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Genetics and Breeding System for Cytoplasmic and Genetic Male Sterility in Rice

Christian De Guzman and James Oard

Abstract

The initial discovery of cytoplasmic male sterile (CMS) three-line system made it possible to produce hybrids that significantly increase rice yields compared to its inbred counterparts. Further genetic and molecular studies help elucidate the mechanisms involved in CMS male sterility. Additional CMS types were also discovered with similar genetic control from wild sources by interspecific hybridization. While the three-line system was a success, the two line system using photoperiod genetic male sterile (PGMS), thermosensitive genetic male sterile (TGMS) and photoperiod and thermosensitive genetic male sterile (PTGMS) were becoming more popular due to the ease in breeding and with more hybrid combinations generated compared to the CMS types. Inheritance and molecular studies showed that the trait is controlled by one or more recessive genes depending on the genetic background and environmental conditions. Due to the sensitivity of the lines to temperature and/or photoperiod, unique breeding procedures were followed. Methods involved the use of growth chamber, timing of planting, and selection of suitable locations. These practices successfully maintained sterility for hybrid seed production or reversion to fertility for seed multiplication of parental male sterile lines.

Keywords: cytoplasmic male sterile (CMS), genetic male sterile, hybrid rice, photoperiod, thermosensitive

1. Introduction

The use of hybrid greatly increased rice production worldwide due to the improved yields, better tolerance to pest, diseases and environmental stress compared to inbred varieties. The discovery of cytoplasmic male sterility was the major milestone to the development of hybrid rice [1]. Further discovery of two line male sterility made hybrid breeding more efficient and further increased the probability in finding the best performing hybrid combinations [2].

Two different male sterility systems are available for hybrid seed production (**Figure 1**). The first is a cytoplasmic male sterility (CMS) which is a three-line system that uses a male sterile line, a restorer line and a maintainer line. The male sterility is more stable albeit more complicated to breed and maintain [3]. The second is the two-line male sterility system that uses a genetic male sterile which is controlled by temperature, photoperiod or both. The use of this system is increasing due to the ease in breeding, finding more heterotic combinations, and in seed multiplication of parental male sterile lines. However, hybrid seed production may

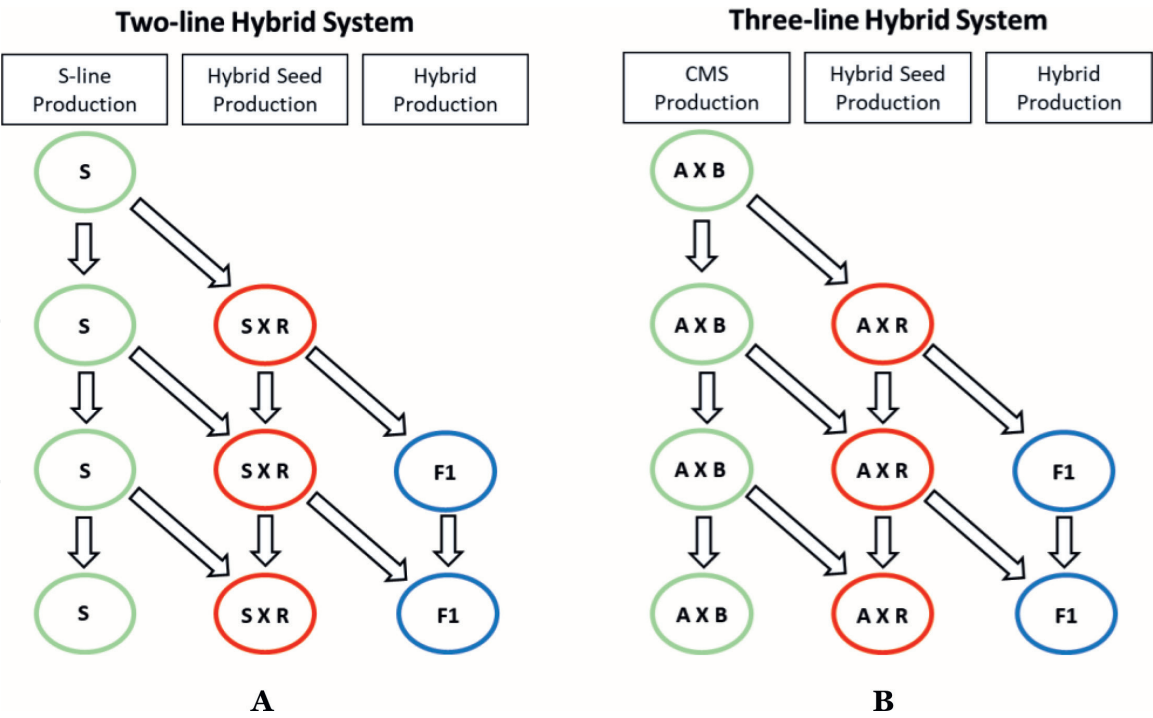


Figure 1. Comparison of two-line and three-line hybrid rice breeding system. A: two line hybrid system; S—genetic male sterile, R—restorer/pollen fertile, F₁—hybrid. B: three line hybrid system A—CMS line, B—maintainer line, R—restorer line with restorer gene, F₁—hybrid.

be catastrophic if there are severe changes in environmental conditions [4]. Both systems proved effective in hybrid rice production which increased yields by up to 20% therefore, increased farm profitability and has contributed significantly in addressing global food security.

