

The effects of *Rf5* and *Rf6* on fertility restoration in Honglian-type cytoplasmic male sterile (CMS) lines of *japonica* rice (*Oryza sativa* L. ssp. *japonica*)

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Abstract

The Honglian (HL)-type cytoplasmic male sterility (CMS) has only been used in the development of three-line *indica* rice hybrids, and the fertility of HL-type *indica* CMS lines can be restored by two nonallelic fertility-restorer (*Rf*) genes, *Rf5* and *Rf6*. For the development of HL-type *japonica* hybrid combinations, it is therefore necessary to determine whether *Rf5* and *Rf6* can restore the fertility of HL-type *japonica* CMS lines. Here, we genetically characterized HL-type *japonica* CMS lines and the ability of *Rf5* and *Rf6* to restore fertility for breeding HL-type *japonica* hybrids. I₂-KI pollen staining revealed that HL-type *japonica* CMS lines and their derived testcross F₁ hybrids had stained abortive pollen grains, unlike HL-type *indica* CMS lines. Crossing experiments showed that *Rf5* and *Rf6* partially restored the fertility of HL-type *japonica* CMS lines, and *Rf6* showed higher restorability than *Rf5*. Furthermore, we found that there were additive and dosage effects of *Rf5* and *Rf6* with respect to fertility restoration in HL-type *japonica* CMS lines. These results give critical insight into the breeding of HL-type *japonica* CMS lines and restorers, which will be helpful for the development of commercial HL-type *japonica* hybrids.

Introduction

Rice is the most important staple food crop that feeds more than half of the world's population, and the breeding of high-yielding rice varieties is very important to meet the requirements of an increasing world population. In the past decade, rice yield has increased significantly with the introduction of semi-dwarf varieties and the exploitation of heterosis, and hybrid rice technology is considered to be a major strategy for increasing the yield potential of rice (Cheng et al. 2007). In breeding practice, three-line and two-line systems are the main strategies for development of hybrid rice. The three-line hybrid breeding system is composed of a cytoplasmic male sterile (CMS) line, a maintainer line and a restorer line (Fujimura et al. 1996; Yuan 1994). Currently, three-line *indica* hybrids are grown on a large scale, but the planting area of *japonica* hybrid rice has been limited to around 100,000 ha, accounting for < 3% of the total *japonica* rice grown in China (Ma and Yuan 2015; Deng et al. 2006). Therefore, the development and application of *japonica* hybrids will be an important way to increase the total rice grain yield in China.

The development of three-line hybrid rice is based on CMS, and Wild abortive (WA), Honglian (HL), and Chinsurah Boro II (BT) are three representative types of CMS used for commercial hybrid rice seed production. The BT-type CMS is mainly utilized in *japonica* hybrid cultivation, while the WA-type and HL-type CMS systems are widely used for three-line *indica* hybrids (Chen and Liu 2014; Huang et al. 2014; Li et al. 2007; Yuan 1994). Seeds resulting from self-pollination in some BT-type CMS lines and the potential genetic vulnerability caused by intensive use of BT-type CMS are considered to be two main constraints in hybrid *japonica* rice breeding. (Li et al. 2007; Tang et al. 2005; Tang et al. 2008; Wang et al. 1991). In order to overcome the deficiencies of the BT-type CMS lines, the incorporation of WA- and HL-type CMS into *japonica* hybrids has been proposed as a selection strategy in the past (Zheng et al. 2020). In a previous study, WA-type and HL-type *japonica* CMS lines were found to have more stable sterility than BT-type *japonica* CMS lines (Zhu et al. 2010). However, the fertility restoration in WA-type *japonica* CMS lines is extremely poor, resulting in the lack of restorer lines in *japonica*, which means that large-scale breeding

and cultivation of WA-type hybrid *japonica* rice is not possible. Compared with WA-type *japonica* CMS lines, fertility in HL-type *japonica* CMS lines can be restored more easily, which means that it is possible to breed three-line *japonica* hybrids based on the HL-type CMS (Tang et al. 2005; Zhu et al. 2010). In addition, the HL- and BT-type CMS lines share similar restoration and maintenance relationships, and the HL-type *japonica* CMS lines can be bred easily with the BT-type *japonica* maintainer by backcrossing (Tan et al. 2008; Zhu et al. 2010). In *japonica* rice varieties, only BT-type restorer lines have been bred and used in the development of hybrids. However, it has been shown that most BT-type *japonica* restorer lines have only partial fertility restorability to HL-type *japonica* CMS lines (Zhang et al. 2016; Zhu et al. 2010). Thus, breeding of HL-type *japonica* restorer lines with strong restorability is the most important and urgent issue in the development of HL-type *japonica* hybrids.

