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Innovation and development of the third-generation hybrid rice technology

Chancan Liao [a], Wei Yan [a,b], Zhufeng Chen [b], Gang Xie [b], Xing Wang Deng [b,c,*], Xiaoyan Tang [a,b,*]

[a] Guangdong Provincial Key Laboratory of Biotechnology for Plant Development, School of Life Sciences, South China Normal University, Guangzhou 510631, Guangdong, China
[b] Shenzhen Institute of Molecular Crop Design, Shenzhen 518107, China
[c] Institute of Plant and Food Sciences, Department of Biology, Southern University of Science and Technology, Shenzhen 518055, China

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ABSTRACT

The breeding and large-scale application of hybrid rice contribute significantly to the food supply worldwide. Currently, hybrid seed production uses cytoplasmic male sterile (CMS) lines or photoperiod/thermo-sensitive genic male sterile (PTGMS) lines as female parent. Despite huge successes, both systems have intrinsic problems. CMS systems are mainly restricted by the narrow restorer resources that make it difficult to breed superior hybrids, while PTGMS systems are limited by conditional sterility of the male sterile lines that makes the propagation of both PTGMS seeds and hybrid seeds vulnerable to unpredictable climate changes. Recessive nuclear male sterile (NMS) lines insensitive to environmental conditions are widely distributed and are ideal for hybrid rice breeding and production, but the lack of effective ways to propagate the pure NMS lines in a large scale renders it impossible to use them for hybrid rice production. The development of "the third-generation hybrid rice technology" enables efficient propagation of the pure NMS lines in commercial scale. This paper discusses the establishment of "the third-generation hybrid rice technology" and further innovations. This new technology breaks the limitations of CMS and PTGMS systems and will bring a big leap forward in hybrid rice production.

1. Introduction

Heterosis indicates the phenomenon that the F_1 hybrid between two parental lines of different genetic backgrounds is often superior to the parental lines in stress tolerance, disease resistance, adaptability, growth, or yield. Heterosis is widely present in the biological world and is the foundation of hybrid crops. The utilization of heterosis has been proved to be highly fruitful in crop improvement. Maize was the first commercialized hybrid crop. Subsequently, hybrid production systems have been developed in crops such as rice, sorghum, rapeseed, and many others [1].

Rice is an important food crop in the world. The successful deployment of hybrid rice is a significant achievement in crop production. The hybrid rice breeding was initiated in China in the 1970s, and now hybrid rice is planted in most of the rice growing countries [2–5]. Cultivation of hybrid rice leads to a great improvement in rice productivity. In general, it increases the grain yield by over 20% compared to the inbred rice varieties [2–5]. The hybrid varieties are generated through either the "three-line system" or the "two-line system" [2–5]. The core of these systems is the large-scale propagation of pure male sterile seeds that can be used for the large-scale production of hybrid seeds.

The "three-line system", which is also known as "the first-generation hybrid rice technology", consists of a CMS line, a maintainer line, and a restorer line (Fig. 1A) [5,6]. The CMS lines are usually caused by abnormal mitochondrial genes encoding cytotoxic proteins [2,6]. There are at least seven CMS genes identified in rice, all of them encoding the mitochondrial proteins [2,6]. Three CMS genes, including CMS-WA, CMS-HL, and CMS-BT, have been used in the breeding of hybrid rice [2,6]. CMS-WA was originally discovered in a wild rice in Hainan Island in 1970 [2,6]. It encodes a mitochondrial protein that interacts with the nuclear-encoded mitochondrial transmembrane protein OsCOX11 to induce male sterility [7]. Most of the *indica* CMS lines currently commercialized in hybrid production use the CMS-WA gene [2–6]. The maintainer line has the same nuclear genes as the CMS line, but it does not have the abnormal mitochondrial gene [6]. The maintainer line has normal fertility and can self-pollinate to reproduce itself [6]. Cross-pollination of the CMS line by the maintainer line can propagate the CMS line [2–6]. The restorer line contains specific restorer of fertility (*Rf*) gene(s) that can inhibit the function of the

* Corresponding authors.
E-mail addresses: txy@frontier-ag.com (X. Tang), deng@pku.edu.cn (X.W. Deng).