Heterosis in hybrid rice minimize the impact of reduced yields brought by diseases compared to the inbred counterpart. However, due to narrow genetic diversity of the male sterile parent, they became vulnerable to pathogens and pests resulting to the loss of its yield potential [5]. This makes it difficult for growers to recover the high cost of seed and F₁ production. It became apparent that discovering new sources of male sterility to increase genetic diversity and further introgression of resistance genes are necessary to secure the yield gain in hybrid rice [6].

This chapter focuses on the discovery of rice male sterility, genetics, mechanisms and procedures in multiplication and handling of male sterile rice for hybrid rice breeding.

2. Cytoplasmic male sterile (CMS) rice lines

Development and cultivation of hybrid rice started in China with the initial work of rice breeder Yuan Longping. As early as 1964, Yuan Longping have tested different male sterile lines however, no stable sterility exists and the group started resorting to making distant hybridization by crossing wild rice with cultivated rice. In 1970, a wild-abortive type cytoplasmic male sterile rice CMS-WA were discovered which eventually leads to the release of the first hybrid rice in 1976. By 1980's, hybrid rice accounts to about 55% of the total rice planting area in China [7, 8]. More CMS types were discovered that further expand the diversity in hybrid rice three-line system. These were developed by direct crossing or backcross breeding from two different species, subspecies or different cultivars [9]. The major type of CMS systems with their cytoplasm and nucleus sources are shown in **Table 1**.

MS type	Male sterility source	
	Cytoplasm	Nucleus
CMS-BT	Chinsurah Boro II (<i>indica</i>)	Liming (<i>japonica</i>)
CMS-HL	Hong lian (<i>Oryza rufipogon</i>)	Liantanzao (<i>indica</i>)
CMS-CW	Chinese wild W1 (<i>Oryza rufipogon</i>)	Reimei (<i>japonica</i>)
CMS-WA	Wild abortive (<i>Oryza rufipogon</i>)	Erjiunan (<i>indica</i>)
CMS-LD	Burmese “Lead rice” (<i>indica</i>)	Fujisaka 5 (<i>japonica</i>)

Table 1.
Primary CMS male sterility systems utilized in hybrid rice production.

There were more than 60 types of CMS systems discovered in China alone but most of them may only be classified in three types CMS-BT (Boro II), CMS-WA (wild abortive), and CMS-HL (Honglian) [10, 11]. The three major types produces pollen that lack starch or are starch deficient while CMS-LD and CMS-CW produces morphologically normal pollen grains but were unable to fully germinate [12]. In CMS-WA, pollen abortion occur at a uninucleate stage primarily during microspore development [13]. The result is an irregularly shaped and lightly stained pollen when treated with 1% iodine potassium iodide solution (I₂KI). The genotype of sporophytic tissues determines pollen abortion. In CMS-BT, pollen abortion occurs at trinucleate stage with pollen lightly stained due to deficiency of starch and spherical in shape rather than irregular [14]. In CMS-HL, pollen abortion appears at binucleate stage and the pollen is spherical in shape but without starch. Restoration to fertility in all CMS type except CMS-WA are all gametophytic therefore producing half of the pollen fertile in the F₁ generation (**Figure 2**).

2.1 Genetics and mechanism of cytoplasmic male sterility (CMS)

Sterility in CMS is controlled by the interaction of genes in the cytoplasm and the nucleus. The sterility factor S is located in the mitochondrial DNA while the *rf* (restorer of fertility) allele is located in the nucleus. The plant is sterile (A line) if it carries both the S factor and the recessive allele *rf*. Maintainer line (B line) carries the *rf* allele but has a different cytoplasmic factor N which allows the plant to be fertile. The B-line has the ability to make the S line produce seeds after crossing but the progeny remains sterile thus useful for S line seed multiplication. Restorers (R line) is the diverse pollen fertile parent that carries the dominant *Rf* gene that when crossed to B line restores fertility in the F₁ (**Figure 3**) [15].