CMS can be restored by the presence of a fertility restorer gene (*Rf*) in the nucleus, and *Rf* genes in restorer lines play a fundamental role in the spikelet fertility of F₁ hybrids. Thus, the genetic characterization of *Rf* genes can provide crucial information for breeding restorer lines. Usually, the CMS/*Rf* systems act in a target-specific manner, meaning that *Rf* genes are highly specific in the fertility restoration of the CMS lines. To date, several *Rf* genes have been identified in rice. *Rf3* and *Rf4*, two major fertility restorer genes located on chromosomes 1 and 10, respectively, have been identified for the recovery of pollen fertility in WA-CMS lines (Ahmadikhah and Karlov 2006; Tang et al. 2014; Zhang et al. 1997). *Rf5* and *Rf6*, are located on chromosomes 10 and 8, respectively, and have been mapped and cloned for HL-CMS (Hu et al. 2012; Huang et al. 2000; Huang et al. 2012; Huang et al. 2015). The restorer genes *Rf1a/Rf1b* on chromosome 10 for BT-CMS (Akagi et al. 1996; Komori et al. 2004; Wang et al. 2006), *Rf17* on chromosome 4 for Chinese wild rice (CW)-CMS (Fujii and Toriyama 2009), and *Rf2* on chromosome 2 for Lead rice-type CMS (Itabashi et al. 2011), have been mapped and cloned. Although the *Rf* genes for HL-type CMS have been extensively investigated in *indica*, there are few related empirical studies describing the characterization and mapping of *Rf* genes for HL-CMS in *japonica* rice varieties (Zhang et al. 2016). Thus, analyzing the restorability of *Rf5* and *Rf6* in *japonica* rice is a reasonable choice for breeding HL-type *japonica* restorer lines, which will be valuable for breeding high yielding HL-type *japonica* hybrids.

In our previous study, near-isogenic lines (NILs) for the *Rf5* and *Rf6* genes and the polygene pyramid lines (PPLs) combining *Rf5* with *Rf6* were developed in *japonica* rice (Zhang et al. 2019). Here, crossing experiments were performed using NILs and PPLs as the male parent and HL-type *japonica* CMS lines as female parents to evaluate the abilities of *Rf5* and *Rf6* to restore male fertility in *japonica* lines. In addition, the functional model and effects of *Rf5* and *Rf6* on the fertility restoration in *japonica* were analyzed. Our discovery will assist in analyzing the differences of fertility restoration in HL-type CMS in *indica/japonica* genetic backgrounds and strengthen the breeding of *japonica* restorer lines for the development of HL-type *japonica* hybrids.

Materials And Methods

Plant materials

The HL-type *indica* CMS line HL-YuetaiA (HL-YTA) and three pairs of isonuclear alloplasmic *japonica* CMS lines; HL-LiuqianxinA (HL-LQXA) and BT-LQXA, HL-NipponbareA (HL-NIPA) and BT-NIPA, and HL-LingfengA (HL-LFA) and BT-LFA were used in this study. *Rf5* and *Rf6* are two major HL-type *Rf* genes identified from the elite restorer line *indica* '93-11' (Hu et al. 2012; Huang et al. 2015). From 2015-2018, we performed marker-assisted selection (MAS) to develop the NILs for *Rf5* and *Rf6* in the HL-YTA background in order to identify the effects of *Rf5* and *Rf6* on fertility restoration in HL-type *indica* CMS lines. The NILs for *Rf5*, *Rf6* designed NIL^{*Rf5*} and NIL^{*Rf6*}, and the polygene pyramid lines PPL^{*Rf5+Rf6*} in the BT-NIPA genetic background were developed in our previous study (Zhang et al. 2019). In 2018, the isonuclear alloplasmic *japonica* lines were used as females in separate crosses with NIL^{*Rf5*}, NIL^{*Rf6*}, and PPL^{*Rf5+Rf6*}, resulting in a testcross population consisting of nine pairs of F₁ hybrids for evaluating the fertility restoring ability of these two *Rf* genes in *japonica* rice lines. Subsequently, three F₂ populations derived from crosses between HL-NIPA and NIL^{*Rf5*}, HL-NIPA and NIL^{*Rf6*}, and HL-NIPA and PPL^{*Rf5+Rf6*} were planted in 2019 to further analyze the effects of the *Rf* genes on fertility restoration in HL-type *japonica* CMS lines. Twenty plants were grown for each CMS line or each testcross F₁ line under natural conditions in the field. These plant materials were all planted at the experimental field of Yangzhou University in Yangzhou, Jiangsu Province (32°23'24"N, 119°25'3"E) and in Lingshui, Hainan Province (18°31'52"N, 110°10'53"E).

Fertility examination

The CMS lines, NILs, and testcross F₁ lines (five plants each) were evaluated for pollen fertility, bagged spikelet fertility and natural spikelet fertility. The natural spikelet fertility levels of all plants in the three F₂ populations were quantified. For pollen fertility, mature anthers were harvested, smeared in 1% iodine-potassium iodide (I₂-KI) solution, and observed using a light microscope. The numbers of normal dark-blue (stainable), clear (unstainable), and typical aborted pollen grains in each individual were counted (Zhu 1979). For bagged spikelet fertility, two major panicles emerging from the sheath on one plant were bagged before flowering. The natural spikelet fertility level and bagged spikelet fertility level were used as the seed-setting rate by counting the filled and unfilled grains from two opening panicles and the two bagged panicles, harvested 25-30 days after flowering.

