

Chapter

Molecular Cloning of Genic Male-Sterility Genes and Their Applications for Plant Heterosis via Biotechnology-based Male-sterility Systems

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Abstract

In this chapter, we summarize the strategies about molecular cloning and functional confirmation of plant genic male-sterility (GMS) genes and their applications for hybrid breeding and seed production via biotechnology-based male-sterility (BMS) systems in crop plants. The main content includes four sections: (1) GMS gene cloning strategies, including forward genetic approaches (e.g., map-based cloning, T-DNA or transposon tagging, and MutMap method) and reverse genetic approaches (e.g., homology-based cloning, anther-specific expression gene screening, and other reverse genetic methods); (2) functional confirmation methods of GMS genes, including transgenic complementation, targeted mutagenesis, allelic mutant test and sequencing, anther spatiotemporal expression analysis, and cytological observation; (3) application value assessment of GMS genes and mutants, such as genetic stability analysis of male sterility controlled by GMS genes under different genetic backgrounds and multiple environments, and genetic effects driven by GMS genes on plant heterosis and analysis of potential linkage with bad traits; (4) development and application of BMS systems based on GMS genes and/or their mutants, including transgenic construct-driven non-transgenic seed systems (e.g., seed production technology (SPT) and multi-control sterility (MCS)), and transgenic male-sterility systems (e.g., roundup hybridization systems (RHS1 and RHS2) and Barnase/Barstar system). Finally, we summarize and provide our perspectives on the studies of GMS genes and development of cost-effective and environment-friendly BMS systems in crop plants.

Keywords: gene cloning, genic male sterility (GMS), biotechnology-based male sterility (BMS), heterosis application, genetically modified plants

1. Introduction

The demand for food supply is increasing exponentially with the human population continuously growing. According to a report, the world population is predicted to increase by 34% by 2050 [1], whereas the area of land for agriculture practices is decreasing consistently over the last few decades because of urbanization and land degradation. Therefore, it is necessary to increase the food production per unit area as cultivated lands are limited [2].

Hybrid vigor (or heterosis) is the superior performance of the heterozygous hybrid progeny over both homozygous parents. Most crops show hybrid vigor, such as maize, rice, wheat, sorghum, rapeseed, and sunflower, but commercial production of hybrids is only feasible if a reliable and cost-effective pollination control system is available. In cereal crops, maize is a monoecious and diclinous species, which makes it very successful in heterosis utilization with relatively feasible emasculation. The emasculation, namely, the physical removal of the male floral structure, usually includes manual and mechanical detasseling. However, emasculation is not only time-consuming, labor-intensive, and expensive but also detrimental to plant growth, thus reducing the yield of hybrid seed. At the same time, it is unfeasible for the crops that have small, bisexual flowers, such as rice, wheat, and barley. Therefore, it is an ideal alternative to use male-sterile line for pollination control in these cereal crops [3, 4].

Male sterility (MS) refers to cases in which viable male gametes (i.e., pollen) are not produced, while female gametes are fully fertile. Male sterility can be generated by either cytoplasmic or nuclear genes. Cytoplasmic male sterility (CMS) is caused by mitochondrial genes together with nuclear genes and has been used in commercial hybrid production in crops (such as maize, rice, and oilseed rape), but this method suffers from the poor genetic diversity, increased disease susceptibility, and unreliable restoration of CMS lines [5]. Genic male sterility (GMS) is caused by nuclear genes alone, and the use of GMS can overcome these drawbacks, but it is difficult to obtain a pure and large-scale increase of male-sterile female lines through self-pollination. Fortunately, with the rapid development of GMS gene isolation methods, plant-transformation techniques, and other new biotechnologies, many efforts have been made to identify and utilize GMS genes and ultimately develop more efficient biotechnology-based male sterility (BMS) systems in crop plants [3, 4, 6].

In this chapter, we systemically described the molecular cloning methods, functional confirmation approaches, application value assessment of GMS genes, as well as the strategies and comprehensive evaluation of BMS systems based on elite GMS genes in cereal crops (**Figures 1** and **2**). This will provide a guideline and shed light on GMS gene cloning and application in hybrid seed breeding and production via BMS systems in major cereal crops.

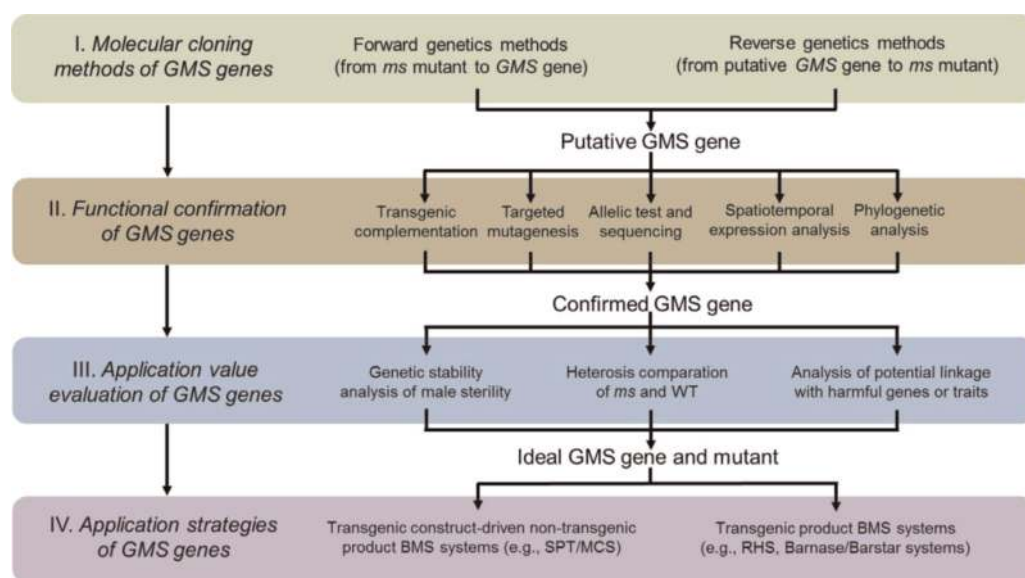


Figure 1. The technology workflow of cloning methods and application strategies of GMS genes in crop plants.