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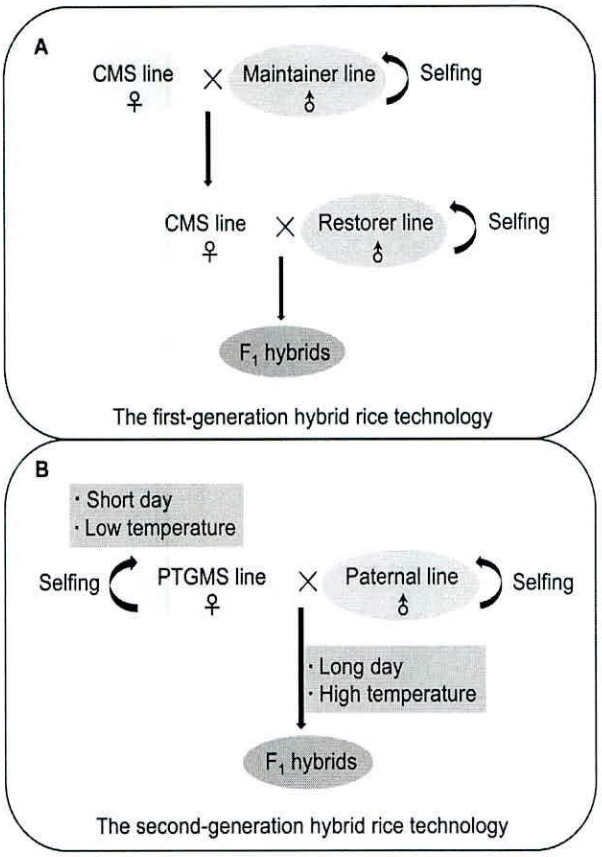

Fig. 1. Diagram of the first-generation and the second-generation hybrid technology in rice. (A) "The first-generation hybrid rice technology" consists of a cytoplasmic male sterile (CMS) line, a maintainer line, and a restorer line. The maintainer line and restorer line self-pollinate to reproduce themselves. Cross-pollination of the CMS line by the maintainer line propagates the CMS line. Cross-pollination of the CMS line by the restorer line propagates the F_1 hybrids. (B) "The second-generation hybrid rice technology" consists of a photoperiod/thermo-sensitive genic male sterile (PTGMS) line and a paternal line. The paternal line self-pollinates to reproduce itself. PTGMS line propagates itself through self-pollination under short day and/or low temperature restoring the male fertility, and outcrosses with the paternal line to produce the F_1 hybrid under long day and/or high temperature inhibiting the male fertility.

corresponding CMS gene by acting on the RNA or protein of the CMS gene [6]. For example, CMS-WA can be specifically inhibited by *Rf3* and *Rf4* genes, CMS-HL can be inhibited by *Rf5* and *Rf6*, and CMS-BT can be inhibited by *Rf1* [7–12]. Because the CMS gene function is suppressed by the *Rf* gene, the fertility is restored in the hybrid between the CMS line and the restorer line [6–12]. The first hybrid rice Nanyou-2 was derived from CMS-WA and was released in 1973. Since then, the CMS hybrid varieties had been quickly deployed for commercial production in China because of their obvious yield advantages over the inbred varieties [2,5,13]. Despite the huge success, CMS systems suffer from several intrinsic problems. The major problem is that the *Rf* genes exist in only 2%–5% of rice germplasms, therefore only a very small number of rice germplasms can be explored as restorer lines for heterosis [2–3,13–14]. Besides, it is also tedious to breed new restorer lines. Because the restorer line needs to carry the very specific *Rf* genes for the CMS genes, breeders not only need to select for the desired traits for plant development and stress tolerance, etc., but also need to make sure not losing the *Rf* genes and the combining ability from generation to generation, and these increase very much the workload and difficulties during breeding. In addition, it is also difficult to breed a new CMS line because it requires a maintainer line for propagation [2–4,13–14]. Thus breeders have to breed all the desired traits into the maintainer line and then change the cytoplasm through several generations of crossing. Furthermore, CMS genes have negative impact on hybrid performance and are unstable under certain environmental conditions [2–4,12–15]. Because of these problems, it is difficult to obtain CMS hybrids of strong heterosis. This is believed to be the main reason that CMS varieties stayed stagnant in yield increase in the last 20 years [2–4,14].