DNA extraction and PCR

Total genomic DNA was isolated from fresh leaves of field-grown plants using a cetyltrimethylammonium bromide (CTAB) method with minor modification (Rogers and Bendich 1985), and the resulting DNA concentrations were adjusted to 100 ng/mL with TE buffer (pH 8.0) for subsequent analyses. Marker loci closely linked to *Rf5* and *Rf6* were developed as described in previous studies (Huang et al. 2012; Zhang et al. 2017). Simple Sequence Repeat (SSR) markers were identified from the Gramene database (<http://www.gramene.org/>). Newly-developed specific functional markers for *Rf5* and *Rf6* were developed based on the sequences (<http://www.ncbi.nlm.nih.gov/>) using the BLAST algorithm to search the sequences of 'Nipponbare' (*rf5*, *rf6*) and '93-11' (*Rf5*, *Rf6*). The primers used in this study are

given in Table S1. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai). The molecular marker assays were performed out in 20 µl reaction mixtures containing 1× PCR buffer, 0.1 mmol/L of each dNTP, 1.0 U *Taq* DNA polymerase, 0.2 µmol/L of each primer, and 20 ng template DNA. The amplification reaction consisted of an initial denaturation cycle of 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 50 s, with a final extension step of 72°C for 5 min. The amplification products were separated by electrophoresis on a 3.0% (w/v) agarose gel containing ethidium bromide, and visualized with a GEL DOC 1000 system (Bio-Rad Company).

Data analysis

Analysis of variance (ANOVA) as implemented in SPSS14.0 was used for statistical analysis of the fertility of the materials and populations used in this study.

Results

HL-type CMS japonica lines and the testcross F₁ hybrids have stainable pollen grains

In 2018, pollen grains of the CMS lines, 93 – 11, the NILs (NIL^{Rf5}, NIL^{Rf6} and PPL^{Rf5+Rf6}) and the testcross F₁ plants were analyzed using I₂–KI staining. The fertile pollen grains of 93 – 11 and the NILs stained dark-blue, and the sterile pollen grains of HL-YTA (*indica*) were spherical and showed no starch accumulation (Fig. 1a, b). The *indica* 93 – 11 cultivar and the NILs displayed normal spikelet fertility (data not shown). The sterile pollen grains of BT-type and HL-type *japonica* lines were filled with starch, similar with those of 93 – 11, and the pollen grains from the BT-type and HL-type *japonica* lines were morphologically similar (Fig. 1c-h). All the CMS lines used in this study had bagged spikelet fertility levels of zero, indicating that the pollen grains from these lines are completely sterile. Because BT-type and HL-type CMS are gametophytic CMS systems, 50% or 75% of pollen grains in the F₁ hybrids (carrying one *Rf* gene or two *Rf* genes) can be theoretically restored. However, the pollen grains of testcross F₁ plants derived from BT-type CMS *japonica* lines and HL-type CMS *japonica* lines stained dark blue with I₂–KI, and the sterile and fertile pollens were morphologically indistinguishable (Fig. 1j-n). Thus, it is obvious that the effect of HL-type CMS on pollen fate differs in the *indica* and *japonica* genetic backgrounds, which is consistent with our previous findings (Zhang et al. 2016). Thus, more detailed in-depth studies are needed to understand the underlying mechanisms behind these differences in CMS in *indica* vs. *japonica* rice lines.

Development of NILs for *Rf5* and *Rf6* in the HL-YTA background

For analyzing the ability of *Rf5* and *Rf6* to restore fertility in HL-type *indica* CMS lines, NILs for *Rf5* and *Rf6* were developed in the HL-YTA background by MAS. In 2015, we first crossed HL-YTA with 93 – 11, a restorer line carrying *Rf5* and *Rf6*. The resulting F₁ plant was backcrossed with HL-YTA to produce a population of 120 BC₁F₁ plants, and the genotypes of these plants at the *Rf5* and *Rf6* loci were screened using two markers RM407 (locus tightly linked to *Rf6*) and RM6100 (locus tightly linked to *Rf5*). Based on

the screening results, five plants carrying only *Rf5* or *Rf6* were selected for successive backcrossing with the recurrent parent HL-YTA, which generated 10 BC₂F₁ and 10 BC₃F₁ lines. In the BC₃F₁ population, the MAS results showed that all of the plants had the linked markers, and two plants carrying *Rf5* or *Rf6* that were phenotypically similar to the recurrent parent were preferentially selected to cross with HL-YTA, resulting in four BC₄F₁ lines, from which two BC₅F₁ lines, here designed as YTA-*Rf5* and YTA-*Rf6*, were obtained (Fig. 2a). YTA-*Rf5* and YTA-*Rf6* had similar plant morphology to HL-YTA, and can be considered to be NILs for *Rf5* and *Rf6* (Fig. 2b).