CMS-BT genes were the first to be identified that has the mitochondrial open reading frame *orf79* [16] and is co-transcribed with *B-atp-orf79*. Similarly, CMS-HL carries the mitochondrial *atp6-orfH79* in which *orfH79* and *orf79* are 98% identical in DNA sequence [9]. In CMS-WA *orf224*, *orf284* and *orf288* were discovered with one still unknown segment. Together they encode a 325-residue protein with three transmembrane segments that are believed to be responsible for CMS trait [17]. A *B-atp-orf79* like structures were also found in CMS-LD that may be link to male sterility but in CMS-CW, no similar structures were identified, thus the specific cytoplasmic factor is still unknown [17].

A total of six restorer of fertility genes (*Rf*) in rice have been discovered. These are *Rf1a*, *Rf1b*, *Rf2*, *Rf4*, *Rf5*, and *Rf17*. These genes were classified as pentatricopeptide repeat (PPR) proteins which are RNA binding and act in post-transcriptional mRNA process in cell organelles [18]. *Rf1a* and *Rf1b* restores fertility in CMS-BT while in CMS-HL, 50% fertile pollen can be restored by either *Rf5* or *Rf6*. If both present in

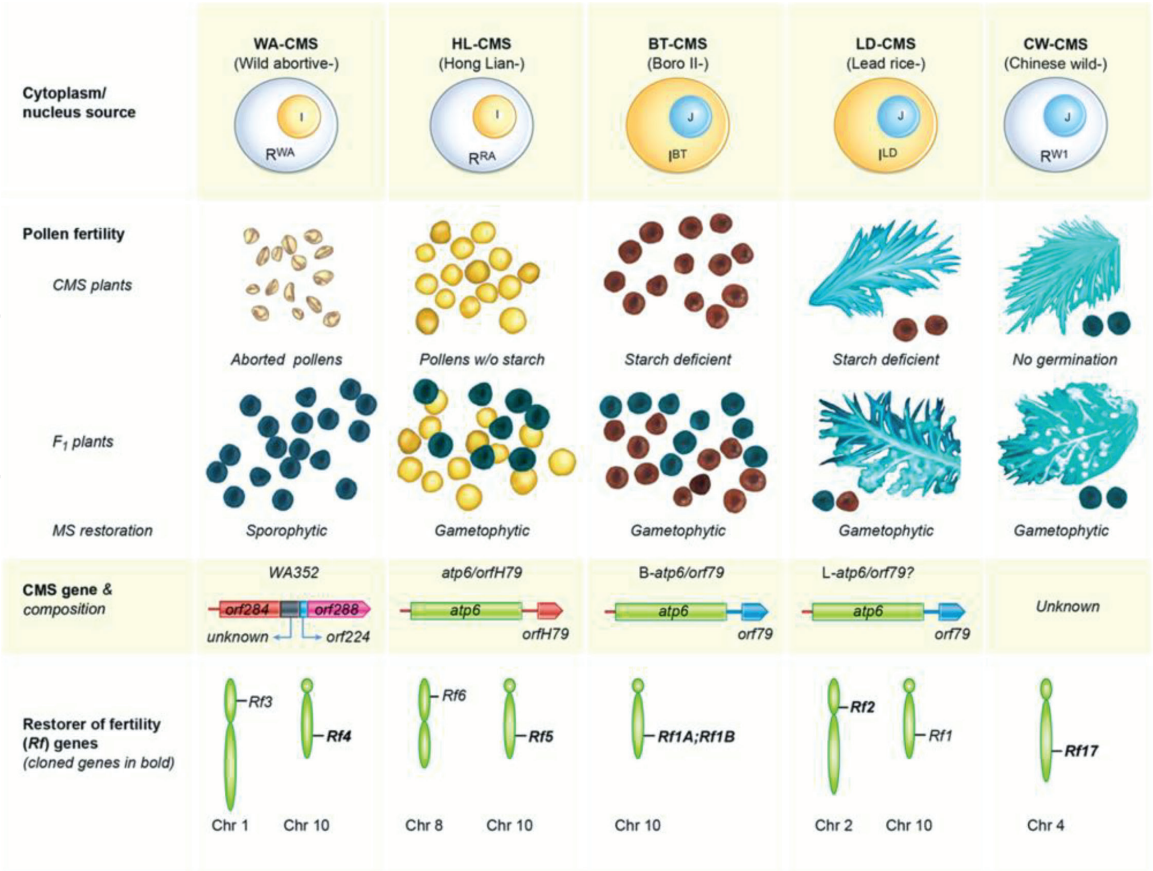


Figure 2. A schematic presentation of the five well-studied rice CMS types. Abbreviations for cytoplasm sources are RWA for wild-abortion *Oryza rufipogon*, RRA for red-awned *O. rufipogon*, and RW1 for Chinese wild rice (*O. rufipogon*) accession W1; IBT and ILD for indica Boro-II type and Lead rice, respectively. Nucleus sources are either indica (I) or japonica (J) [7].