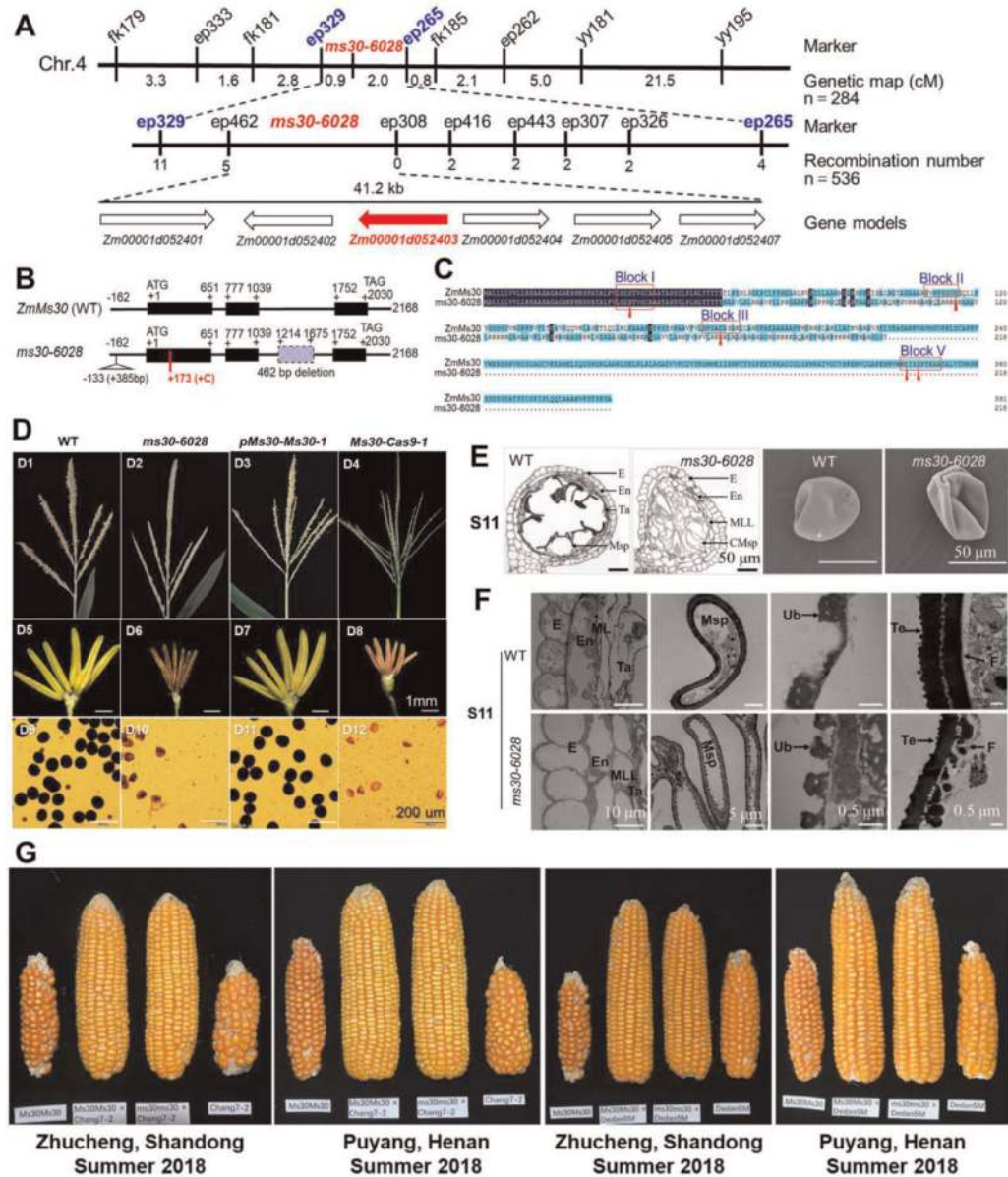


Figure 2. A typical example of maize ZmMs30 gene: from map-based cloning and functional confirmation to application value assessment (adapted from Ref. [18]). (A) Map-based cloning of ZmMs30 gene; (B) gene structure and mutation site comparison between WT and ms30-6028 mutant; (C) predictive protein sequence comparison of ZmMs30 between WT and ms30-6028 mutant; (D) transgenic complementation and CRISPR-Cas9 targeted mutagenesis confirmation of ZmMs30 gene in maize; (E and F) cytological observation of anther and pollen development in WT and ms30-6028 mutant at stage 11 (S11) by using transverse section light microscopy, SEM (E) and TEM (F); (G) heterosis comparison of WT and ms30-6028 mutant by using two representative hybrid combinations with male lines Chang 7-2 and Dedan5M in two locations, respectively.

2. Molecular cloning strategies of GMS genes in crop plants

There are basically two ways to clone GMS genes in crops: forward and reverse genetics. Forward genetic approaches require the cloning of sequences underlying the male-sterile phenotype, such as map-based cloning, T-DNA or transposon tagging, and MutMap method (Figure 3), whereas reverse genetic strategies seek to identify and select mutations in a known sequence, such as homology-based cloning, anther-specific expression gene screening, and other reverse genetic cloning strategies.

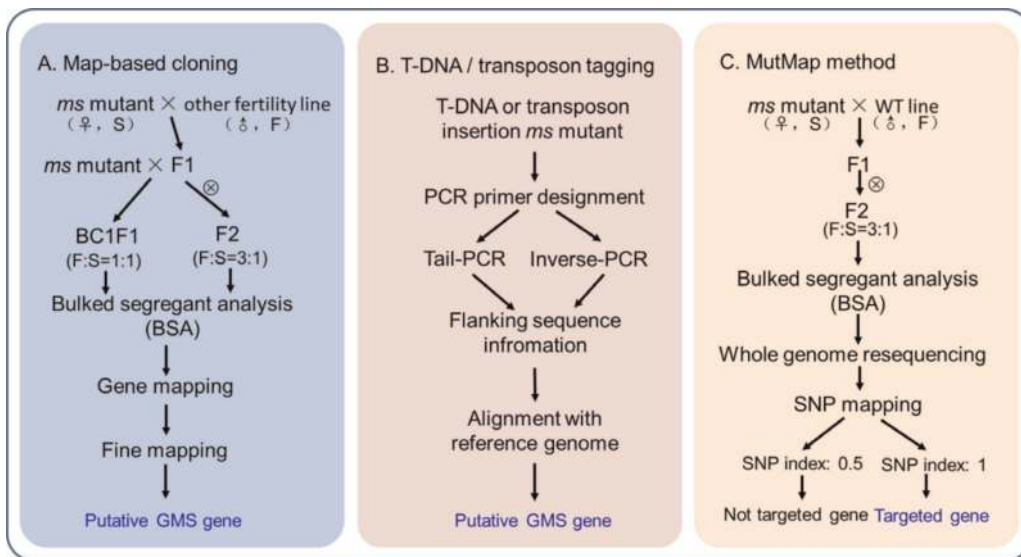


Figure 3.
The forward genetic approaches of GMS gene cloning in crop plants.

2.1 Forward genetic approaches

2.1.1 Map-based cloning

Map-based cloning or positional cloning is a classical forward genetic strategy of GMS gene cloning. Map-based cloning strategy relies on linkage disequilibrium between markers and the gene of interest, that is, as distances between the gene of interest and the analyzed markers decrease, so does the frequency of recombination. In general, the procedure of map-based cloning method includes the following steps (**Figure 3A**). First is the construction of segregating population of F₂ or BC₁F₁ by crossing male-sterile mutant with a distant male-fertile line followed by self-pollination or backcrossing with the male-sterility mutant line. Second is primary mapping of GMS gene based on bulked segregant analysis (BSA) and molecular marker (such as SSR, SNP, and INDELS) linkage analysis using the F₂ or BC₁F₁ segregating populations which have high levels of linkage disequilibrium. Third is fine mapping of GMS gene by developing more polymorphic markers and enlarging the segregating population. Finally, the GMS gene will be narrowed down to a small interval on the targeted chromosome. Under the help of bioinformatic analysis, the putative GMS gene will be identified.

So far, there are at least 38 GMS genes in major cereal crops that have been cloned via map-based cloning strategy, including 20, 15, and 3 GMS genes reported in rice, maize, and wheat, respectively (**Table 1**). As the genome sequence information of more crop plants is available, there will be more GMS genes isolated by map-based cloning strategy in crop plants.

2.1.2 T-DNA or transposon tagging

T-DNA or transposon tagging are efficient and straightforward approaches for GMS gene cloning based on the T-DNA or transposon insertion male-sterile mutants (**Figure 3B**), PCR, and bioinformatic analysis. If the male-sterile mutant comes from a T-DNA or transposon insertion, rapid identification of the GMS gene is at least theoretically possible by locating the sequence tag and analyzing its neighboring sequences by using thermal asymmetric interlaced (TAIL) PCR,