Rf5 and Rf6 partially restore fertility in HL-type japonica lines

In 2018, YTA-*Rf5* and YTA-*Rf6* were used as the controls to analyze the effects of *Rf5* and *Rf6* on fertility restoration in HL-type *japonica* CMS lines. The natural spikelet fertility levels of the plants were used as indicators for evaluating the ability of *Rf5* and *Rf6* to restorer fertility in HL-type *japonica* CMS lines. YTA-*Rf5* and YTA-*Rf6* plants showed spikelet fertility levels of ~ 80%, and no significant differences were observed between these two types of plants (Fig. 3a). The results revealed that *Rf5* and *Rf6* exhibited similar restorability and were effective at restoring fertility to HL-type *indica* lines, which was in agreement with previous reports (Huang et al. 2012; Huang et al. 2015).

In the testcross population, natural spikelet fertility levels of the testcross F₁ hybrids between BT-type *japonica* CMS lines and NIL^{*Rf5*}, NIL^{*Rf6*}, and PPL^{*Rf5+Rf6*} were > 79%, indicating that these two genes are effective at restoring fertility in BT-type CMS *japonica* lines (Fig. 3b). In contrast, the testcross F₁ hybrids between HL-type CMS *japonica* lines and NIL^{*Rf5*} showed natural spikelet fertility levels of ~ 30% (ranging from 27.92–31.31%), and the natural spikelet fertility levels of the testcross F₁ hybrids between HL-type CMS *japonica* lines and NIL^{*Rf6*} were ~ 50% (ranging from 47.77–56.77%), which is significantly lower than that of the corresponding testcross F₁ hybrids with BT-type CMS lines (Fig. 3b). These results indicate that *Rf5* and *Rf6* restore partial fertility to HL-type *japonica* CMS lines, and the effect of *Rf6* is larger than that of *Rf5* on fertility restoration in HL-type *japonica* CMS lines. Moreover, the natural spikelet fertility levels of the testcross F₁ hybrids between the HL-type CMS *japonica* lines and PPL^{*Rf5+Rf6*} were > 60%, which is considerably higher than the crosses with NIL^{*Rf5*} and NIL^{*Rf6*}, implying that *Rf5* and *Rf6* exert an additive effect on fertility restoration in HL-type *japonica* CMS lines (Fig. 3b). In addition, similar results were observed for the bagged spikelet fertility levels of the testcross F₁ hybrids, although they were significantly reduced compared with the natural spikelet fertility levels (data not shown).

Rf5 and Rf6 display dosage effects on fertility restoration in HL-type japonica CMS lines

In 2019, there were 40, 40, and 100 plants in the HL-NIPA/NIL^{*Rf5*}, HL-NIPA/NIL^{*Rf6*} and HL-NIPA/PPL^{*Rf5+Rf6*} F₂ populations, respectively. Due to outcrossing, the HL-NIPA plants exhibited very low natural spikelet fertility levels (< 2%) under natural conditions without isolation. Plants in the three F₂ populations exhibited natural spikelet fertility levels of ≥ 20.43% at the same time, indicating that all of these F₂ plants were fertile. Also, we screened the plants in the three F₂ populations with two molecular markers,

Rf1a-M1 (*Rf5* functional-marker) and 1870CF (*Rf6* functional marker). There were 17 *Rf*-homozygous individuals and 23 heterozygous individuals, and 25 homozygous individuals and 15 heterozygous individuals in the HL-NIPA/NIL^{*Rf5*} and HL-NIPA/NIL^{*Rf6*} F₂ populations, respectively, and the segregation ratios of the homozygous and heterozygous genotypes were 1:1 ($\chi^2 = 0.34$ and 0.11 in the HL-NIPA/NIL^{*Rf5*} and HL-NIPA/NIL^{*Rf6*} F₂ populations, respectively, which were $< \chi^2_{0.05} = 3.84$). Moreover, we failed to detect plants with the genotypes *rf5rf5*, *rf6rf6*, and *rf5rf5rf6rf6* in the HL-NIPA/NIL^{*Rf5*}, HL-NIPA/NIL^{*Rf6*}, and HL-NIPA/PPL^{*Rf5+Rf6*} F₂ populations, respectively. These results indicate that fertility restoration in HL-type *japonica* CMS lines by *Rf5* and *Rf6* is consistent with a gametophytic restoration model.

In the HL-NIPA/NIL^{*Rf5*} F₂ population, plants harboring the genotype of *Rf5rf5* had an average natural spikelet fertility level of 35.60%, and plants with the *Rf5Rf5* genotype displayed an average natural spikelet fertility level of 58.09% (Fig. 4a). In the HL-NIPA/NIL^{*Rf6*} F₂ population, the average natural spikelet fertility levels of the plants carrying the *Rf6rf6* and *Rf6Rf6* genotypes were 47.33% and 71.79%, respectively (Fig. 4b). In the HL-NIPA/PPL^{*Rf5+Rf6*} F₂ population, plants carrying the *Rf5rf5rf6rf6* genotype had an average natural spikelet fertility of 28.54%, which was the lowest among all of the F₂ plants. In contrast, plants carrying the *Rf5Rf5Rf6Rf6* genotype had the highest natural spikelet fertility of 78.07% (Fig. 4c). These results indicate that there are dosage effects associated with *Rf5* and *Rf6* on fertility restoration in the HL-CMS *japonica* lines.