The "two-line system", which is also known as "the second-generation hybrid rice technology", depends on the male sterile lines controlled by the recessive photoperiod/thermo-sensitive genic male sterile (PTGMS) genes (Fig. 1B) [1–5]. There are at least 13 PTGMS loci identified in rice, but only three PTGMS genes have been cloned [16]. *PMS1* and *PMS3* encode phased small-interfering RNAs and long noncoding RNA, respectively, and both mutants are responsive to both photoperiod and temperature [17–19]. *TMS5* encodes RNase Z^{S1}, and *tms5* mutant is responsive mainly to temperature [20]. Over 70% of commercial two-line hybrid rice cultivars in China were bred with the *tms5*-containing male sterile lines [20]. Compared with the three-line systems, the PTGMS systems have the following advantages. First, the PTGMS systems do not need a maintainer line for propagation of the male sterile lines, which simplifies the breeding and production procedures. Because male fertility of the PTGMS lines is reversible in response to environmental conditions, PTGMS lines can propagate themselves through self-pollination under conditions restoring the male fertility. Under conditions that inhibit the male fertility, PTGMS lines outcross with paternal lines to produce hybrid seeds [1–4]. Second, because the male sterility in PTGMS lines is controlled by recessive nuclear genes, they can cross with any plants carrying the wild type fertility gene to restore the fertility in hybrids. Therefore, most rice germplasms can be explored for breeding of hybrids of superior heterosis [1–4]. In addition, the two-line systems are free of the cytoplasmic negative effects imposed by the abnormal mitochondrial genes. Because of these advantages, the PTGMS systems were quickly adopted for farming since the initial application in the 1990s, and hundreds of environment-sensitive genic male sterile lines and two-line hybrids have been released for commercial production [2,16,21]. At present, two-line hybrids exceed three-line hybrids in cultivation acreage, and the highest yielding varieties currently cultivated in China are mostly PTGMS hybrids [16,21]. Despite the huge success, the PTGMS systems also have intrinsic problems. Most of the commercial PTGMS lines are sterile when grown at long day with temperature above 25 °C, but fertile when grown at short day with temperature below 23 °C during the booting stage [2,21–22]. To meet these requirements, strict environmental conditions must be used for propagation of the PTGMS seeds as well as production of hybrid seeds. The small window of the critical temperatures for fertility transformation (CTFT) also brings high risks to the production of PTGMS seeds and hybrid seeds under unpredictable weather conditions [2–3,21–22]. For example, during propagation of the PTGMS seeds, an increase of environmental temperature can reduce the fertility of PTGMS lines and decrease the yield of PTGMS seeds, while during the hybrid seeds production, a drop in temperature can render the PTGMS line fertile, and self-pollination can cause impurity of the hybrid seeds [2–3,21–22]. The failure in hybrid seeds production happened quite frequently in recent years in China, which brought huge losses [2,21]. In addition, the CTFT in PTGMS lines often shifts up after a few generations of propagation [22]. Because the increase in fertile temperature brings higher risk to hybrid seeds production, PTGMS individuals of suitable CTFT must be re-isolated repeatedly during production [3,22]. Furthermore, the CTFT trait is influenced by minor QTL, and this significantly increases the

difficulty and uncertainty to breed the PTGMS genes into different genetic backgrounds using marker-assisted selection [16,22].

2. Establishment of the third-generation hybrid rice technology

Male sterility caused by nuclear genes that are insensitive to environmental conditions is common in flowering plants. However, commercial application of these mutants is limited because of the difficulty to propagate the pure male sterile lines in a large quantity. In 1990, Rao et al. [23] presented a number of ways for propagation and selection of nuclear male sterile lines in various crop species based on the genetic linkages between male sterility and special phenotypes such as leaf morphology, flower morphology, anther color, seedling color, endosperm color, and shrunken endosperm, etc. However, most of these methods have not been widely deployed for agricultural practices because of the late expression of the phenotype in plant development or the poor linkage between the male sterility and the phenotype. In 1993, Williams and Leemans [24] proposed an idea to obtain a transgenic maintainer by transforming a fertility-restoration gene linked with a pollen-lethality gene. In 2002, Perez-Prat and van Lookeren Campagne [25] discussed two strategies to obtain maintainer lines and pure nuclear male sterile lines. One strategy is to transform the fertility restoration gene linked with a seed-color gene into the male sterile plant to obtain a color maintainer. Cross-pollination of the color-maintainer to the male sterile line would generate 50% of male sterile seeds and 50% of color maintainer seeds that can be separated based on the seed color. The other strategy is to transform the fertility-restoration gene linked with a pollen-lethality gene into the male sterile plant. Cross-pollination of the pollen-lethality maintainer to the male sterile line would generate pure male sterile seeds.

Based on these ideas, DuPont-Pioneer devised Seed Production Technology (SPT) in 2006 to produce the transgenic maintainer line in maize by transforming the male sterile mutant *ms45* with the wild type gene *MS45* linked with the maize α-amylase gene *ZmAA1* to disrupt the transgenic pollen and the red florescence protein gene *DsRed* to mark the transgenic seed (Fig. 2) [26]. *ZmAA1* was placed under the pollen-specific promoter *PG47* to prevent the accumulation of starch in the transgenic pollen grains, which specifically inactivated the pollen grains carrying the transgene [26–27]. *DsRed* was placed under the aleurone-specific *LTP2* promoter to make the transgenic seeds produce red florescence [26–27]. The hemizygous transgenic plant recovered the male fertility and produced two kinds of pollen grains, the viable pollen grains without the transgene and the nonviable pollen grains with the transgene, in 1:1 ratio [27]. Because the transgene had no effect on the female organ, self-pollination of the transgenic plant produced two kinds of seeds, those with the transgene and those without the transgene, in 1:1 ratio [27]. The transgenic seeds were labeled with red florescence because of the function of *DsRed* gene, and the non-transgenic seeds were color-free. The two kinds of seeds could be mechanically separated based on the seed color. The non-transgenic seeds are the male sterile line, and the transgenic seeds are the maintainer line. Cross-pollination of the male sterile line by the maintainer produced the pure male sterile seeds in a large quantity [27]. The SPT has been applied in hybrid corn production in the United States since 2012, and the produced hybrid corn was certified as non-GMO (genetically modified organisms) by regulatory agencies in the United States, Australia and Japan [27]. Application of SPT in maize saves the costs for mechanical detasseling, which is the predominant method for commercial maize hybrid seed production.