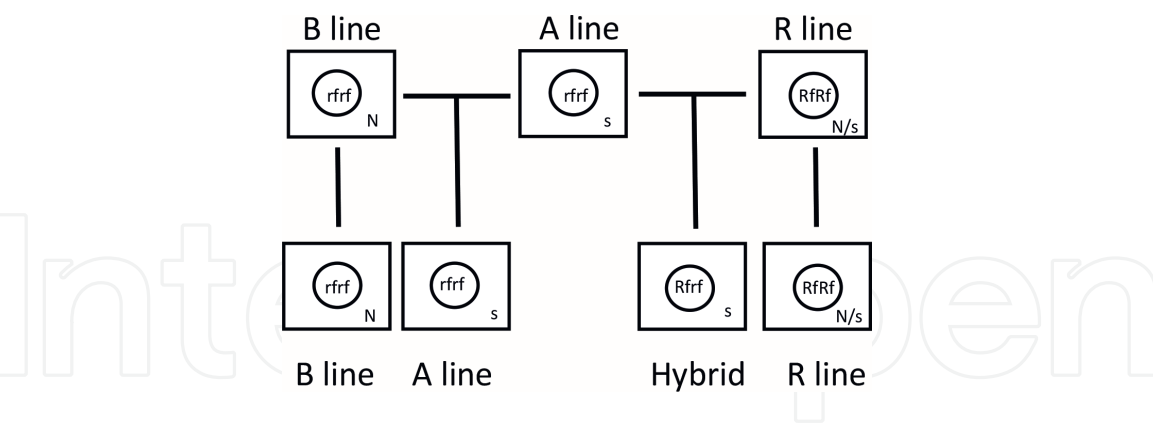


Figure 3. Schematic diagram of CMS three line system. *Rfrf* = nuclear gene homozygote recessive, *RfRf* = nuclear gene homozygote dominant, *Rfrf* = nuclear gene heterozygous, N = cytoplasmic factor (fertile), s = cytoplasmic factor (sterile).

CMS-HL, *Rf5* and *Rf6* restores 75% of fertile pollen in the F₁. Further analysis by sequencing and cloning concluded that *Rf1* is the same gene as *Rf5* located in chromosome 10 [19]. *Rf3* and *Rf4* restores fertility in CMS-WA and are located in chromosome 1 and 10 respectively with the latter recently cloned [20]. CMS-LD fertility can be restored by *Rf1* or *Rf2* while CMS-CW can only be restored by a single gene *Rf17* [12].

2.2 Breeding and diversification of CMS sources

Diversification of both CMS maintainers and restorer lines are very important to guarantee the continued progress of finding the best hybrid combinations.

Extensive evaluation of lines and backcross breeding were employed to improve the lines and adapt to a particular environment [21]. Furthermore, new maintainers and restorers were developed from the original donors. A new CMS source was discovered in Dongxiang wild rice by continuous backcrossing to the *indica* variety Zongzao 35 [10]. In another study, a new source was found from interspecific cross of an *indica* with an African rice (*Oryza glaberrima* Steud.) that showed similarity to the sporophytic type CMS-WA [22].

3. Discovery of genetic male sterile lines

Although the CMS three-line system greatly increased yields in hybrid rice, there are difficulties and limitations on its use. One of the difficulties is the need to simultaneously develop maintainer lines (B lines) by subsequent nucleus substitution of the original CMS line with the B lines through repeated backcrossing. Furthermore, there are also limited choices available for restorer lines (R lines) with only about 5% of the current existing lines can be used that carries the restorer gene [7]. The discovery of genetic male sterility or photoperiod and/or thermosensitive male sterile lines addresses these problems. These lines responds to photoperiod, temperature or a combination of both which cause the plant to be fertile or sterile depending on the critical daylength or temperature [23]. With the two-line system using genetic male sterile, there is no need to develop a maintainer line and any fertile line can be used as a restorer. This greatly reduce the time and resources in making hybrid combination and parental seed production. Moreover, it broadens the available choices of restorers that can generate more combinations which in turn increases the probability of finding the best hybrids [4].

Extensive studies suggest that genetic male sterile lines can be broadly classified into three categories; photoperiod genetic male sterile (PGMS), thermosensitive genetic male sterile (TGMS), and photoperiod and thermosensitive genetic male sterile (PTGMS) [15, 24, 25]. The first reported genetic male sterile came from spontaneous mutant in a japonica cultivar Nongken 58 discovered in Hubei China and were later called as Nongken 58S [2]. Further studies after its discovery revealed that the male sterility is regulated mainly by photoperiod and thus referred to as photoperiod genetic male sterile (PGMS). Nongken 58S showed complete pollen sterility when grown under long day conditions (>14 h), fertility was restored when subjected to <10 h of light under controlled environment [26].