Discussion

Development of three-line hybrid rice depends on the breeding of elite CMS lines and restorers. In China, three-line *japonica* hybrids have been available since 1976 and are based mainly on the BT-type CMS (Shinjyo 1969). To accelerate the development of three-line *japonica* hybrids, breeders have attempted to develop three-line *japonica* hybrids using other CMS systems, such as HL-type and WA-type CMS. However, the genetic characters of the HL-type and WA-type CMS *japonica* lines and restorers have not been well studied, resulting in the failure to breed HL-type and WA-type CMS *japonica* hybrids. In the present study, we showed that the HL-type *japonica* CMS lines have aborted pollen grains that stain dark blue with I₂-KI. Two HL-type *Rf* genes, *Rf5* and *Rf6*, both partially restored fertility of HL-type *japonica* CMS lines, and we identified a dosage effect of these two genes in the fertility restoration of HL-type *japonica* CMS lines.

HL-type CMS is derived from common red-awned wild rice (*Oryza rufipogon*), and has been successfully used for developing three-line *indica* hybrids, which have been planted in China and Southeast Asia for many years (Li et al. 2007). In *indica*-type rice, the spherical aborted pollen grains are considered to be one of the representative characteristics of HL-type CMS lines (Li et al. 2007; Rao 1988; Zhu 1984). In this study, we found that the HL-type *japonica* CMS lines and their derived testcross F₁ hybrids all have blue-staining aborted pollen grains, which is in line with our previous observations (Zhang et al. 2016; Zhu et al. 2010). These results further confirm that it is the HL-type CMS that causes the blue-staining aborted

pollen grains in the *japonica* genetic background, which is different from the effect of HL-type CMS in *indica* rice. It has generally been thought that the morphology of aborted pollen grains is determined by the sterile cytoplasm, not the nuclear genetic backgrounds of the CMS lines. Our results clearly show that this is not the case. Thus, we hypothesize that there is a different mechanism underlying the development of aborted pollen grains caused by the HL-type CMS in *indica* vs. *japonica* genetic backgrounds, and that different non-restorer genes might exist in *japonica* and *indica* maintainer lines. Further studies are needed to test this hypothesis.

In *O. sativa* ssp. *indica*, *Rf5*, an HL-type *Rf* gene that is present in many different restorers, has been mapped to the same region of chromosome 10. *Rf5* has been cloned, showing that it is the same gene as *Rf1a*, a fertility restorer gene for BT-type CMS (Hu et al. 2012; Huang et al. 2003; Huang et al. 2000; Liu et al. 2004). *Rf6*, the HL-type *Rf* gene in the *indica* variety '9311' is located on chromosome 8 (Huang et al. 2012). In a previous study, *Rf5* and *Rf6* were shown to restore 50% pollen fertility and normal spikelet fertility to F₁ plants from a cross between the *indica* CMS lines and restorers carrying one of the two genes (Huang et al. 2012). In the present study, we constructed NIL populations for *Rf5* and *Rf6* in the YTA background, and the natural spikelet fertility levels of the NILs were ~ 80%. Our observations indicated that *Rf5* and *Rf6* are indeed effective for fertility restoration in the HL-type *indica* CMS lines, which is generally consistent with previously reported results. In contrast, the crossing experiments revealed that *Rf5* and *Rf6* restore only partial fertility to the HL-type *japonica* CMS lines, indicating that the ability of these two genes to restore fertility in HL-type CMS differs between the *indica* and *japonica* genetic backgrounds, which lends some support for the different non-restorer genes in the *japonica* and *indica* maintainer lines mentioned above. However, the crossing experiments also showed that the recoverability of HL-type *japonica* CMS lines was lower than that of HL-type *indica* CMS lines and BT-type *japonica* CMS lines. In rice breeding, it is generally thought that the CMS lines with aborted pollen grains that stain blue can be restored more easily than the CMS lines with either spherical aborted or typical aborted pollen grains. Thus, our current observations provide new insights for recognizing the recoverability of CMS lines, which is of value for further breeding and utilization of CMS lines in the future.

The abilities of *Rf5* and *Rf6* to restore fertility in HL-type *indica* CMS lines are similar. In the present study, we initially found that *Rf6* shows higher restorability than *Rf5* in HL-type *japonica* CMS lines, although *Rf5* and *Rf6* are both weakly effective in HL-type *japonica* CMS lines. *Rf5* and *Rf6* encode proteins that belong to the pentatricopeptide repeat (PPR) family, and these two genes share a high degree of sequence homology (Hu 2012; Huang 2015). For processing the HL-CMS-associated transcript *atp6-orfH79*, RF5 is needed to interact with the RNA-processing factor GRP162, but RF6 works with OsHXXK6. Thus, RF6 and RF5 function through distinct mechanisms to restore the fertility of HL-type CMS in *indica* rice lines. It is reasonable to speculate that *Rf5* and *Rf6* play different roles in the fertility restoration of HL-type *japonica* CMS lines, and this is the most likely reason behind their different effects. Therefore, it will be necessary to test whether *Rf6* and *Rf5* function through the same mechanism in *japonica* rice varieties.