Although the SPT technology was first developed in maize, it is fundamentally more useful for crops that have bisexual flowers not amenable to manual emasculation, including rice, wheat, sorghum, and rapeseeds, etc. In 2010, Wang et al. [28] adopted the same idea and transformed the rice *MS26* (*OsCYP704B2*) gene linked with the maize α-amylase gene *ZmAA1* under the pollen-specific promoter *PG47* and the red florescence protein gene *DsRed* under the aleurone-specific *LTP2* promoter into the *oscyp704b2* male sterile mutant derived from Wuyungeng, a *japonica* rice variety [29–30]. As expected, the resulted transgenic line carrying a single transgene insertion produced 1:1 florescent fertile seeds and non-florescent male sterile seeds, which verified the application potential of this technology in rice [28–29]. However, because Wuyungeng is a *japonica* rice that is neither suitable for hybrid rice production nor a good material for commercial breeding of new male sterile lines, and the red florescence marker was not highly stable for seed sorting, the transgenic line was not further deployed for application [28].

In 2016, Chang et al. [31] published another work in rice by using the recessive *osnp1* male sterile mutant derived from the *indica* rice Huanghuazhan (HHZ) and the corresponding wild type gene *OsNP1* for construction of the nuclear male sterile (NMS) system. HHZ is an elite *indica* cultivar that is semi-dwarf with super high yield and good eating-quality and is widely cultivated in diverse geographical regions in China. It is a core germplasm for commercial rice breeding along Yangtze River and southern part of China [32]. With the purpose to develop a practical NMS system for commercial breeding of hybrid varieties, the group started from constructing the HHZ mutant library and screening for the male sterile mutants that can be developed into a male sterile line of commercial value [31]. From ~300 male sterile mutants, they selected the *osnp1* mutant for construction of the NMS system, because this mutant had normal vegetative growth, high stigma extrusions, high outcrossing rate, no pollen, and stable male sterility under diverse environment conditions [31]. *OsNP1* was cloned using the SIMM method based on the whole genome resequencing of the mutant plants [31,33]. *OsNP1* is a novel gene specifically expressed in the tapetum and microspores and is required for pollen exine formation [31]. The tissue-specific expression pattern of *OsNP1* further ensures that the *osnp1* mutation affects only the male fertility but not any other developmental processes. By transforming the *OsNP1* gene linked with *ZmAA1* under *PG47* promoter and *DsRed* gene under *LTP2* promoter plus the *35S* enhancer into *osnp1* mutant, the team obtained a maintainer line named Zhen18B carrying one copy of the transgene [31]. Zhen18B has no difference from the wild type HHZ plant in morphology, growth and development, and seed setting. As expected, self-pollination of Zhen18B propagated itself and the male sterile Zhen18A seeds in 1:1 ratio, while cross-pollination of Zhen18B to Zhen18A propagated pure Zhen18A seeds to a large quantity [31]. The transgene in Zhen18B stayed stable in at least 12 generations of millions of plants tested by far. It also stayed stable when crossed into various other rice backgrounds during the breeding of new NMS lines [31]. Approximately 85% of the hybrids between Zhen18A and other rice germplasms out-performed their parents in the per-plant yield [31]. The seeds of Zhen18A were distributed to many rice breeders in China for test crossing. A number of hybrids with very high yield and excellent grain quality were obtained. In 2018, Zhen18A was officially certified to meet the national standard of commercialization in China; and in 2020, permit for production test was assigned to Zhen18B by the Genetically Modified Organisms Safety Committee in China (Table 1). These marked the successful establishment of the NMS system in rice, which was also called "the third-generation hybrid rice technology" (Fig. 2) [29].

Compared with the CMS and PTGMS systems, the NMS system has several obvious advantages. First, since the male sterility phenotype is controlled by a recessive nuclear gene, any rice germplasm containing the wild type gene can restore the fertility of