A thermosensitive type of male sterility was discovered in a spontaneous mutant AnnongS-1 (Ans-1) in 1997. The pollen remained sterile at both long and short day when exposed to 33°C and reverts back to fertile when the temperature reached 24°C [27]. Additional lines exhibiting thermosensitivity were also discovered in Zhu1S, Hengnong 1S and Guangzhang 63S where the fertility rates vary at different controlled temperatures regardless of daylength [28–30].

The third classification of genetic male sterility affects both photoperiod and temperature. Pei'ai 64S is a line derived from the original male sterile mutant Nongken 58S with genetic backgrounds such as *indica* and *javanica* [31]. A study conducted on the response of Pei'ai 64S and another line 8902S showed fertility under long daylength (>14.5 h) and low temperature (24°C) or short daylength (10 h) and high temperature (28°C) conditions, but were consistently sterile at long daylength (14 h) and high temperature conditions (28°C) [32].

3.1 Genetics of male sterility

Numerous genetic studies concluded that genetic male sterility can be controlled by single, two genes or multiple genes depending on the genetic background and

the environment. The original Nongken 58S when crossed to conventional *indica* and *japonica* lines produced F_1 's that are all fertile [15]. The F_2 reciprocal crosses to Nongken 58S concluded that that male sterility is controlled by a single recessive gene [33]. Further studies showed similar results when Nongken 58S (sterile) and Nongken 58 (fertile) were crossed showing a typical single gene recessive segregation in the F_2 population under long day conditions [34, 35]. A single locus segregation of genetic male sterile lines was also reported in several TGMS lines under long day and high temperature field conditions [29, 30, 36, 37]. Previous research also discovered two gene recessive segregation when Nongken 58S was crossed to an *indica* variety. F_2 segregation exhibited a ratio of 15 fertile: 1 sterile pollen fertility [2]. Several *indica* derived male sterile lines such as Pei'ai 64S displayed similar genetic ratios [31, 38, 39]. Additional studies detected segregation that followed either a bimodal or a continuous distribution in some populations of genetic male steriles [26, 40].

Studies conducted under US conditions on *indica* male sterile line 2008S originated from China showed that male sterility is controlled by two or three recessive genes depending on the location and year of planting (**Figure 4**). When planted in

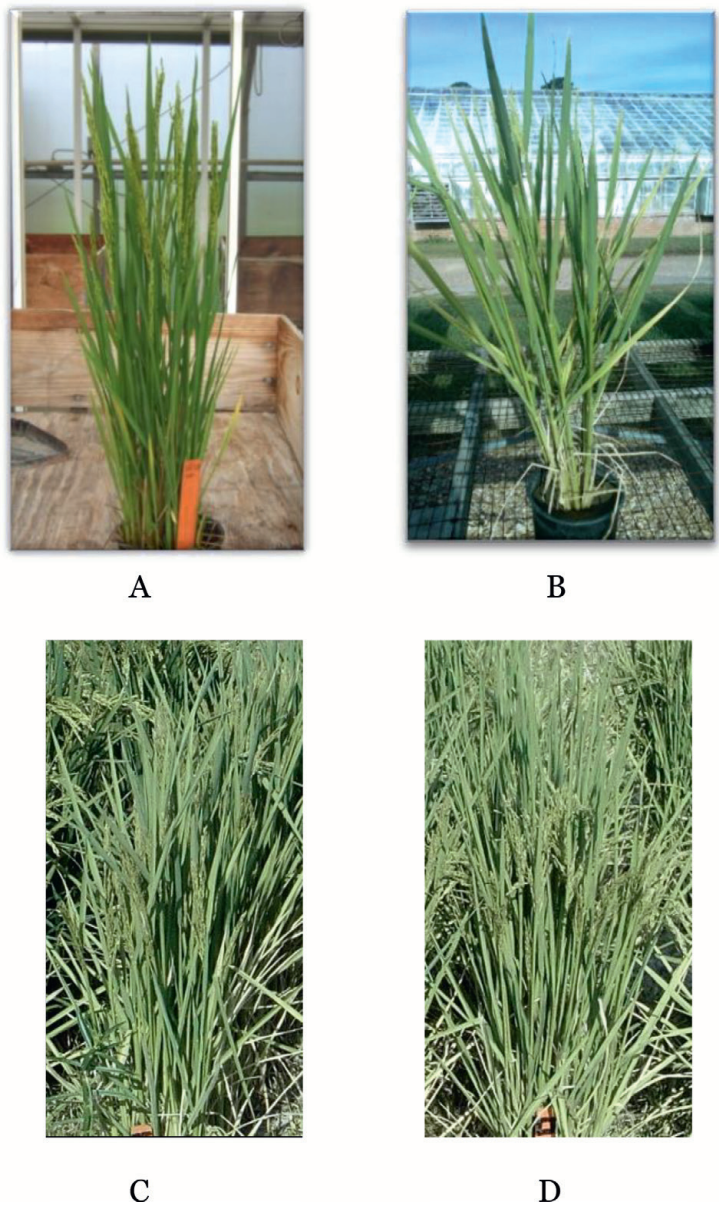


Figure 4. Male sterile line 2009S grown in the greenhouse (A) and in the field (C). 2008S male sterile line grown in the greenhouse (B) and in the field (D). Both lines exhibited 100% sterility when grown at H. Rouse Caffey Rice Research Station in Crowley, Louisiana USA under high temperature and long day conditions.

