemented to compare differentially expressed genes. Presently, RNA-seq, a next-generation sequencing method, is prominently used to reveal the transcriptome between the sterile line and its maintainer. It is because the microarray-based chip is unable to find unique transcripts implicated in targeted pathways. Compared to micro-array, RNA-seq offers expression profiling results with high accuracy and minimum background signal at a faster and less expensive rate. Expression level detection in distinct allelic variants forms its prominent application [51].

The differences in gene expression between anthers of fertile and sterile lines in several species were explored by proteomic studies, that help in decoding the MS mechanism. Numerous proteins and their corresponding transcripts concerned with cytoskeleton formation, energy conversion, stress tolerance and defence mechanisms, have been reported in angiosperm mature pollen.

Down-regulation of proteasome and protein 5B, both of which effectuate in degeneration of tapetum in 7B-1 male sterile mutants of tomato have been reported. They also had high-level of cystatins, which are endogenous proteolytic regulators of programmed cell death (PCD), besides seed development and germination [52]. In addition, another proteomic analysis disclosed that proteins linked to photosynthesis, flavonoid synthesis, energy and carbohydrate metabolism have adequate contributory roles in pollen development. Down-regulation of these proteins caused CMS in *Brassica napus* [53]. In upland cotton, two enzymes linked to photosynthesis and carbohydrate metabolism, viz., glutaminyl-tRNA synthetase and cytosolic ascorbate peroxidase 1, were found to be low in the mutant anthers of the GMS line implicating their role in development of pollen [54].

Studies based on RNA-seq unlocked the exact mechanism regarding CMS in cotton. Signalling pathway and gene enrichment analysis indicate that abnormal regulation of tubulin, actin and myosin-related genes may be the cause of the early developmental defects in cytokinesis of pollen. It was also found that some fertility restorer genes, such as the PPR family, were downregulated. Enhanced expression of NAC, HSP and WRKY and down-regulation of transcription factors like MYB, bHLH and TCP is associated with pollen development and circadian clock. Furthermore, aberrant pollen development and definitive male sterility could be caused by the down-regulation of genes taking part in cell wall development and energy metabolism in pollen maturation [55]. RNA sequencing could also be used to identify candidate genes for fertility restoration by using bulked segregant analysis (BSA) in  $F_2$  individuals of CMS and restorer lines. According to one such report by [56] the genes namely *GH\_D05G3183*, *GH\_D05G3384*, and *GH\_D05G3490* identified in the restorer line R186, restores fertility in the CMS line, 2074 A.

By using light and electron microscopy, it was possible to determine the primary causes of male sterility phenotype in PGMS mutants with uneven exine, lack of tryphine, and immature anther cuticle. It may be a consequence of the down-regulation of genes, ABA and MYB transcription factors, involved in the assembly of tryphine and anther cuticle. The functional expression of these genes may be related to the conversion of fertility in various photoperiods [57].

Other male reproduction genes include four homoeologous pairs of *GhFAD2* gene family (*GhFAD2-1* to *GhFAD2-4*), among which *GhFAD2-3* play a major role in anther development in cotton. When its RNA is silenced and expression is inhibited, anther developmental stages after meiosis are affected until the maturation of pollen, resulting in male sterility [58]. Pollen exine is one of the important structures in pollen grains, with role in its viability. The gene *GhTKPR1\_8* has been identified as a crucial gene in the synthesis of sporopollenin in exine. It plays an integral role in catalyzing the reduction of tetraketone carbonyl to hydroxylated  $\alpha$ -pyrone. [59]. According to the authors, expression of *GhTKPR1\_8* is localized in the endoplasmic reticulum and anther developmental tetrad stage. Dehiscence of anther and viability of pollen are affected by the knockdown of the gene. Apart from this, some flavonoids play a role in pollen fertility and plant growth. The contribution of 4-Coumarate CoA ligase (4CL) and flavonoids in cotton anther remains understudied. A homologous gene pair, *Gh4CL20A* and *Gh4CL20*, have been reported to express in petals as well as stamens. Mutants for the genes exhibited white petals, aborted pollen in reduced number and indehiscent anthers. This male sterility was caused by tapetum degradation at the tetrad stage and abnormal development of pollen at the maturation stage, which was revealed in histological studies [60].

Since post-meiotic abnormalities are the root cause of male sterility in cotton, microspore development and pollen maturation are the crucial phases to study for male sterility. The ill effects of environmental stresses during the above sensitive stages are overcome by the anther cuticle and pollen exine. Glycerol-3-phosphate acyltransferase (GPAT) is involved in cuticle formation and hence critical for fertility studies. *GhGPAT12/25* (a paralogs pair on A12/D12 sub-chromosome of cotton) is engaged in the formation of cuticle, development of exine and degradation of tapetum. The mutant developed by knocking down both the genes, exhibited all the symptoms of male sterility, viz. swollen tapetum, abnormally developed anther cuticle and irregularly shaped inviable microspores with exine defect. It was also reported that *GhMYB80s* enhance the expression of these genes [61].