No.	Cloning strategy	GMS genes	Crops	Functional confirmation methods	Application value evaluation	References
1	Map-based cloning	<i>ZmMs7</i>	Maize	1, 3, 4, 5	MCS system	[9]
		<i>ZmMs8</i>	Maize	4	No data	[10]
		<i>ZmMs9</i>	Maize	3, 4	No data	[11]
		<i>ZmMs10/APV1</i>	Maize	3, 4	No data	[12]
		<i>ZmMs22/MSCA1</i>	Maize	1, 3, 4, 5	No data	[13, 14]
		<i>ZmMs23</i>	Maize	3, 4, 5	No data	[15]
		<i>ZmMs26</i>	Maize	1, 2, 3, 4	SPT system	[16, 17]
		<i>ZmMs30</i>	Maize	1, 2, 3, 4	MCS, sterility stability, and heterosis analysis	[18]
		<i>ZmMs32</i>	Maize	3, 4, 5	No data	[19]
		<i>ZmMs33</i>	Maize	1, 2, 3, 4, 5	MCS, MAS	[20, 21]
		<i>Zmms44</i>	Maize	4	Maize SPT-like	[22]
		<i>ZmMs6021</i>	Maize	1, 3, 4, 5	No data	[23]
		<i>IG1</i>	Maize	3, 4	No data	[24]
		<i>MAC1</i>	Maize	2, 3, 4	No data	[25]
		<i>IPE1/ZmMs20</i>	Maize	3, 4	No data	[26, 27]
		<i>MIL1</i>	Rice	1, 4	No data	[28]
		<i>MIL2</i>	Rice	3, 4	No data	[29]
		<i>TIP2/bHLH142</i>	Rice	1, 4, 5	No data	[30, 31]
		<i>TIP3</i>	Rice	1, 2, 3, 4	No data	[32]
		<i>CYP704B2</i>	Rice	1, 4, 5	No data	[33]
		<i>CYP703A3</i>	Rice	4, 5	No data	[34]
		<i>PTC1</i>	Rice	1, 4, 5	No data	[35]
		<i>TDR</i>	Rice	1, 4, 5	No data	[36]
		<i>OsNP1</i>	Rice	1, 4, 5	No data	[37]
		<i>OsGPAT3</i>	Rice	1, 3, 4, 5	No data	[38]
		<i>DPW</i>	Rice	1, 4, 5	No data	[39]
		<i>DPW2</i>	Rice	1, 3, 4	No data	[40]
		<i>OsDEX1</i>	Rice	1, 3, 4, 5	No data	[41]
		<i>CSA</i>	Rice	1, 4	No data	[42]
		<i>OsABCG26</i>	Rice	1, 2, 4, 5	No data	[43]
		<i>OsPKS2</i>	Rice	1, 4, 5	No data	[44]
		<i>EAT1</i>	Rice	1, 3, 4, 5	No data	[45]
<i>PDA1/OsABCG15</i>	Rice	3, 4, 5	No data	[46]		
<i>MTR1</i>	Rice	1, 4	No data	[47]		
<i>OsERS1</i>	Rice	1, 4	No data	[48]		
<i>TaMs1</i>	Wheat	1, 2, 3, 4	Wheat SPT-like	[49]		
<i>TaMs2</i>	Wheat	1, 2, 3, 4	No data	[50, 51]		
<i>TaMs5</i>	Wheat	1, 2, 3, 4	No data	[52]		

No.	Cloning strategy	GMS genes	Crops	Functional confirmation methods [*]	Application value evaluation	References
2	T-DNA tagging	<i>bHLH142</i>	Rice	1, 4, 5	No data	[31]
		<i>OsAPI5</i>	Rice	1, 3, 4	No data	[53]
		<i>OsGT1</i>	Rice	3, 4	No data	[54]
		<i>UDT1</i>	Rice	3, 4, 5	No data	[7]
		<i>RIP1</i>	Rice	2, 3, 4	No data	[55]
		<i>WDA1</i>	Rice	2, 3, 4, 5	No data	[56]
		<i>OsCP1</i>	Rice	4,	No data	[57]
		<i>GSL5</i>	Rice	2, 3, 4, 5	No data	[58]
		<i>DTM1</i>	Rice	3, 4	No data	[59]
		<i>DTC1</i>	Rice	3, 4	No data	[60]
3	Transposon tagging	<i>ZmMs45</i>	Maize	1, 2, 3	STP system	[8, 61, 62]
		<i>OCL4</i>	Maize	2, 3, 4	No data	[63]
		<i>OsGAMYB</i>	Rice	1, 3, 4, 5	No data	[64]
		<i>MSP1</i>	Rice	1, 4, 5	No data	[65]
		<i>CAP1</i>	Rice	1, 4, 5	No data	[66]
4	MutMap cloning	<i>OsLAP6/OsPKS1</i>	Rice	2, 3, 4, 5	No data	[67]
		<i>OsABCG26</i>	Rice	1, 2, 4, 5	No data	[68]
		<i>OsNP1</i>	Rice	1, 2, 3, 4, 5	Rice SPT-like	[69]
		<i>TaMs1</i>	Wheat	1, 2, 3, 4	No data	[70]
5	Homology-based cloning	<i>OsTDF1</i>	Rice	1, 2, 4, 5, 6	No data	[71]
		<i>OsACOS12</i>	Rice	1, 3, 4, 5, 6	No data	[72]
		<i>OsIG1</i>	Rice	2, 3, 4, 5, 6	No data	[24, 73]
		<i>OsCER1</i>	Rice	2, 3, 4, 5, 6	No data	[74]
		<i>OsRAFTIN</i>	Rice	2, 4, 5, 6	No data	[75]
		<i>TaMs45</i>	Wheat	1, 2, 3, 4, 5	No data	[76]
		<i>TaMs26</i>	Wheat	1, 2, 4, 5, 6	No data	[77]
6	Anther-specific expression gene screening	<i>OsC6</i>	Rice	2, 4, 5, 6	No data	[78]
		<i>OsG1</i>	Rice	2, 4, 6	No data	[79]
		<i>OsADF</i>	Rice	2, 4, 6	No data	[80]
		<i>OsUAM3</i>	Rice	2, 4, 6	No data	[81]
		<i>TaRAFTIN</i>	Wheat	2, 4, 5, 6	No data	[75]
7	Other reverse genetic cloning	<i>OsSTRL2</i>	Rice	2, 4, 5, 6	No data	[82]
		<i>OsFTIP7</i>	Rice	2, 3, 4	No data	[83]
		<i>OsTGA10</i>	Rice	2, 3, 4, 5, 6	No data	[84]
		<i>OsAGO2</i>	Rice	2, 3, 4, 6	No data	[85]

^{*}Notes: (1) functional complementation, (2) knockout by using CRISPR-Cas9 or knockdown by using RNAi, (3) allelism test and allelic mutant sequencing, (4) anther-specific expression analysis, (5) phylogenetic analysis and orthologous analysis with known GMS gene, (6) cytological observation

Table 1. Cloning, functional confirmation, and application value evaluation of GMS genes in crops.

inverse PCR, and genomic PCR methods. There are at least 10 GMS genes cloned by using T-DNA tagging in rice, such as *API5*, *bHLH142*, *OsGT1*, *UDT1*, *RIP1*, *WDA1*, *OsCP1*, *GSL5*, *DTM1*, and *DTC1* (**Table 1**). For instance, rice *UDT1* (*Undeveloped Tapetum1*) gene was isolated from a T-DNA insertional rice male-sterile mutant by using T-DNA tagging method [7]. The flanking region of the inserted T-DNA in mutant line was amplified by TAIL-PCR. Sequence analysis of that region revealed that T-DNA was inserted into a gene located on chromosome 7. BLAST analysis indicated that the most similar proteins are the *Brassica napus* bHLH transcription factor CAD54298 and the *Arabidopsis* bHLH protein AMS (At2g16910), each of which shares 32% overall identity with the rice protein. These results indicated that *UDT1* encodes a putative bHLH transcription factor in rice [7].

In addition, there are at least two and three GMS genes cloned via transposon tagging in maize (*Ms45* and *OCL4*) and rice (*OsGAMYB*, *MSP1*, and *CAP1*), respectively (**Table 1**). For example, maize *Ms45* gene is isolated by an activator transposon tagging, the tassel-specific transcription of *Ms45* gene is shown by RNA hybridization analysis, and genetic transformation of *ms45* mutant with a copy of *Ms45* gene can restore the fertility phenotype in maize [8].