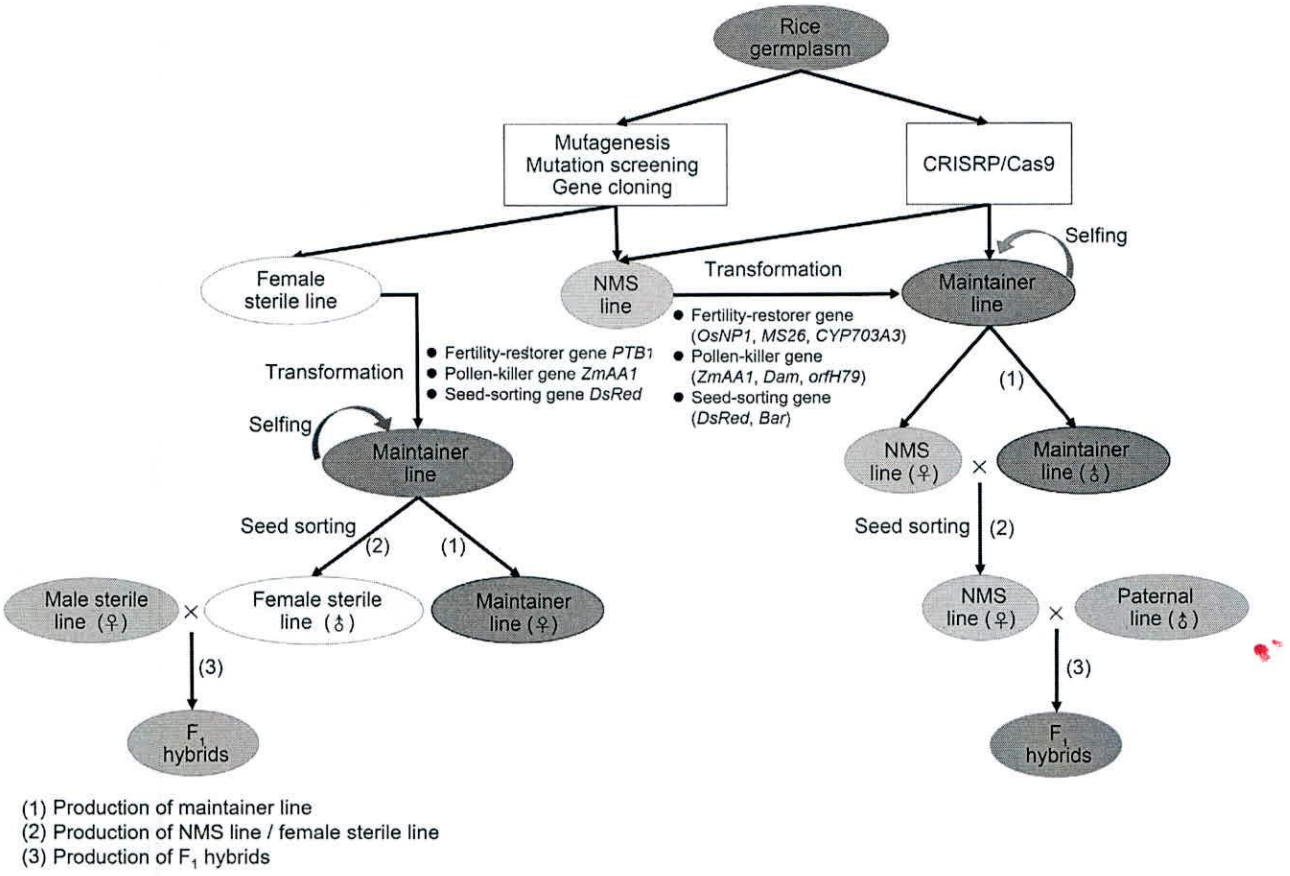

Fig. 2. Diagram of the third-generation hybrid technology in rice.

Table 1
Summary of NMS lines derived from the *OsNP1*-maintainer.

Name	Parental lines	Combining ability (high/middle/low)	NMS yield (kg ha^{-1})	Hybrid yield (kg ha^{-1})	Certified
Zhen18A	HHZ	Middle	100	120	Yes
Zhen20A	(Zhen18B/Guangzhan63s)//(You1/*H11*)	Middle	180	200	Yes
Zhen21A	(Zhen18B/Guangzhan63s)//Ping138	Middle	180	200	Yes
Zhen6A	Zhen18B/Helisimiao	High	120	150	No
Zhen9A	Zhen18B/Lixiang18s	High	180	200	No
Zhen68A	Zhen18B/Guangzhan63s	High	200	220	No
Zhen136A	Zhen18B/Guangzhan63s	High	200	220	No

the hybrid. Thus, the NMS system allows broader choices of germplasms as paternal lines to breed hybrids of superior heterosis. Second, the male sterility of the NMS line is insensitive to environmental conditions, thus both the male sterile line and the hybrid seeds can be propagated under regular farming conditions. This significantly lowers the demand on specific environmental conditions for seed production, which are often difficult and costly to satisfy with the PTGMS systems. In addition, it can also reduce the risk induced by unpredictable weather changes on hybrid seed production. Third, the male sterility and fertility restoration are each controlled by a single genetic locus, and both traits can be stably inherited in different genetic backgrounds. Thus, both loci can be easily crossed into other cultivars to generate new maintainers and male sterile lines using marker-assisted breeding. Fourth, because the fertility restoration gene is closely linked to the pollen-killer gene, it blocks the transmission of transgenic components into environment through pollen, which significantly increases the environmental safety. Finally, although the technology involves transgenics, only the maintainer line carries the transgene. Both the male sterile seeds and the hybrid seeds are non-transgenic. Thus, transgenic oversight is applicable only to the maintainer line, which requires only a small acreage for cultivation. The production of hybrid seeds and cultivation of hybrid rice do not involve transgenics and thus do not require transgenic oversight.