### Development of molecular markers for GMS in cotton

It is vital to create hybrids by functional male sterility, to increase the financial worth of commercial hybrid seed production. For rapid identification and elimination of individuals in a population, the use of molecular markers becomes inevitable, especially in GMS. The marker development in cotton for the MS trait improves the efficiency of hybrid

production by decreasing the time and increasing the accuracy in selection.

The map placements of GMS genes, *ms5* and *ms6*, were determined in backcross populations by [10], by utilizing higher-density SSR markers. Molecular markers for each gene, indicating dominant and recessive alleles, were reported by the authors by testing these patterns in male sterile (*ms5ms5ms6ms6*) and male fertile (*Ms5ms5Ms6ms6*) progenies. The markers that were linked to the genes were compared to other molecular markers to determine their genetic distance. An alternative strategy for the above is to exploit the populations, that are selective for only one of the two alleles, with the second gene fixed to recessive homozygote [26].

It was anticipated that *ms5* and *ms6* genes are homeologs and perhaps duplicated during polyploidization. Further genomic polymorphisms from the second homoeologous chromosome in flanking regions can be employed as flanking markers, giving the two haplotypes a higher rate of precision in phenotypic prediction. With 98% accuracy, two haplotypes made up of two SNPs each linked to *ms5* and *ms6*, were found to explain male sterility and fertility [26].

In order to uncover chromosomal variations at *ms5* and *ms6* loci, between GMS and wild-type fertile inbred lines, sequence analysis was performed. Sequence polymorphism showed an association of *ms5* and *ms6* on A12 and D12, respectively. Four SNPs, MOGH583971, MOGH582973, MOGH211275, MOGH493571, that target the *ms5* and *ms6* genic regions, made up of a haplotype marker-set was developed by [26] and it was validated for association with the GMS trait in cotton. Over 99% of the GMS trait phenotype could be predicted with this haplotype SNP collection [26]. Using this marker set, molecular breeding at a high-throughput level could be attained to select sterile individuals and increase the reliability of hybrid production. It also encourages introducing male-sterility genes into elite lines or cultivars quickly and efficiently, through marker-assisted backcrossing. A rapid approach to find GMS gene markers in the interspecific biparental population using bulk segregant analysis (BSA) has also been reported in cotton. Recently, the *Ms5* and *Ms6* genes have also been discovered to be associated with four SNP markers. The *Ms5* gene associated with markers i23493*Gh* and i46470*Gh* and *Ms6* gene associated with i08605*Gh* and i08573*Gh* were reported [62]. These markers would be helpful for rapid screening of numerous individuals and facilitate precise marker-assisted selection (MAS), contributing to accelerated hybrid cotton seed production (Table 2).

**Table 2** List of molecular markers developed for MS in cotton

Type of MS system	Gene name	Markers identified	Remarks	Source
CMS-D2 system	$Rf_1$	OPV15-300	Linked with a recombination value of 13.0+2.57%	[63]
	$Rf_1$	UBC6592	Converted as RFLP marker	[64]
		2 RAPDs 3 SSR	Among the five markers, three were co-dominantly inherited.	[65]
	$Rf_1$	1 RAPD	Converted as sequence tagged site (STS) marker	[66]
		4 RAPDs	Converted as STS markers with specific primer pairs	[67]
		5 SSRs	Mapped the $Rf_1$ locus with 15 markers within a genetic distance of 0.9 Cm	[47]
		2 STSs		[47]
CMS-D8 system,	$Rf_2$	2 RAPDs	One among the two converted as a STS marker	[66]
	$Rf_2$	3 RAPDs 2 AFLPs 1 SSRs	One of the RAPDs converted to a CAPS marker	[48]
CMS-D2-2 (Sporophytic system)	$Rf_1$	InDels	Chromosome_D05 carries $Rf_1$ , in which majority of the InDels are distributed	[68]
CGMS	$Rf$	3 STS (Y1107, UBC 147 and UBC 607)	Twenty four molecular markers known to be associated with fertility restorer (10 RAPD, 7 SSR, 6 STS, and 1 TRAP) were studied	[69]
GMS	$ms_5$	2 SNPs (MOGH583971, MOGH582973)	C/T polymorphism Markers are located on PHD-zinc-finger-like domain protein on chromosome A12	[26]
GMS	$ms_6$	2 SNPs (MOGH211275, MOGH493571)	C/G, A/G polymorphisms. Markers are located on $AP2$ gene on chromosome D12	[26]
GMS	$ms_{15}$	2 SSRs (NAU2176 and NAU1278),	Genetic distance of 0.8 and 1.9 cM respectively flanking the gene.	[10]
	$ms_5$	2 SSRs (NAU3561, NAU2176 and NAU2096)	Genetic distances of 1.4, 1.8 and 1.8 cM, respectively	[10]
GMS	$ms_6$	2 SSRs (BNL1227 and NAU460)	Genetic distance of 1.4 and 1.7 cM respectively.	[10]
	$ms$	RAPD – Diploid GMS lines (OPB04, OPZ14) Tetraploid GMS lines (OPB04)	The primers OPZ14 and OPB04 showed 25 per cent polymorphism in all pairs of near isogenic diploid GMS lines	[70]