2.1.3 MutMap method

MutMap method is based on whole-genome sequencing of pooled DNA from a segregating population of plants that show a useful phenotype [86], for example, male sterility resulted from ethyl methanesulfonate (EMS) mutagenesis (**Figure 3C**). In classic MutMap method, a GMS mutant is crossed directly to the original wild-type line; the resulting F₁ is self-pollinated to obtain F₂ progeny segregating for the GMS mutant and wild-type phenotypes. DNA of F₂ displaying the mutant phenotype is bulked and subjected to whole-genome sequencing followed by alignment to the reference sequence. SNPs with sequence reads composed only of mutant sequences (SNP index of 1; SNP index is defined as the ratio between the number of reads of a mutant SNP and the total number of reads corresponding to the SNP) are closely linked to the causal SNP for the mutant phenotype [86]. The MutMap method was used for rice GMS gene cloning, e.g., *OsLAP6/OsPKS1* [67]. Recently, a modified MutMap method was developed in rice male-sterility gene (*OsMs55/MER3*) cloning [87]. Different from the original MutMap method that aligns the mutant pool DNA sequence with the assembled WT genome, the modified MutMap method was to align the re-sequencing data of the mutant pool DNA and WT DNA with the Nipponbare reference genome. The resulting SNPs of mutant/Nipponbare and WT/Nipponbare were further compared to determine the candidate mutant gene. This modified method does not need an assembled WT genome as reference and thus is more cost-effective and widely applicable. The modified MutMap method was used for GMS gene cloning in rice and wheat, such as *OsABCG26* [68], *OsNP1* [69], and *TaMs1* [70] (**Table 1**). As the next-generation sequencing technology advances and cost of sequencing decreases rapidly, the MutMap method will be applicable for more crop plants except for rice and wheat.

2.2 Reverse genetic approaches

Reverse genetic approach means from gene to phenotype and relies upon sequence information as retrieved from genome, cDNA library, and/or expressed sequence tag (EST) sequencing. The scientist starts with the selection of a specific

sequence and tries to gain insight into the underlying function by selecting for mutations that disrupt the sequence and thereby its function. The reverse genetic approaches for GMS gene cloning include homology-based cloning, anther-specific expression gene screening, and other methods.

2.2.1 Homology-based cloning

Homology-based cloning is a simple and straightforward method of GMS gene cloning, and it relies on the conservation in sequence and function of the reference GMS gene among different species, mainly through the sequence alignment and phylogenetic analysis of the related GMS genes. As a lot of GMS genes have been cloned in model plants and the genome sequencing information become available for most important crops [88], there are several GMS genes that have been identified through homology-based cloning approach, such as *OsTDF1*, *OsACOS12*, *OsCER1*, *OsIG1*, *OsRAFTIN*, *TaMs26*, and *TaMs45* (**Table 1**). In *Arabidopsis* and rice, the molecular, genetic, and biochemical pathways regulating anther and pollen development have been extensively studied [89, 90], revealing the same number of developmental stages (14) and relatively conserved regulatory pathways in both species [91, 92]. The information about GMS obtained in *Arabidopsis* and rice provides opportunities to identify and utilize male sterility in economically important crops such as maize, barley, and wheat where GMS systems are not as well characterized [88, 93]. Based on this gene cloning strategy, about 62 putative maize GMS genes have been predicted and analyzed recently [3], and this will greatly enlarge the GMS gene number after functional confirmation via multiple methods (refer to Section 3).

2.2.2 Anther-specific expression gene screening

Given that most of GMS genes show anther-specific expression pattern, some putative GMS genes can be isolated by differential screening of the anther cDNA library, such as the GMS genes *OsC6*, *OsG1*, *OsADF*, and *OsUAM3* in rice and *TaRAFTIN* in wheat (**Table 1**). *OsC6*, encoding a lipid transfer protein, was reported to be abundantly expressed in tapetal cells of the anther and played a crucial role in the development of lipidic orbicules and pollen exine during another development in rice [78, 94]. *OsG1* was originally cloned from a rice anther cDNA library, encoding a β -1,3-glucanase and belonging to the defense-related subfamily A. *OsG1* was essential for callose degradation in tetrad dissolution, and its silencing results in male sterility [79]. *OsADF*, encoding an anther development F-box protein, was obtained from a rice panicle cDNA clone and played a critical role in rice tapetum cell development and pollen formation [80]. *OsUAM3* (UDP-arabinopyranose mutase 3) was identified by screening the expression patterns of the *OsUAM* genes in various vegetative and reproductive tissues and found to be a unique gene required for pollen wall morphogenesis in reproductive development [81]. *TaRAFTIN* was identified from an anther cDNA library of hexaploid wheat and cloned by using the RACE-PCR method, encoding a sporophytically produced structural protein that is essential for pollen development [75].

2.2.3 Other reverse genetic approaches

Once a GMS gene is cloned and characterized, its interaction protein or targeted gene may be involved in male-sterility regulation, too. Therefore, some of the GMS genes could be isolated by other reverse genetic approaches, such as chromatin immunoprecipitation sequencing (ChIP-Seq), genome-wide expression analysis of

a specific gene family, targeted mutagenesis of candidate GMS genes, etc. For example, *OsTGA10* encoding a bZIP transcription factor was identified as a target of the MADS box protein *OsMADS8* by using the ChIP-seq technique, and mutation of *OsTGA10* resulted in male sterility [84]. *OsSTRL2* was identified based on the genome-wide expression analysis of rice STR-like (*OsSTRL*) gene family and its anther-specific expression pattern [82]. *OsFTIP7* was identified as GMS gene through targeted mutagenesis of the rice genes encoding multiple C2 domain and transmembrane region proteins (MCTPs) using the clustered regularly interspaced short palindromic repeats (CRISPR)—CRISPR-associated nuclease 9 (Cas9) technology and targeted mutation of *OsFTIP7* lead to complete male-sterility phenotype in rice [83].

3. Functional confirmation methods of GMS genes in crop plants

As described above, once the putative GMS gene has been cloned, it should be tested by using a series of experiments, including transgenic complementation, targeted mutagenesis, allelism test and allelic mutant sequencing, anther-specific expression analysis, phylogenetic analysis, and cytological observation (Figure 4).

3.1 Transgenic complementation

Transgenic complementation is an essential tool and an effective way to confirm the function of a putative GMS gene. Based on the difference of transformation receptors, it includes two ways. The first one is transformation of the male-sterile mutants from which the GMS gene has been cloned, and then observation of the male-fertility phenotype in transgenic plant. For example, maize *ZmMs7* gene was confirmed by the transformation of *proZmMs7-ZmMs7* construct into maize Hill hybrid line.

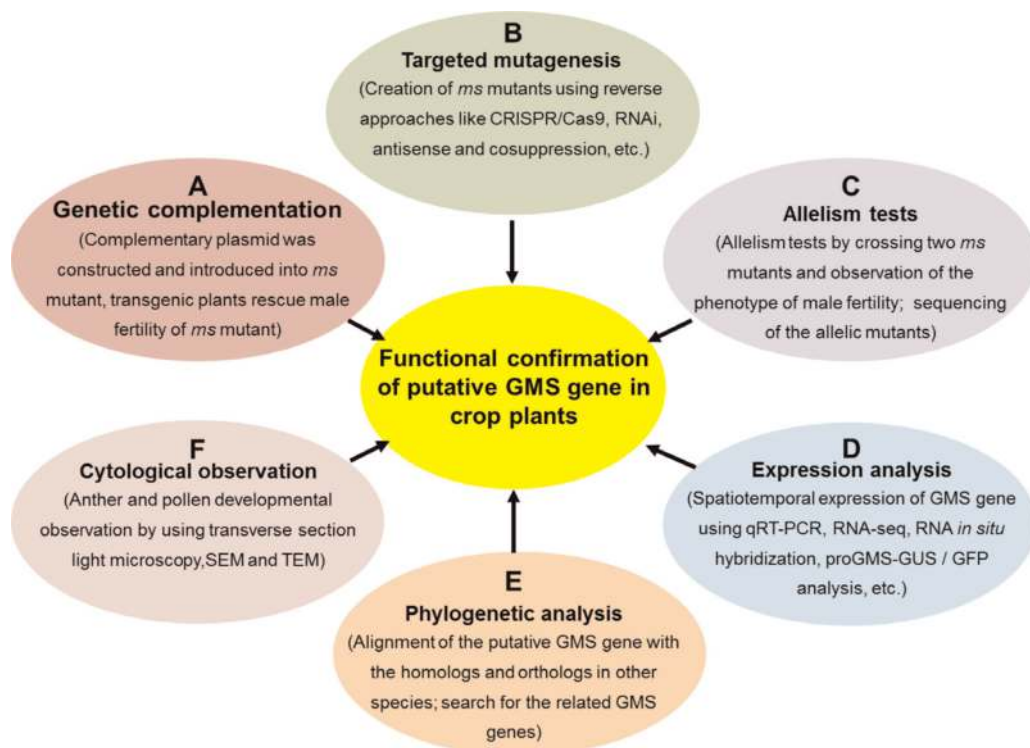


Figure 4.
The functional confirmation approaches of GMS genes in crop plants.