In this study, we observed dosage effects of the *Rf* genes on fertility restoration of HL-type *japonica* CMS lines in plants carrying different genotypes in the F₂ populations; this shows that more *Rf* genes combined in the HL-type *japonica* F₁ hybrids is helpful to increase the seed-setting rate. Usually, the seed-setting rate in three-line hybrids should reach 80% in rice production. However, our present observations showed that pyramiding the *Rf5* and *Rf6* gene is still insufficient to restore a normal level of fertility to HL-type CMS *japonica* lines. Thus, to breed HL-type *japonica* restorers, other new major *Rf* genes will be required for pyramiding with *Rf5* and *Rf6* to recover full male fertility.

In summary, the results of our study will have important implications for the breeding of *japonica* hybrids that carry HL-type CMS, and should greatly facilitate the further exploitation of heterosis in rice breeding.

Declarations

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All data supporting the conclusions of this article are provided within the article (and its Additional files).

Author Contributions: HZ performed the data analysis and drafted the manuscript. RW and ZX performed the phenotypic evaluation and analyzed the data. HG participated in the construction of the NIL populations. QL participated in the design of the study. HZ, ST and XZ designed the study and revised the manuscript. All of the authors have read and approved the final manuscript.

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Tables

Table 1 Number of plants with different genotypes at the *Rf5* and *Rf6* loci in the three F₂ populations

Population	Genotype	Number of plants
HL-NIPA/NIL ^{<i>Rf5</i>} F ₂	<i>Rf5Rf5</i>	17
	<i>Rf5rf5</i>	23
HL-NIPA/NIL ^{<i>Rf6</i>} F ₂	<i>Rf6Rf6</i>	25
	<i>Rf6rf6</i>	15
HL-NIPA/PPL ^{<i>Rf5+Rf6</i>} F ₂	<i>Rf5Rf5Rf6Rf6</i>	12
	<i>Rf5Rf5Rf6rf6</i>	23
	<i>Rf5rf5Rf6Rf6</i>	17
	<i>Rf5rf5Rf6rf6</i>	21
	<i>rf5rf5Rf6Rf6</i>	10
	<i>rf5rf5Rf6rf6</i>	7
	<i>Rf5Rf5rf6rf6</i>	4
	<i>Rf5rf5rf6rf6</i>	6

Figures

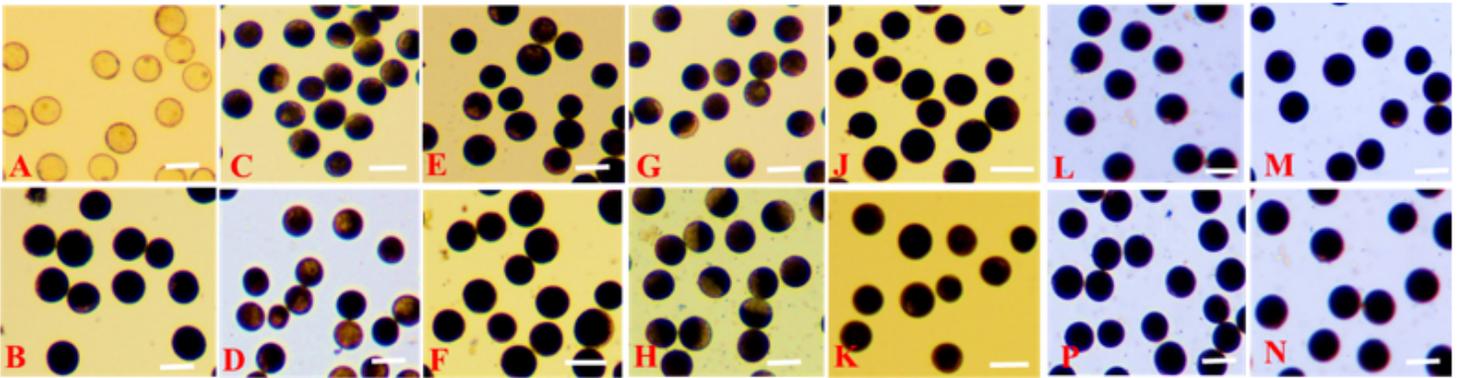


Figure 1

Pollen grains from the parental lines and F1 hybrids stained with I2-KI. (a) HL-YTA (indica). (b) 93-11 (indica). (c-h) Pollen grains from HL-NIPA, BT-NIPA, HL-LqxA, BT-LqxA, HL-LFA, and BT-LFA, respectively. (j-n) Pollen grains from the F1 hybrids HL-NIPA/NILRf5, BT-NIPA/ NILRf5, HL-NIPA/NILRf6, HL-NIPA/PPLRf5+Rf6, BT-NIPA/ PPLRf5+Rf6, and BT-NIPA/NILRf6, respectively (data from other F1 hybrids were similar but are not shown). Scale bars=50 μ m.