Stuttgart Arkansas USA, 2008SxCL131 F₂ population fit a three-gene model, while the same population planted in Crowley, Louisiana USA segregated in two-gene model during 2013. In 2014, the 2008S/Cypress population fit both 15:1 and 63:1 ratios in Arkansas, whereas 2008S/CL131 only fit a 15:1 segregation ratio. In the same year at Crowley, Louisiana a single 15:1 ratio exhibited in 2008SxCypress, and only a three-gene model was found for 2008SxCL131 [4].

Another male sterile line 2009S currently used by the LSU Agcenter for their hybrid rice breeding program shows a single gene recessive inheritance (**Figure 4**). Field trials carried out in Crowley Louisiana over 2 years (2013–2014) in two F₂ and four BC₁F₂ populations showed consistent results both in seed and pollen fertilities [25, 41]. A comparison of the pollen sterility frequency distribution is presented in **Figure 5** for 2008S and 2009S F₂ populations.

3.2 Candidate genes and mechanism of genetic male sterile rice

Several candidate genes were identified in controlling male sterility in PGMS lines. More recent studies using Nongken 58S discovered that the gene in LOC_Os12g36030 which was previously mapped as pms3 and is also allelic to p/tms12-1 located on chromosome 12 regulates photoperiod genetic male sterility. The single nucleotide polymorphism (SNP) mutation located in non-coding region increased the methylation on the promoter which in turn reduced transcription of LOC_Os12g36030. The reduced transcription caused the pre-programmed cell death in developing anthers causing pollen sterility [42, 43].

A second study conducted in PTGMS line Pei'ai 64S revealed that the gene LOC_Os07g12130 previously mapped as pms1(t) encodes a protein containing Myb-like DNA binding domain that affect the transcription of a protein responsible for the photo-thermosensitive response. RT-PCR results showed that mRNA levels of LOC_Os07g12130 changes at different photoperiod and temperature conditions [31]. However, the gene is yet to be cloned and further study needs to be conducted. De Guzman [4] sequenced both locus in male sterile line 2008S and found both SNPs present. When QTLs were analyzed in two segregating populations using both single marker analysis and interval mapping, each locus and their interaction gives significant effects [25].

For lines exhibiting thermosensitivity, candidate genes associated with TGMS lines were mapped on chromosome 2. QTL mapping using bulk segregant analysis approach (BSA) identified the ptgms2-1 locus [30]. Further analysis showed

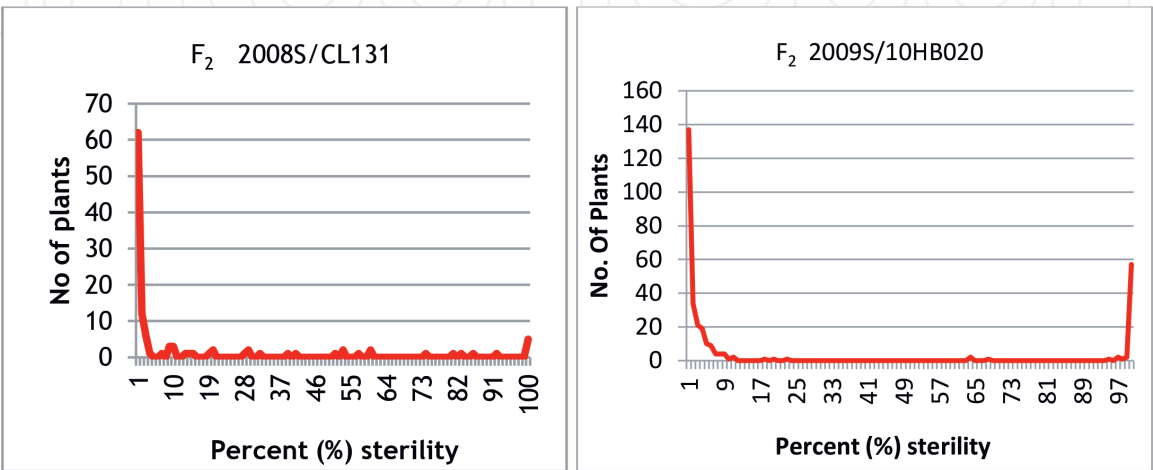


Figure 5.
Pollen sterility distribution of F₂ plants in populations of 2008S/CL131 and 2009S/10HB020 male sterile lines planted in 2013 at H. Rouse Caffey Rice Research Station, Crowley LA USA.