The transgenic plants were then crossed with the *ms7-6007* mutant, and transgenic plant in the *ms7-6007* homozygous mutant background can rescue the male-sterility defect of *ms7-6007* mutant and recovered the fertility phenotype (**Table 1**) [9]. The second one is transformation of the corresponding heterozygous male-sterility mutants with the orthologous GMS gene in model plants (such as *Arabidopsis*) and segregation analysis of complementation by the transgene. The putative GMS ortholog needs to be fused to a promoter to drive its expression in the *Arabidopsis* mutant, either by a constitutive, overexpression promoter, or via the *Arabidopsis* native gene-specific promoter. Although the first option is usually quicker, the results are not always satisfactory, due to the temporal and cell-specific regulation observed in some genes. For instance, anther and pollen transcription factors such as *AtMs1* orthologs in rice (*PTC1*) and barley (*HvMs1*) did not recover *Arabidopsis ms1* mutant fertility when driven by the CaMV35S overexpression promoter. However, once the rice and barley ortholog genes were fused to the *Arabidopsis AtMs1* native promoter, fertility was restored in the *ms1* homozygous mutant [35, 93].

3.2 Targeted mutagenesis

Targeted mutagenesis of the putative GMS gene includes two ways: knockdown and knockout approaches. Knockdown strategy, such as RNA interference (RNAi) silencing, is very helpful to those genes in which null mutant is lethal. RNAi silencing is a useful technique to characterize gene function; however, this approach may not generate clear phenotypes due to the threshold level needed for effective silencing [61]. RNAi target genes generally have reduced expression rather than being fully silenced; thus enough transcript may remain to maintain wild-type function. This partial reduction in gene expression was seen in several GMS gene RNAi silencing [63, 73, 80, 81, 93], where pollen development was affected by the silencing and showed a partial male-sterility phenotype. In addition, RNAi silencing has been shown to be unreliable after successive generations [95].

Knockout strategies, such as zinc finger nucleases (ZFNs), customized homing endonucleases (meganucleases), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 technology, have been shown to significantly increase the frequency and precision of genome editing. Especially, CRISPR-Cas9 has quickly become the technology of choice for genome editing and functional confirmation of GMS gene due to its simplicity, efficiency, and versatility [96]. For instance, rice *OsLAP6/OsPKS1*, maize *ZmMs30*, *ZmMs33*, and wheat *TaMs45* gene are confirmed as GMS genes by using the CRISPR-Cas9 technology, respectively. Targeted mutagenesis of these genes leads to complete male-sterility phenotype [18, 20, 67, 76].

3.3 Allelism test and allelic mutant sequencing

Allelism test (complementation test for functional allelism) is a test to determine whether two mutants are caused by the same gene. If there is more than one mutant of a specific GMS gene, allelism test should be carried out. A male-sterile (*ms*) homozygote is pollinated by a fertile heterozygote (*+/ms*) from the putative allelic line. If the progeny exhibits a fertile/sterile segregation ratio of 1:1, the two mutants are allelic with each other. If all the progenies display male fertile, suggest that the two mutations complement each other and they are not allelic. Furthermore, the allelic mutant gene can be confirmed based on sequencing and alignment with each other. If different *ms* mutants come from the mutation of the same GMS gene, the GMS gene function in male-sterility will be confirmed. For instance, the function of maize *ZmMs33* has been confirmed by allelism test and sequencing of several *ms33*

allelic mutants, such as *ms33-6019*, *ms33-6029*, *ms33-6024*, *ms33-6038*, and *ms33-6052* [20, 97]. Most of the cloned GMS genes have allelic mutants and are confirmed by allelic mutant sequencing (**Table 1**), so it is a usefully functional confirmation strategy besides the genetic complementation and targeted mutagenesis.

3.4 Anther-specific expression analysis

The anther-specific expression analysis is another important method for functional confirmation of putative GMS gene. In general, the expression pattern of GMS gene could be analyzed by using the following approaches: semiquantitative reverse transcription (RT)-PCR, quantitative real-time RT-PCR (qRT-PCR), northern blotting, promoter-GUS or GMS-GFP transgenic plant analysis, RNA in situ hybridization, and immunoblotting (or western blotting). For instance, the spatio-temporal expression pattern of *Ms6021* was analyzed by qRT-PCR, RNA in situ hybridization, and western blotting, and the results indicated that *Ms6021* is mainly expressed in the tapetum and microspore in maize [23]. The anther- and tapetum-specific expression pattern of rice *PTC1* was analyzed by using RT-PCR, qRT-PCR, and *PTC1_{pro}-GUS* transgenic rice anther staining [35]. Spatiotemporal expression pattern of *TaMs2* was analyzed by using RT-PCR, RNA in situ hybridization, and *TaMs2:GFP* transgenic anther microscopy, indicating that *TaMs2* is an anther-specific expression and dominant GMS gene [50].

3.5 Phylogenetic analysis

In order to get more functional information of the putative GMS gene, phylogenetic analysis should be carried out for expounding the evolutionary relationship with other putative orthologs. The detailed method is as follows: protein sequences of the putative orthologs of the targeted GMS can be obtained from Gramene (<http://www.gramene.org>) or NCBI (<https://www.ncbi.nlm.nih.gov/>) and aligned using ClustalX program [98]. A phylogenetic tree can be generated using molecular evolutionary genetics analysis (MEGA6) program based on a Poisson model with the maximum likelihood method [99]. Support values are estimated by 1000 times of bootstrap replicates. For instance, by using phylogenetic analysis, maize *Ms23* and its paralog *bHLH122* fall in the same clade with two rice GMS proteins, *TIP2* and *EAT1*. *TIP2* is the rice ortholog of *Ms23*, whereas maize *bHLH122* is the ortholog of rice *EAT1*. Maize *bHLH51*, rice *TDR*, and *Arabidopsis* *AMS* fall in the same clade, while maize *Ms32*, rice *UDT1*, and *Arabidopsis* *DYT1* fall in the same clade [15]. These results not only confirm the function of *Ms23* and *Ms32* in regulating male sterility but also predict that their paralogs *bHLH122* and *bHLH51* may be involved in male sterility, and this needs to be confirmed by targeted mutagenesis analysis and/or other strategies.

3.6 Cytological observation

As to the forward genetic cloning of the GMS gene, cytological observation is one of the phenotypic analyses of the male-sterile mutant. When the candidate GMS gene is cloned by reverse genetic approaches, cytological observation is one of the most important strategies for functional confirmation of the putative GMS gene. Cytological observation methods include light microscopy of transverse sections, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) of anther and pollen development. For instance, the functions of rice *OsTGA10* and *OsAGO2* in male sterility were confirmed by targeted mutagenesis of these genes

with antisense and CRISPR-Cas9 systems and cytological characterization of mutants by transverse section observation and TEM analysis of anthers at different stages [84, 85]. The functions of wheat *TaMs26* in anther and pollen wall development in bread wheat were tested by targeted mutagenesis of all the three homologs and cytological analysis using SEM method [77]. Cytological observation is helpful to confirm the function mechanism of the putative GMS genes in the cellular level.