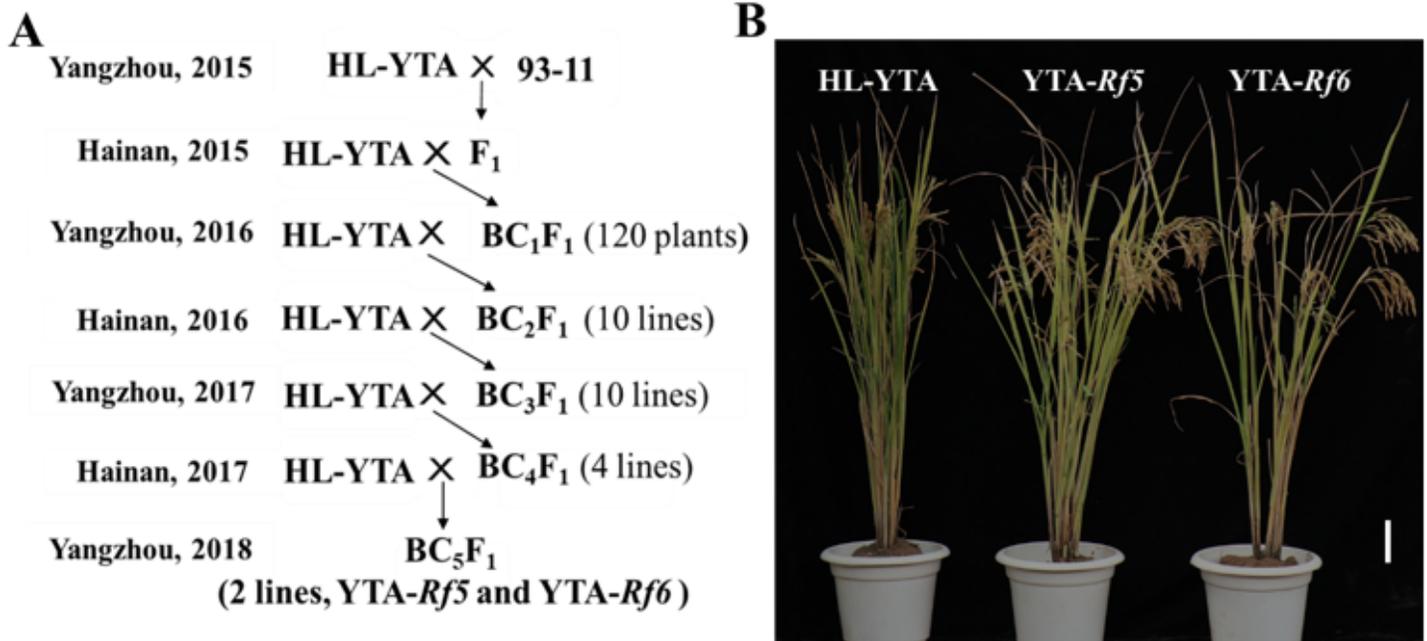


Figure 2

Breeding scheme and gross plant morphology of the parental lines and the newly developed NILs (near isogenic lines) in the YTA genetic background. (a) Breeding scheme showing the development of the YTA-

Rf5 and YTA-Rf6 NILs. (b) Mature plants of HL-YTA, YTA-Rf5, and YTA-Rf6. Scale bar = 15 cm.