LOC_Os02g12290 encode a ribonuclease Z gene that when the SNP is present, it created a premature stop codon rendering the RNase Z^{S1} defective. The mechanism described that when the mutant male sterile is exposed under high temperature (28°C), it induced the accumulation of mRNA ribosomal protein UBL40 in microspore mother cell that were not processed by the defective RNase Z^{S1} enzyme consequently causing pollen degeneration. At lower temperatures (23°C), UBL40 mRNAs levels remained low allowing production of normal pollen. This mutation was reported in TGMS varieties Guangzhang 63S, Ans-1 and Zhu1S [28]. Zhang [44] discovered that the locus tms5, ptgms2-1 and tms9 were allelic and were all mapped to chromosome 2 that contains the similar ribonuclease Z gene. De Guzman [25] sequenced the locus LOC_Os02g12290 in line 2009S and discovered the same SNP present in TGMS lines. Inheritance studies on F₂ and BC₁F₂ showed similar segregation ratios. SNP marker were developed using CEL1 nuclease to identify association of the marker to the trait and showed that the markers were able to predict 95–100% of male sterile lines in F₂ and BC₁F₂ population [41].

3.3 Breeding and production of genetic male sterile rice

Since PTGMS and TGMS have different responses to temperature and photoperiod, methods in seed multiplication and breeding varies including selection of specific location and time of planting. On PTGMS lines, weak and strong photoperiodic responses were reported [45]. In strong photoperiodic response such as in long daylength, the critical sterility inducing temperature (CST) is low (21°C) and at short daylength high (25–26°C).

For a PTGMS lines with weak photoperiodism, the (CST) is about 22°C under short daylength. In China, Chen [45] suggested that seed production of this type should be bred during autumn in Guangdong and Guangxi, and in winter in Hainan province. Cold water irrigation treatment has been used extensively in lines with both weak and strong photoperiod. This solved the problem of low yields in multiplication of PTGMS lines such as Pei'-ai 64 s with low CST [46].

For TGMS lines, there is no weak or strong photoperiodism, thus timing and selection of location is important for seed production of male steriles and hybrids. On most TGMS lines, the ideal CST is about 22.5°C. Seed production are also treated with continuous cold water irrigation to increase seed yield during the winter [46].

In the US, RiceTec of Alvin Texas successfully used both ptgms and tgms lines however, seed production locations are unknown and specific methods of seed multiplications of male sterile lines are undisclosed.

In Louisiana USA, the LSU AgCenter initiated the hybrid rice breeding program in 2009 using ptgms and cytoplasmic male sterile lines obtained via a Material Transfer Agreement with the Guangxi Academy of Agricultural Sciences, Nanning, China. Test crosses made from these lines showed equal or superior grain and head rice yield compared to the current RiceTec commercial varieties. However, high chalk, lodging, and late maturities were observed that warrant the development of male sterile with improved agronomic traits and are suitable to the southern US conditions [47]. The breeding cycle starts with crosses of adapted lines to male sterile lines to produce F₁'s. Seeds from hybrids then harvested and spaced planted. Single plant selections of male sterile lines were performed by looking at sterile pollen stained with 1% I₂KI under the microscope during early heading. Plants suitable for generation advance were selected with the following characteristics: 98–100% pollen sterile, 60–80 cm in height, short flag leaf, intermediate tillering and with compact growth habit. Selected plants were uprooted, placed in one gallon pots and transferred in the greenhouse. The plants were ratooned by cutting ~9–10 cm from the soil line. The ratoons are allowed to grow up to early booting stage where the