4. Application value evaluation of GMS genes and mutants in crop plants

Compared to CMS and environment-sensitive genic male sterility (EGMS), GMS has many advantages such as the high germplasm utilization efficiency, higher male-sterility stability under various environments, and lower linkage rate with adverse traits. As more and more GMS genes have been cloned in crops, the BMS systems by using GMS gene have been developed in several crop plants and come into commercial utilization, such as SPT and MCS systems [3]. However, before utilization in the BMS systems, many characteristics of GMS gene and its mutant should be assessed systemically, such as genetic stability analysis of male-sterility, heterosis comparison, and analysis of potential linkage with bad traits.

4.1 Genetic stability analysis of male-sterility

Firstly, the genetic stability of the male-sterile mutant should be appraised in different genetic backgrounds and various environments. The general procedure is as follows (**Figure 5A**; the recessive *ms* mutant is taken as an example): a homozygous *ms* mutant is used as female parent and crossed with hundreds of inbred lines with broad genetic backgrounds to get the heterozygous F_1 hybrids. Then one of the F_1 hybrids is self-pollinated to produce F_2 seeds. Thereafter 50–100 of the F_2 seeds from each cross are grown in various environments. The fertility segregation ratios

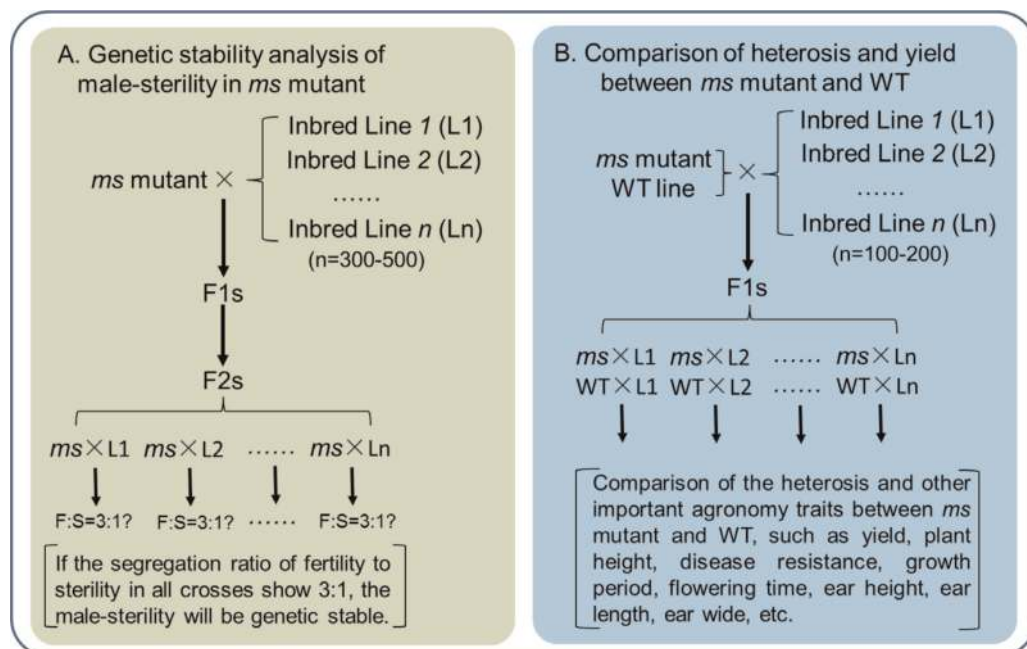


Figure 5.
The application value evaluation of GMS genes and mutants in crop plants.

of the F₂ populations are investigated, and anthers of three sterile individuals in each F₂ population are collected and stained with 1% I₂-KI solution to examine male-sterility status of pollen grains. If the segregation ratio of fertility to sterility in all crosses shows 3:1 as expected, we can say that the male sterility is genetically stable in different genetic backgrounds and various environments. Otherwise, if the ratio is not always 3:1 and confirmed by the molecular marker-assisted selection results, we can say that the male sterility is unstable in different genetic backgrounds and/or various environments. For instance, the male-sterility stability of maize *ms30-6028* mutant was analyzed by crossing with 329 maize inbred lines and observation of the segregation ratio of fertility to sterility in F₂ populations, suggesting that *ms30-6028* is a stable male-sterility mutant under diverse genetic backgrounds [18].

4.2 Heterosis comparison between *ms* mutant and wild type

Secondly, the effects of *ms* mutant on heterosis should be analyzed by comparing the yield and related agronomy traits between F₁ hybrid plants produced by using *ms* mutant and wild type (WT) as female parents and crossing with the same inbred line (Figure 5B). For instance, to test whether *ms30-6028* gene affects maize heterosis and grain yield, *ms30-6028* mutant and its homozygous WT line were used as female parents and crossed with 30 maize inbred lines, respectively. The harvested F₁ hybrids and their corresponding parental lines were grown according to the planting model of maize field production in two different locations. Eighteen agronomic traits such as plot yield, whole growth period, plant height, ear height, and hundred-kernel weight were investigated to compare the differences of heterosis and field production performance of 30 pairs of hybrid combinations using *ZmMs30* and *ms30-6028* homozygous plants as female parents, respectively. The results indicated that *ms30-6028* mutation has no obvious negative effects on maize heterosis and field production, suggesting that *ZmMs30* gene and its mutant *ms30-6028* are applicable for hybrid maize breeding and hybrid seed production [18].

4.3 Analysis of potential linkage with disadvantage genes and traits

Furthermore, other than the male-sterility stability analysis and heterosis comparison described above, the potential linkage with bad traits of *ms* locus should be analyzed. There are at least two ways to get this target: one is phenotypic observation of the hybrid plants that come from the homozygous *ms* mutant used as female parent, while those of the fertile sibling used as control. If the field production performances of the hybrid plants between *ms* mutant and WT are similar with each other, we can say that the *ms* mutation is not linked with disadvantage traits and thus can be applicable in hybrid seed breeding and production. For instance, maize *Ms44* hybrids showed no yield penalty in any of the tested environments, indicating that it is desirable for commercially viable products [22]. The other is sequencing of the putative genes near the *ms* locus and screening for the potential disadvantage genes based on bioinformatic analysis.

5. Application potential analyses of BMS systems by using GMS genes in crop plants

As described above, cloning and characterization of plant GMS genes have contributed significantly to our understanding of the molecular mechanisms of

anther and pollen development in crop plants and have provided an important basis for developing BMS lines. Several attempts have been made to utilize GMS genes in combination with new technologies to achieve more feasible BMS systems in crop plants [3, 4, 6, 100]. Here, we introduce briefly the strategies, assessment, and applications of some typical BMS systems in crop plants (**Table 2**).

5.1 Development strategies of the BMS systems

The development strategies of BMS systems based on GMS genes and other new technologies have been reviewed thoroughly and systemically in our laboratory [3]. In general, there are two kinds of strategies to develop BMS systems that have an application potential in hybrid seed production: transgenic construct-driven non-transgenic seed systems and transgenic male-sterility systems. The former includes the SPT and MCS systems in maize, SPT-like systems in maize, rice, and wheat,

Strategy	BMS system	Crop	Application assessment	Application status	Ref.
Transgenic construct-driven non-transgenic product systems	MCS	Maize	1. Genetic stability and heterosis of <i>ms</i> line 2. Genetic stability analysis of MCS maintainer lines	Product test	[9, 18, 21]
	SPT	Maize	1. Genetic stability of SPT maintainer lines 2. SPT transgene transmission rate through pollen	Commercial application in maize	[62]
	SPT-like (based on dominant <i>ms44</i> gene)	Maize	1. <i>Ms44</i> plants increase kernel number 2. <i>Ms44</i> hybrids showed no yield penalty	Product test	[22]
	SPT-like (based on <i>OsNP1</i> gene)	Rice	The <i>osnp1</i> line was crossed with ~1200 rice germplasms, ~10% out-yielded the best local cultivars	Product test	[69]
	SPT-like (based on <i>TaMs1</i> gene)	Wheat	No data	No data	[49]
Transgenic male-sterility systems	RHS1: glyphosate-mediated male sterility	Maize	1. The consistency in performance across inbreds 2. The RHS inbreds showed no yield penalty	Commercial application in maize	[101]
	RHS2	Maize	1. The endogenous mts-siRNAs are present widely 2. The elite event was introgressed into 15 maize germplasms	Product test	[102]
	Barnase/Barstar system	Oilseed rape	1. It is feasible in some crop plants 2. Double female parent seeds needed	Commercial application in canola	[103, 104]

Table 2. Strategy and application assessment of BMS systems in crop plants.

while the latter includes the RHS1 and RHS2 systems in maize and Barnase/Barstar system in oilseed rape (**Table 2**).