3. Innovations to the third-generation hybrid rice technology

Zhen18B was the first NMS system constructed in rice. Although the resulting Zhen18A line displayed good application potential, there are still traits requiring improvements, such as

disease resistance, stress tolerance, and particularly the yield trait following cross-pollination and the combining ability to produce super heterosis. More importantly, as a technology with a big application potential, genetic diversity and technical perfection of the NMS systems are both necessary to ensure the wide and sustainable application in large scales. Following the publication of Zhen18B in 2016, a number of valuable innovations to the NMS systems have been published. Some of these innovations have been done in maize, but they are also valuable to the improvement of the NMS technology in rice.

3.1. Innovations of the pollen-inactivation function

For NMS technology, inactivation of the transgenic pollen grains is critical, because it not only ensures propagation of pure male sterile seeds during cross-pollination of the maintainer line to the male sterile line, but also prevents the transmission of transgenic components to the environment. The *ZmAA1* gene under *PG47* promoter in the maize SPT system and rice Zhen18B line can effectively kill the transgenic pollen grains, but cross-pollination of the maintainers to the male sterile lines still showed transgene transmission rates of 0.002%–0.518% in different transgenic lines of the maize SPT system and 0.01%–0.08% in Zhen18B line growing at different seasons, based on the numbers of red florescent seeds produced [27,31]. Therefore, there are still rooms for improvement of the pollen-killing function.

The deployment of new pollen-killer genes is an important innovation to the NMS technology. Besides *ZmAA1*, several other genes have been reported with pollen-killing functions in various plants, including a new rice α-amylase [34], the cytotoxin barnase [35–36], and the rice CMS gene *orfH79* [37]. The new rice α-amylase gene driven by the *PG47* promoter is able to kill the transgenic pollen grains in rice [34]. This result implicated that many other α-amylase genes in other plant species may deliver the pollen-killing function in rice as well. The *Barnase* gene has been used for genetic engineering of male sterility in various plants either by disrupting the tapetum or pollen grains [38]. Because barnase has strong cytotoxic activity, to reduce the impact of its leaky expression, people have tried to split the gene into two parts and then use two different pollen-specific promoters to drive the N-terminal part and C-terminal part in the same T-DNA, or to use two different pollen-specific promoters to drive *barnase* and its inhibitor gene *barstar* in the same T-DNA to inactivate the transgenic pollen grains [35–36]. *orfH79* encodes a mitochondrial protein inducing pollen sterility in HL-CMS rice [39]. ORFH79 acts on the electron transport chain in mitochondria, causing abnormal energy supply and abnormal accumulation of reactive oxygen species, which leads to male gamete infertility [40]. When the recombinant gene of ORFH79 fusion with the RF1b mitochondrial signal peptide was expressed in *indica* rice 93-11 under the *PG47* promoter, the transgenic plant displayed the 1:1 ratio of normal and abnormal pollen grains, confirming the pollen-killing activity of the transgene [37]. This pollen-killer gene was then linked with the seed-marker gene *DsRed2* and the fertility-restoration gene *OsCYP703A3* and transformed into 93-11^{03a3}, a male sterile mutant of 93-11 with the *OsCYP703A3* gene knocked out. A stable maintainer line was obtained and named 93-11-3B [37]. The 93-11-3B plant displayed ~1:1 ratio of normal pollen grains and abnormal pollen grains, and self-pollination of the 93-11-3B plant produced ~1:1 segregation of seeds with red florescence and seeds without florescence, which further verified that *orfH79* can be used as a pollen-killer gene in the NMS system [37]. Cross-pollination of 93-11-3B to the male sterile lines showed the transgene transmission rates of 0.14%–0.17%, which was >2-fold higher than that displayed by the *ZmAA1* gene in Zhen18B [37]. In addition to *orfH79*, many other CMS genes have been cloned from rice and several other plant species [6]. Further exploration of these CMS genes may identify other genes that can be developed into pollen-killing tools for the construction of NMS systems in rice and other crops as well.