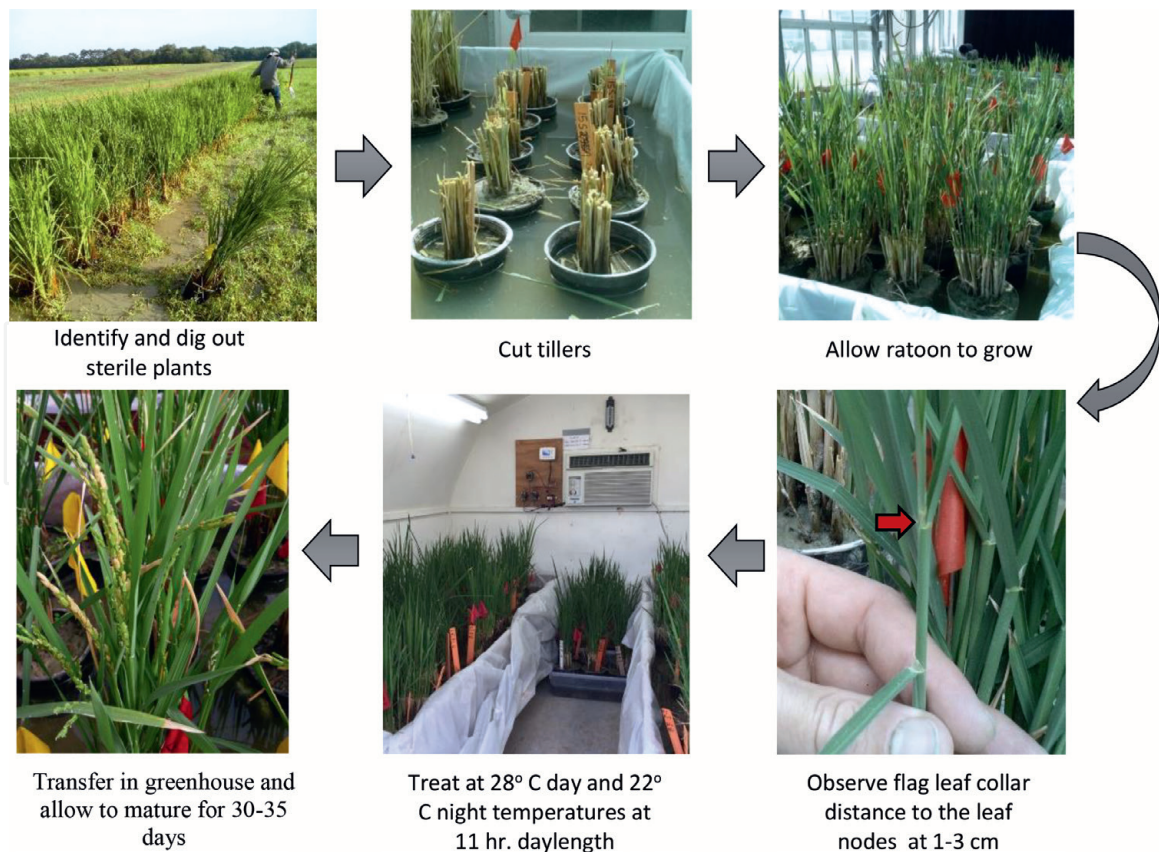


Figure 6.
 Methods of selection and production of male sterile lines in LSU Agcenter hybrid rice breeding program.

majority of the shoots have the measurement distance of about of 1–3 cm between the flag leaf collar to the leaf node or at about the early booting stage (meiotic division of pollen mother cell). Plants are then subsequently treated in a growth chamber with temperatures 22°C during the night and 28°C during the day at 11 h daylength for 10 d. After treatment, plants are then transferred to the greenhouse for 30–35 days for the seed to mature (**Figure 6**). Seed multiplication were carried out in Puerto Rico agricultural experiment station planted during October to November [48, 49].

4. Introgression of disease resistance in hybrid rice

Introgression of disease resistance traits in hybrid rice becomes a necessity largely due to the narrow genetic diversity of both CMS and genetic male sterile sources. A study showed leading hybrids from China that was introduced in Africa were out yielded by inbred checks due to non-adaptability and susceptibility to diseases and pests [50]. There were also reported insect and disease incidence in China that are more frequent in hybrid rice than on inbred varieties [5]. Research institutes such as the International Rice Research Institute (IRRI) was aware of these issues and has continued to develop new CMS, maintainer, restorers and genetic male steriles in diverse background. Current improvement on hybrid rice focuses on incorporation of resistance gene identified from inbred and wild sources. For instance, blast resistance genes have been introgressed in maintainer, restorer and S lines through hybridization, backcross and marker assisted selection (MAS) [6]. In the early 2000, varieties with multiple bacterial panicle blight resistance were released in Indonesia and China. These varieties were developed using MAS and produced significant yield gain demonstrated in farmer's field [51].

5. Future aspect

CMS and genetic male sterility revolutionize rice production due to its contribution to the development of hybrid rice. Elucidation of physiological and molecular mechanism leads to the establishment of the process involved in breeding male sterile and hybrids. The increase in yield and tolerance to biotic and abiotic stress are largely due to the effect of heterosis. However, the narrow genetic diversity presents a challenge as very few sources of male sterility are used. Improving parental lines by incorporation of genes with resistance to biotic and abiotic conditions are essential to secure the yield advantage of hybrids over inbreds. Different methods can be used to add genetic diversity to the hybrids. Mutation, MAS and interspecific hybridization are a proven approach to incorporate targeted traits as well as discovering new genes from wild relatives. Genomic selection as well as gene editing will likely play a significant role in future improvement of rice hybrids.

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Conflict of interest

The authors declare that they have no conflict of interests.

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