5.1.1 SPT- and SPT-like systems in crops

The SPT system is one of the representatives of transgenic construct-driven non-transgenic seed systems initially developed in maize by using a transgenic maintainer strategy [62]. The SPT transgenic maintainer line is created by transforming the plant of interest with an SPT construct consisting of three components: (i) a wild-type male-fertility gene (e.g., *Ms45*) to restore fertility, (ii) a pollen lethality gene (e.g., *ZmAA*) to disrupt normal pollen development, and (iii) a fluorescent seed color marker gene (e.g., *DsRed2*) for seed sorting. Of the pollen grains produced by the SPT maintainer line, all have the *ms45* genotype, 50% are non-transgenic, and 50% have the SPT transgenic elements. The latter grains are unable to germinate due to expression of the *ZmAA* gene. Thus, self-pollination of the SPT maintainer line produces both seeds with the same genotype of the SPT maintainer line (*ms45/ms45* + SPT-T-DNA) and seeds with the male-sterile genotype (*ms45/ms45*). The two types of seeds can be efficiently separated by mechanical color sorting, since the 50% of seeds contain the SPT elements showing a red color under green excitation light. When the male-sterile line (*ms45/ms45*) is pollinated with the SPT maintainer line, almost 100% of the resulting seeds have the *ms45/ms45* genotype and can be used as male-sterile female lines for crossbreeding and hybrid seed production.

Since the maize SPT system was developed and applied successfully [62], several SPT-like systems have been developed in maize, rice, and wheat based on *ZmMs44*, *OsNP1*, and *TaMs1* genes, respectively [22, 49, 69]. Although there are potentially many advantages of the SPT system, the rate of transgene transmission through pollen was found to vary with the highest rate being 0.518% [62]. Therefore, there is an increased risk of transgenic pollen flow during the male-sterile line propagation phase, thus resulting in greatly limited application in the countries and regions with strict biotechnology regulatory oversight.

5.1.2 MCS System

To decrease the rate of transgene transmission through the pollen of SPT maintainer lines, our laboratory developed a MCS system by transforming a single MCS construct into the maize *ms7*, *ms30*, or *ms33* mutant [9, 105, 106]. The MCS construct contains five functional modules: (i) a male-fertility gene (e.g., *ZmMs7*, *ZmMs30* or *ZmMs33*) to restore fertility, (ii) two pollen disruption genes (e.g., *ZmAA* and *Dam*) to disrupt the production of transgenic pollen, (iii) a fluorescent color marker gene (e.g., *DsRed2* or *mCherry*) for seed color sorting, and (iv) an herbicide-resistant gene (e.g., *Bar*) to prevent sophistication of seeds because it is beneficial for the propagation of high-purity MCS transgenic maintainer line seeds through herbicide spraying during specific stages of production. As the MCS construct harbors two pollen disruption modules, both of which can inhibit transgenic pollen formation or function, the transgene transmission rate through pollen is greatly decreased. Furthermore, the *Bar* gene in the MCS construct is helpful for propagating highly pure seeds of the transgenic maintainer line. Compared with the SPT construct, the MCS construct, which harbors two additional functional modules, the *Bar* and *Dam* genes, can produce maintainer and male-sterile lines with higher purity and greatly decrease the transgene transmission rate as well as the risk of transgene flow in commercial maize hybrid seed production. To promote commercial application, a field test of the MCS system in China is currently underway.

As described above, although the final product of transgenic construct-driven male-sterility systems is non-transgenic, the use of these systems is often limited by the lack of GMS mutants and male-fertile genes in many crops. Consequently, many biotechnology strategies have been developed in the past 30 years to produce artificially dominant male-sterile plants independent of GMS mutants and male-fertility genes, such as the RHS systems in maize and Barnase/Barstar system in oilseed rape.

5.1.3 RHS system

The RHS system, which is based on glyphosate-mediated male sterility, is deployed for hybrid seed production by Monsanto [101]. The RHS system consists of RHS and RR transgene constructs. The RHS transgene cassette contains the CP4-EPSPS gene (encoding 5-enolpyruvyl-shikimate 3-phosphate synthase, which is insensitive or resistant to glyphosate) driven by the enhanced 35S promoter, which has been shown to be poorly expressed in tapetum cells and microspores, and thus the resulting RHS plant demonstrates male sterility following glyphosate application with little/no injury to the rest of the plant. The RR transgene construct comprises a double expression cassette providing high constitutive expression of CP4-EPSPS resulting in robust resistance to glyphosate. In hybrid seed production fields, rows of an RHS female line are interplanted with rows of an RR male line, and over-the-top sprays of glyphosate induce male sterility in RHS female plants, which are subsequently pollinated by RR male plants. By withholding glyphosate, the RHS plants remain fully fertile and are capable of self-pollination for propagation of female line without the need for a maintainer line.

The RHS system replaces mechanical detasseling with glyphosate spray and greatly simplifies the process of hybrid seed corn production. Recently, this system has been improved as RHS2 by using endogenous maize male tissue-specific small interfering RNAs to trigger cleavage of the CP4-EPSPS mRNA specifically in tassels, resulting in glyphosate-sensitive male cells [102].

5.1.4 Barnase/Barstar system

The Barnase/Barstar system was the first dominant BMS system developed in rapeseed and tobacco [103, 104] and then has been tested and tried in wheat and rice [107, 108]. The *barnase* and *barstar* genes are fused with the tapetum-specific TA29 promoter and then transformed individually into plants. The *TA29-barnase* transformed plants are completely male-sterile and are crossed with *TA29-barstar*-expressing fertile plants, which results in the co-expression of *barnase* and *barstar* genes in the anther tapetal cell layer. The inactivation of *barnase* by *barstar* leads to the restoration of fertility in the hybrid F1 plants [109].

5.2 Application assessment of the BMS systems

Although several BMS systems had been developed during the past decades, only three of them were thoroughly assessed and applied in commercial hybrid seed production in some crops, such as SPT, RHS, and Barnase/Barstar systems.

In SPT system, the stability of transgenes in prospective SPT maintainer lines were examined based on Southern blot analyses of genomic DNA from T0 to T4 plants to assess the integration and structural fidelity of the transgenes. No changes in hybridization patterns with three different restriction enzyme digestions of the SPT transgenes indicated that the transgenes are stable over multiple generations. More importantly, the transgene transmission through pollen was tested by using

the pollen of transgenic SPT maintainer to pollinate non-transgenic plants. Then ears were harvested from the non-transgenic female parent plants and examined under visible light for the presence of pink seeds expressing the DsRed2 protein. The transgene transmission rate through pollen varied from 0% to 0.518% with different constructs and transformants. As the transformant DP-32138-1 showed the lowest transgene transmission rate that was maintained across generations and in different inbred backgrounds, it was selected as the SPT maintainer line for use in maize male-sterile parent seed increase [62].