Inactivation of transgenic pollen requires a pollen-specific promoter to drive the pollen-killer gene at the late stage of pollen development. Thus, deployment of a very strong promoter for pollen-inactivation can also improve the NMS system in rice. A number of late-stage pollen-specific promoters have been identified from various plant species, however, only a few have been tested for pollen-inactivation thus far, including the maize *PG47* promoter [26–28,31,35–37,41–42]. The *PG47* gene is specifically expressed in pollen grains at the stage of first mitosis to pollen maturity (stage 11–12) [43]. To obtain more promoters suitable for construction of the NMS technology in rice, Wang et al. [44] conducted an RNA-seq analysis to identify genes that are specifically expressed in mature pollen grains. They then used qRT-PCR analysis to determine the relative RNA expression levels of these genes in anthers at different developmental stages. They tested six *LSP* (late-stage pollen-specific) promoters of genes that showed relatively high levels of RNA expression, and found that the top three promoters (*OsLSP3*, *OsLSP5*, and *OsLSP6*) very active at stages 11 and 12 could drive *ZmAA1* to inactivate pollen in rice, while the promoters of relative lower activities could not. The promoter of *OsLSP4*, which showed higher gene expression at stage 12 but lower expression at stage 11, could not drive *ZmAA1* to inactivate pollen, indicating that strong promoter activity at stage 11 was critical for pollen inactivation. The *OsLSP3*, *OsLSP5*, and *OsLSP6* provide additional tools for genetic engineering of the rice NMS systems. It is expected that promoters stronger than these three gene promoters can produce even better pollen-killing results.

With more pollen-killer genes and late-stage pollen-specific promoters identified, combinational use of these components can produce even better outcome in pollen-inactivation, as recently demonstrated by the multi-control sterility (MCS) system in maize [45–46]. The MCS system involves co-expression of two pollen-killing genes, *ZmAA1* and DNA adenine methylase gene (*Dam*) in pollen. The *ZmAA* gene is placed under the promoter *PG47* to prevent the starch accumulation in transgenic pollen grains. The *Dam* gene is constructed under the pollen-specific promoter *Zm13* to catalyze the methylation of adenine residues in pollen DNA, which affects the cell viability of transgenic pollen [47–49]. The MCS vector containing the fertility restoration gene, two pollen-killer genes, the red fluorescence seed-marker gene, and a herbicide resistance gene was transformed into the male sterile mutant. Compared with transgenic plants expressing only *ZmAA1* under the *PG47* promoter, the MCS transgenic plants showed 7 ~ 8-fold reduced rates of transgene transmission [45–46]. This strategy is also expected to reduce the transgene transmission from the maintainer line into the male sterile line through pollination in rice.

3.2. Innovations to the seed sorting function

The NMS systems can propagate the male sterile seeds by self-pollination of the maintainer line. Because this also produces 50% maintainer seeds, a highly efficient seed sorting machine must be used for large scale production. Without an efficient seed sorting machine, male sterile seeds can be propagated by cross-pollination of the male sterile lines by the maintainer lines, which would significantly reduce the demand on seed sorting to clean the contaminated maintainer seeds from transgene transmission. However, to achieve a maximal yield of the male sterile seeds, it is necessary to use pure maintainer lines for cross-pollination. The pure maintainer seeds can be obtained if a herbicide resistance gene is added to the transgenic cassette, so the male sterile seeds

can be selectively killed by herbicide treatment (Fig. 2). This strategy was tested by An et al. [50] who transformed the fertility restorer gene, pollen-killer gene, color selection gene, and the herbicide resistance *Bar* gene linked in one T-DNA cassette into the maize male sterile *ms7* mutant. Because only the transgenic maintainer lines carry the herbicide resistance gene, the mixed male sterile lines were removed by herbicide treatment [50]. This strategy is expected to work well in the rice NMS system as well.

3.3. Combination of CRISPR/CAS9 with the NMS technology to create the male sterile line and maintainer line in one step

To convert a rice material into a NMS maintainer line, cross the established NMS maintainer line with this material as the recurrent pollen donor is a good choice for the sake of simplified transgenic regulation. However, it would take at least four generations of backcrossing and then two generations of self-pollination to get a stable NMS maintainer line. This is a time-consuming and labor extensive process. Besides, the resulted NMS maintainer line may contain more or less the genetic materials from the donor transgenic parent.

To solve these problems, Qi et al. [51] proposed a strategy that combines the CRISPR/Cas9 gene editing technology with the NMS technology to create the male sterile line and maintainer line in one step (Fig. 2). They constructed two plant transformation vectors. One vector contains the CRISPR/Cas9 gene editing tool targeting two non-coding regions of the maize sterility gene *ZmMS26*. The function of this vector is to delete an exon of *ZmMS26* to create the male sterile mutation. The other vector contains the linked genes of *ZmMS26* cDNA under the *ZmMS26* native promoter, *ZmAA1* gene under *PG47* promoter, and *DsRed* gene under *LTP2* promoter. The function of this cassette is to create the NMS maintainer for the *zmms26* mutant. They co-transformed the two constructs into a normal maize line. The CRISPR/Cas9 gene editing tool created deletion in *ZmMS26* gene causing male sterility, but the plant co-transformed with the NMS construct restored the fertility and set non-florescent male sterile seeds as well as the fertile florescent seeds. Fertile progeny carrying the homozygous *zmms26* mutation and the NMS cassette but lacking the CRISPR/Cas9 cassette was identified by PCR as the NMS maintainer line, and the sterile progeny lacking the CRISPR/Cas9 cassette was the *zmms26* male sterile line. This method greatly shortens the time for construction of NMS systems based on the known male sterile genes.