## Functional marker development

The selection of morphological features of interest in precision breeding is an unambiguous method of plant breeding. It is accomplished accurately only by locating a marker region, that is derived directly from the trait-controlling genetic sequences. The above-stated marker regions are designated as functional markers (FMs), or DNA markers, originating from sequence motifs that are functionally characterized [71]. Random DNA markers (RDMs) form the majority of the available marker types like RAPD, RFLP, AFLP, SSR, and SNP, which can be lost during recombination. On the other hand, FMs are impervious to recombination events and evolutionary changes and hence are highly advantageous.

Consequently, compared to genic molecular markers (GMMs) and Random DNA markers (RDMs), SNPs as FMs are more helpful in plant breeding. Even though GMMs might prevail functionally within a desirable gene, they might not be associated with the desired phenotypic character, resulting in false selection in MAS. Wherefore, RDMs

have been referred to as “non-perfect markers,” whereas FMs have been termed as “perfect markers”, “precision markers” or “diagnostic markers” [72].

Finding a gene of interest that influences a phenotypic feature, determining its nucleotide sequence, and characterizing its function are the initial steps in the construction of FMs. Many methods, including QTL mapping, expression profiling, positional cloning, and transposon tagging, can be utilised to identify the genes [73]. To functionally characterize a potential gene, plants must be transformed for over-expression or knockdown study [74]. The investigation of allelic variation within identified genes is the second stage in the development of FMs. Then, in order to determine which polymorphism causes variance of the phenotypic trait, allele sequencing between genotypes must be carried out. The allelic or genic sequence, associated with the desirable trait, must be understood critically in order to develop FMs [74].



## Need of the hour – FMs

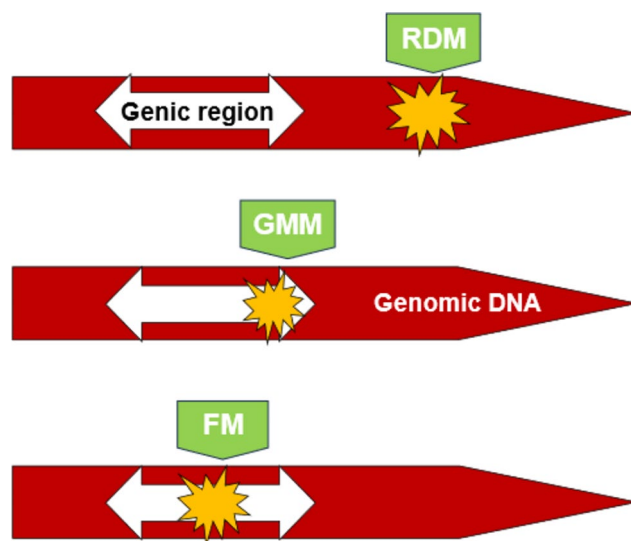
Random DNA markers do not always result in predicted selection for desirable traits as they are produced from the polymorphic regions surrounding the gene of interest. Nevertheless, the selection by FMs can now solve the issues related to the usage of RDMs, by virtue of 100% predictivity of the appropriate phenotype [75].

Owing to the fact that FMs are found inside the target genes (Fig. 3), they are well connected to the morphological characteristics, that makes it highly effective and reliable. In numerous genetic backgrounds, functional sequence motifs (FSMs) fix the alleles without further calibration once genetic effects have been attributed to them. This marker application is useful for selecting parental materials to create segregating populations and for selecting advanced breeding lines. They are resistant to recombination, which acts as the major reason to accurately and quickly characterize germplasm for allele diversity [73]. They also make it possible to lessen the probability of information loss and incorrect selection in employing MAS.

Furthermore, QTL validation in MAS is required when exploited in diverse genetic backgrounds, whereas, FMs do not need validation. Breeders can detect unusual recombinants by using FMs to help select remarkable phenotypic features in a large population.