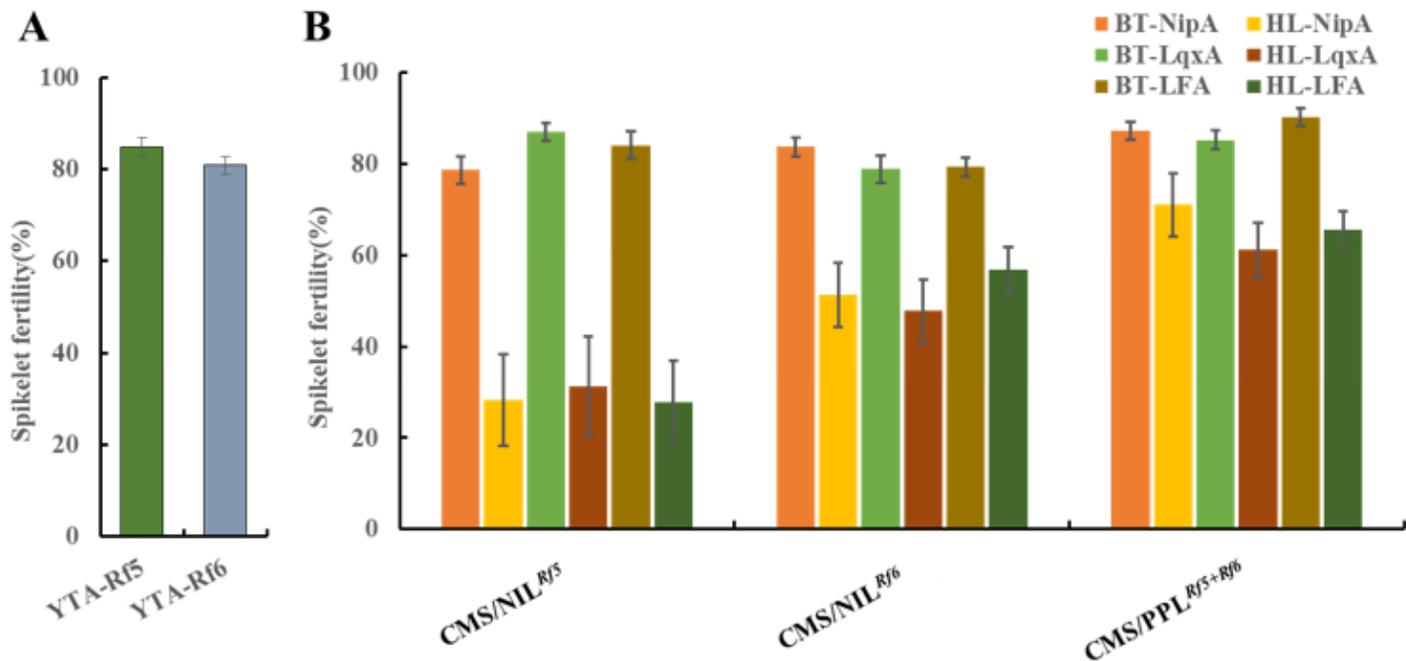


Figure 3

Natural male fertility levels of plants developed in this study. (a) NILs for Rf5 and Rf6 in the YTA background. (b) Testcross F1 plants from crosses between six male-sterile japonica lines, BT-NIPA, BT-LQXA, BT-LFA, HL-NIPA, HL-LQXA, and HL-LFA, and the three parental lines CMS/NILRf5, CMS/NILRf6, and CMS/PPLRf5+Rf6.

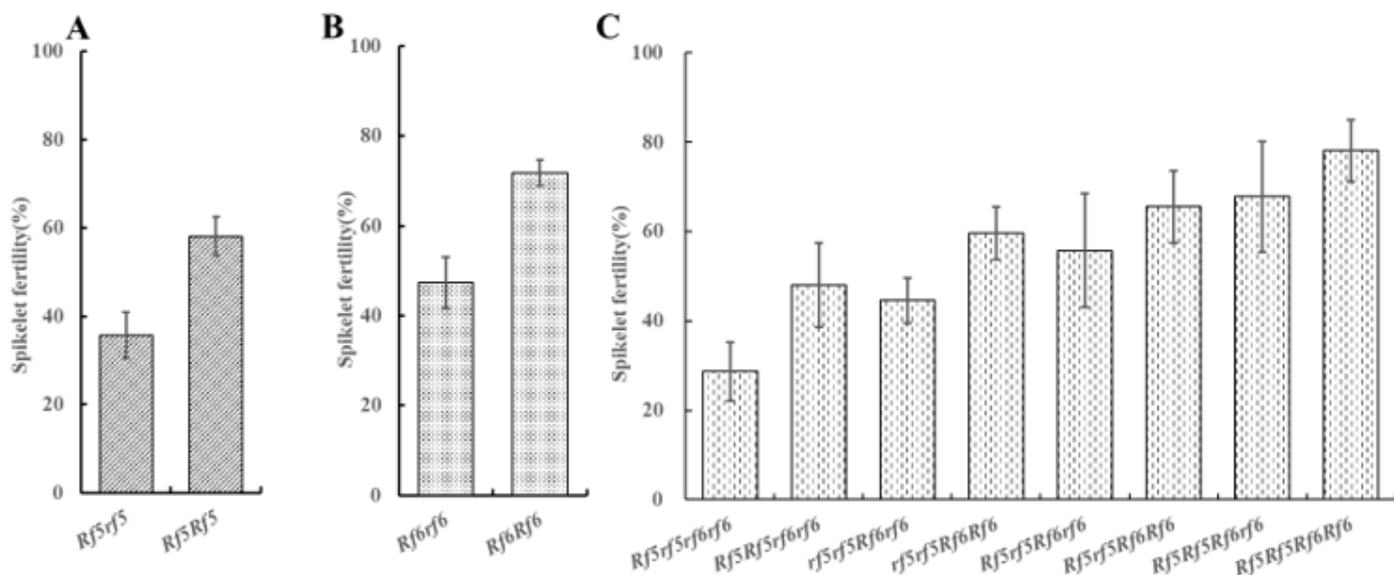


Figure 4

Natural fertility levels of plants with different Rf genotypes in the F2 populations. (a) The HL-NIPA/NILRf5 F2 population, (b) the HL-NIPA/NILRf6 F2 population, and (c) the HL-NIPA/PPLRf5+Rf6 F2 population.