The RHS system was developed by minimizing the CP4-EPSPS expression in the tassel and maximizing glyphosate delivery to the tassel resulting in consistent male sterility for hybrid seed corn production [101]. Therefore, the glyphosate spray timing, dose, target, and mode should be examined firstly in different maize varieties. Subsequently, the consistency in performance of the RHS technology across a broad range of inbred varieties is essential and has been examined in field trials. The performance standard for RHS is 0.5% anther extrusion or >99.5% tassel sterility to insure high purity in hybrid seeds. The field data show that RHS has consistently surpassed the performance standard since 2007 when examined across an increasing number of inbred varieties. In fact, in 2011 and 2012, 100% sterility or zero anther extrusion was observed across 46 and 47 inbred varieties, respectively. The RHS inbreds have shown comparable yield to mechanical detasseling; furthermore, the F1 hybrid seeds produced by crossing RHS with RR showed full resistance to glyphosate and comparable performance to hybrid seeds produced by mechanical detasseling [101].

The Barnase/Barstar system was the first BMS system developed in tobacco and oilseed rape plants based on tapetal cell-specific expression of *TA29-barnase* and *TA29-barstar* genes [103, 104]. *TA29-barnase* transgene leads to dominant male sterility due to selective destruction of anther tapetal cells, while *TA29-barstar* transgene can suppress the expression of *TA29-barnase* transgene and restore the male fertility. The feasibility of this system was confirmed in many crop plants, such as oilseed rape, tobacco, lettuce, chicory, cauliflower, tomato, cotton, and maize. However, the dominant male-sterility line must be maintained in heterozygous plants by crossing with a wild-type line in the same (isogenic) genetic background. The elimination of the fertile segregants by herbicide spray doubles the amount of female parent seed needed for hybrid seed production, which could limit the applicability of the system in crops with low multiplication factor and relatively low plant density [4].

5.3 Commercial application of the BMS systems

As shown in **Table 2**, there are only three BMS systems that have been applied in commercial hybrid seed production, including SPT, RHS, and Barnase/Barstar systems. The SPT system has been deregulated by USDA APHIS in 2011 and is thus available for commercial hybrid seed production in maize [110]. Unlike other BMS systems, the maize inbred parent lines produced using the SPT system do not inherit SPT transgene from the SPT maintainer line and thus are non-transgenic. Furthermore, both the commercial maize hybrid seed produced from the SPT system and the resulting commodity maize grain harvested from these hybrid plants are non-transgenic. Subsequently, acknowledgement of the non-transgenic status of progeny produced by the SPT system is also supported by regulatory agencies in Australia and Japan [111, 112]. Therefore, hybrid maize and commodity grain produced from the SPT system are non-genetically modified (non-GM) and subject only to those regulations applicable to conventional non-GM maize. The performance of RHS system has been evaluated by Monsanto manufacturing group

which produces the hybrid seeds that are sold in the marketplace. A few modifications have been implemented to make the RHS system more practical and manageable in the field [101]. Most recently, the second-generation RHS (RHS2) technology combines the relative simplicity and convenience of a systemic herbicide spray methodology with targeted protein expression to create an inducible male sterility system for industrial production of maize hybrid seeds in an environmentally independent manner [102]. The Barnase/Barstar system has been used successfully for the commercial production of canola hybrids (*Brassica napus*) in Canada [4]. However, most of the BMS systems have not been used in commercial hybrid seed production, maybe because of lacking the cost-effective and environment-friendly BMS systems and/or the regulatory acceptance of using BMS systems among different countries.

6. Conclusions

In this chapter, we focus on the molecular cloning, functional confirmation, and application value assessment of GMS genes as well as their application in hybrid seed production via several BMS systems in cereal crops, such as rice, maize, and wheat. With the rapid development of the next-generation sequencing technology, more genome information of cereal crops are available, leading to plenty GMS genes cloned and characterized in crop plants. As shown in **Table 1**, there are more than 70 GMS genes cloned in cereal crops, and most of them (57/73) are identified by using forward genetic approach, including 38 genes isolated by map-based cloning, 15 genes identified by T-DNA/Transposon tagging and 4 genes isolated by MutMap method. Whereas the rest of GMS genes are identified *via* reverse genetic approach, including 7 genes isolated through homology-based cloning, 5 genes identified by anther-specific expression gene screening and 4 genes cloned by other reverse genetic methods. Among them, there are 49 GMS genes cloned in rice, 17 GMS genes in maize, 6 GMS genes in wheat, and 1 GMS gene in barley. From these data, we conclude that the forward genetic approaches, especially map-based cloning, are the most popular method for GMS gene cloning in crops; most GMS genes have been cloned in rice and maize, whereas only a few GMS genes are cloned in wheat and barley. Consider that the conserved role of GMS genes in different species and the sequence information of GMS genes in rice and maize can be used for cloning of the orthologs in wheat and barley through reverse genetic approaches. For example, the functions of *TaMs26* and *TaMs45*, the wheat homologs of maize *Ms26* and *Ms45*, were confirmed via a custom-designed homing endonuclease and CRISPR-Cas9-targeted mutagenesis in wheat, respectively [76, 77], while the role of *HvMs1*, the barley homolog of *Arabidopsis Ms1* and rice *PTC1*, was analyzed by using RNAi silencing in barley [93].

Although there are a lot of GMS genes identified in cereal crops up to now, less than 10 GMS genes are assessed for the value in heterosis utilization and hybrid seed production (**Table 1**). For example, *ZmMs7*, *ZmMs30*, and *ZmMs33* are tested in maize MCS system [9, 18, 21]; *ZmMs26*, *Zmms44*, and *ZmMs45* are tested in maize SPT (or SPT-like) system [16, 22, 62]; *OsNP1* is tested in rice SPT-like system [69]; and *TaMs1* is tested in wheat SPT-like system [49]. All these BMS systems belong to transgenic construct-driven non-transgenic product strategies, leading to potential application of these systems in commercial hybrid seed production, especially in the countries and/or regions with strict regulatory policy. These systems have many advantages, such as non-transgenic final products, environment-friendly without application of herbicide in hybrid seed production fields, and deregulated by the regulatory authority in some countries, whereas they are limited by using

transgenic maintainer line based on completely male-sterile mutants and the male-fertility genes and requirement of fluorescent seed color-sorting machine. Therefore, the transgenic male-sterility systems, such as RHS system based on glyphosate-mediated male sterility, have also been developed and used in commercial hybrid seed production in maize [101, 102]. This system is independent of male-sterility mutants and male-fertility genes, no need for transgenic maintainer line and seed color sorting, and the herbicide-resistant male-sterility lines are helpful to highly efficient and mechanized hybrid seed production. However, this transgenic male-sterility system is limited by the “zero tolerance” regulatory policy preventing transgenic planting in many countries, need for application of herbicide in hybrid seed production fields, and potential risk of gene flow. In summary, both SPT and RHS systems have advantages and disadvantages compared with each other.

With the advance of molecular cloning methods including both forward and reverse genetic approaches, especially the next-generation sequencing technology and genome-editing technology (e.g., CRISPR-Cas9), more GMS genes in cereal crops with large and complex genome (e.g., wheat and barley) will be identified and characterized by using multiple strategies as described in this chapter. At the same time, the application value of the putative GMS genes should be assessed systematically in genetic stability of male sterility, effects on heterosis performance, and potential linkage with detrimental traits. This will not only boost our understanding in the molecular mechanism of anther and pollen development but also give great opportunity to develop novel BMS systems for commercial hybrid seed production in cereal crops.

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Conflict of interest

The authors declared that they have no conflict of interest.

Abbreviations

BMS	biotechnology-based male sterility
BSA	bulked segregant analysis
CMS	cytoplasmic male sterility
CRISPR	clustered regularly interspersed short palindromic repeats
EGMS	environment-sensitive genic male sterility
EMS	methanesulfonate
EST	expressed sequence tag
GM	genetically modified
GMS	genic male sterility

MCS	multi-control sterility
MS	male sterility
MEGA	molecular evolutionary genetics analysis
qRT-PCR	quantitative real-time PCR
RHS	roundup hybridization systems
RT	reverse transcription
SEM	scanning electron microscopy
SPT	seed production technology
TAIL-PCR	thermal asymmetric interlaced-polymerase chain reaction
TEM	transmission electron microscopy
WT	wild type

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