## Unearthing the FM discovery in plant breeding

The FM development is thus made possible by finding SNPs and indels associated with a variety of economically significant traits. Large-scale genomic impacts caused by



**Fig. 3** Pictorial representation of marker position. (FMs: Functional Markers; RDMs: Random DNA Markers; GMMs: Genic Molecular Markers)

alleles have the potential to eliminate phenotypic variation through natural selection. SNP-derived FMs are advantageous compared to indel-derived markers, because, FMs are extensively dispersed across the genome. They have been generated for an array of agronomic, qualitative and stress resistance traits, to exploit MAS, MARS, MABB and GS methods.

However, for the male sterility gene, the FMs discovered are not ample in many crop species (Table 3) and unavailable in the case of cotton. Despite that, functional markers were developed in cotton for cellulose synthase genes, that play a crucial role in fibre development and architecture of plant type. Identification of SNPs was done *in silico* via Primer-BLAST gene-specific marker design. As a result, 10 SNP markers and 82 gene-specific markers were created in total by [76]. These markers were capable of recognizing genotypes from several species [76].

## Concluding remarks and future perspectives

Research on male sterility has a wider scope as the mechanisms are complicated in each of the crop species. In cotton, the male sterile and fertile anthers undergo a similar microsporogenesis process. But, shortly after tetrad formation, the sterile anthers start to differ in pollen development by resisting the release of pollen from the callose wall. During the developmental process, the pollen wall collapses in sterile anthers. Consequently, the malformed microspores disintegrate, resulting in GMS. It has been discovered that the cause of sterility in case of CMS is tapetum abnormalities. The meiocytes starve and their normal path of development is interrupted when early degeneration of the tapetum occurs in the sterile lines. As such, it becomes ever more crucial to explore the genes underlying these processes and to develop markers for the rapid production of hybrid seeds.

Despite availability of many random markers, FMs have been widely employed in many crops due to their accuracy in selection. As FMs are mostly produced by coding DNA within genes, it is possible to use these markers to directly choose a phenotypic trait. FMs can also be transferred across species and hence can be applied to those for which there are insufficient genetic resources. They are also used in phylogenetic investigations, QTL mapping, diversity analysis, germplasm screening and gene discovery. Furthermore, the creation of affordable FMs is an added advantage for their successful application in precision breeding. This versatile tool needs to be exploited for complex traits like male sterility, for the effortless hybrid development. The future outlook is the creation of allele-specific FMs, which will further enhance the effectiveness of direct selection. In addition, the full potential of next-generation breeding methods

**Table 3** List of functional markers developed for candidate genes for male sterility in other crops

Trait	Gene	Location in chromosome	Primer Name/Sequence	Crop	References
WA-CMS system	<i>WA352</i>	Mitochondrial chromosome	RMS-CMSWA352 RMS-MNTWA352 RMS-3-WA352	Rice	[77]
Male sterility	<i>CDMs399-3</i>	7 C	F-TCCCTTTCACATCGT CCACA R-TGCAGCCCAGAACA GTGATA	Cabbage	[78]
Early and late flowering	<i>BoFLC1.C9</i>	Unmapped	F-GGAAAGCAACATGG TGATGA R-CATGGTGTGAACCA GAGTCC	Cabbage	[79]
Male sterility	<i>ms3</i>	5	F-GGTACTTTGACCCTC ATAATTGG R-TTGTTTGTGGTGTAC GTGCT	Capsicum	[80]
Sex identification	<i>MYB35</i>	5	F-TTGCTTGGCGGATCA TATTATG R-TTGCTTGGCGATGC CCTTTTG	Asparagus	[81]
Genic Male Sterility	<i>Bnms3</i>	12	RCP170	<i>Brassica napus</i>	[82]
Photoperiod response	<i>Phd-H1</i>	2	F-ACGCCTCCCACTAC ACTG R-CACTGGTGGTAGCT GAGATT	Wheat	[83]
Photoperiod response	<i>Phd-H1</i>	7	F-CCTCTTCGCTATTAC GCCAG R-GCCCTTCCCAACAG TTGCG	Barley	[84]
Photoperiod-thermo-sensitive genic male (PGMS and TGMS) sterility	<i>pms3 (p/tms12-1)</i>	12	F-GAATGCCATCTAAA CACT R-ATTTTACTCTTGATG GATGGTC	Rice	[85]
Controlling flowering time	MADS-box, Constants and flowering locus T/Terminal Flower1		F-ATGCACCTAGCCCA AGTGAC R-TGTTTGCATTCATGG CGTGT & R-ATCTGTTGTGCCGG GAATGT F-AACCGAAATGCAAA ACAGGTGA	Pea, soybean, and burclover	[86]

such as GWAS and GS could be achieved through the development and application of functional markers. In summary, considering the critical role of GMS and CGMS in exploitation of heterosis in cotton, research focus on development of functional markers for male sterility needs intensification.

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