3.4. Construction of nuclear female sterile line of normal male fertility

Currently, commercial production of rice hybrid seeds is carried out by planting the two parental lines side-by-side in separate rows, and then using manual assistance to facilitate cross-pollination. When pollination is finished, the paternal lines are removed manually to avoid contamination of the hybrid seeds by the paternal seeds. This process is very laborious and unsuitable for mechanized production of hybrid seeds, and it is the major contributor to the high price of hybrid seeds. If the paternal line is female sterile but with normal male fertility, then the two parental lines can be mix-planted for hybrid seed production, which would facilitate the mechanized production of hybrid seeds.

Xia et al. [52] attempted to innovate the technology for propagation of female sterile seeds by using the rice recessive *ptb1* mutant and the corresponding wild type gene (Fig. 2). *PTB1* (*POLLEN TUBE BLOCKED 1*) gene is required for pollen tube growth in transmitting track after pollen germination, and *ptb1* mutant blocks the pollen tube growth, resulting in female sterility [53]. The *PTB1* was constructed together with the pollen-killer gene *ZmAA1* and seed-marker gene *DsRed*, and the construct was transformed into the *ptb1* mutant plant [52]. The function of *PTB1* restored the female fertility of the transgenic plant, but pollen grains carrying the transgene were inactivated by the pollen-killer gene. Self-pollination of the transgenic plant produced seeds carrying the transgene that are fertile with red florescence, and non-transgenic seeds that are female sterile without red florescence. The two kinds of seeds are sorted out based on the red florescence. The female sterile line can be used for hybrid seed production, and the transgenic line can be used as the maintainer line for propagation of the female sterile line through self-pollination. Although the work proved the concept, however, because the *ptb1* mutant is not completely sterile and has a seed setting rate of ~1.8% [53], this system cannot be used for commercial application. Further improvement of this technology should use a mutant of complete female sterility.

3.5. Synthetic apomixis as a strategy to fix the heterosis

Apomixis is an asexual reproduction process in which clonal seeds are produced without meiosis and fertilization [54]. Through apomixis, a hybrid of strong heterosis can reproduce itself without chromosomal recombination and segregation, thus the heterosis can be passed on through generations [54]. This method has enormous advantages over the hybrid systems because it needs neither the male sterile seeds nor cross-pollination for production of hybrid seeds. Apomixis does not exist naturally in crops such as rice, maize, wheat, etc. However, it was found recently that CRISPR knockout of three genes important for meiosis (*PAIR1*, *REC8*, and *OSD1*) can generate a *MiMe* (mitosis instead of meiosis) mutant with the meiosis replaced by a mitosis-like division [55,56]. The *MiMe* mutant can generate diploid gametes identical to the mother genome. Further expression of the *BBM1* (*BABY BOOM1*) in egg cells or disruption of *MATRILINEAL* (*MTL*) in the *MiMe* background can induce the production of clonal seeds [55,56]. By simultaneous engineering *MiMe* with egg-expression of *BBM1* gene or disruption of *MTL* gene in the F_1 hybrid, synthetic apomixis can be established to produce the F_1 clonal seeds (Fig. 3). Although this strategy is very attractive, currently it can only produce a few diploid seeds and thus is still at the research stage.

4. Future perspectives of the third-generation hybrid rice technology

The development of the NMS systems enables the use of recessive nuclear genes for hybrid rice breeding and production, which overcomes the problems of traditional CMS and PTGMS systems. This new technology is a significant breakthrough in the field of hybrid rice breeding and will bring a huge step forward in hybrid rice production. With the rapid development of molecular biology, more genes will be identified that can be used for further improvement and perfection of the NMS systems. Decades of efforts in traditional rice breeding have accumulated numerous excellent rice germplasms with superior traits in yield, disease resistance, stress tolerance, grain quality, and many others [57]. These materials plus the abundant rice genomic data and tools for molecular markers assisted selection will enable the rapid breeding of new NMS lines with improved traits based on the established NMS systems through crossbreeding [57].

With the CRISPR/Cas9 technology available and many male sterile genes cloned, it is convenient for *de novo* construction of new NMS systems. Several factors should be considered in selecting rice materials and male sterile genes for *de novo* construction of NMS systems. First, it is better to use the elite rice lines amenable to transformation and with big stigma and high seed-setting rates upon cross-pollination, because these reproductive traits are necessary for the high yield during the production of hybrid

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