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

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**Abstract** Honglian (HL)-type cytoplasmic male sterility (CMS) has only been used in the development of three-line \textit{indica} rice hybrids, and the fertility of HL-type \textit{indica} CMS lines can be restored by two non-allelic fertility-restorer (\textit{Rf}) genes, \textit{Rf5} and \textit{Rf6}. For the development of HL-type \textit{japonica} hybrid combinations, it is therefore necessary to determine whether \textit{Rf5} and \textit{Rf6} can restore the fertility of HL-type \textit{japonica} CMS lines. Here, we genetically characterized HL-type \textit{japonica} CMS lines and the ability of \textit{Rf5} and \textit{Rf6} to restore fertility for breeding HL-type \textit{japonica} hybrids. \textit{I}_{2}-KI pollen staining revealed that HL-type \textit{japonica} CMS lines and their derived testcross \textit{F}_{1} hybrids had stained abortive pollen grains, unlike HL-type \textit{indica} CMS lines. Crossing experiments showed that \textit{Rf5} and \textit{Rf6} partially restored the fertility of HL-type \textit{japonica} CMS lines, and \textit{Rf6} showed higher restorability than \textit{Rf5}. Furthermore, we found that there were additive and dosage effects of \textit{Rf5} and \textit{Rf6} with respect to fertility restoration in HL-type \textit{japonica} CMS lines. These results give critical insight into the breeding of commercial HL-type \textit{japonica} hybrids.

**Keywords** \textit{japonica} rice · HL-type CMS · Restorer-of-fertility gene (\textit{Rf}) · Restoration ability · Marker-assisted selection

**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, 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, 2012, 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 (Fuji 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-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 \(R_f5\) and \(R_f6\) to restore male fertility in \(japonica\) lines. In addition, the functional model and effects of \(R_f5\) and \(R_f6\) on the fertility restoration in \(japonica\) were analyzed. Our discovery will assist in analyzing the differences of fertility restoration in HL-type CMS in \(indical\)/\(japonica\) 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. \(R_f5\) and \(R_f6\) are two major HL-type \(R_f\) genes identified from the elite restorer line indica ‘93-11’ (Hu et al. 2012; Huang et al. 2015). In order to identify the effects of \(R_f5\) and \(R_f6\) on fertility restoration in HL-type \(indica\) CMS lines, we performed marker-assisted selection (MAS) to develop the near isogenic lines for \(R_f5\) and \(R_f6\) in the HL-YTA (indica variety) background, named as YTA-\(R_f5\) (\(R_f5R_f5\)) and YTA-\(R_f6\) (\(R_f6R_f6\)). The near isogenic lines for \(R_f5\), \(R_f6\) designated as NIL\(^{\text{R}_f5}\) (\(R_f5R_f5\)) and NIL\(^{\text{R}_f6}\) (\(R_f6R_f6\)), as well as the polygene pyramid lines PPL\(^{R_f5+R_f6}\) (\(R_f5R_f5R_f6R_f6\)) in the BT-NIPA (\(japonica\) variety) genetic background were developed in our previous study (Zhang et al. 2019). In 2018, the isonuclear alloplasmic \(japonica\) lines (HL-LQXA and BT-LQXA, HL-NIPA and BT-NIPA, and HL-LFA and BT-LFA) were used as females in separate crosses with NIL\(^{R_f5}\), NIL\(^{R_f6}\), and PPL\(^{R_f5+R_f6}\), resulting in a testcross population consisting of nine pairs of \(F_1\) hybrids for evaluating the fertility restoring ability of these two \(R_f\) genes in \(japonica\) rice lines. Subsequently, three \(F_2\) populations derived from crosses between HL-NIPA and NIL\(^{R_f5}\), HL-NIPA and NIL\(^{R_f6}\), and HL-NIPA and PPL\(^{R_f5+R_f6}\) were planted in 2019 to further analyze the effects of the \(R_f\) genes on fertility restoration in HL-type \(japonica\) CMS lines. Twenty plants were grown for each CMS line or each testcross \(F_1\) 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 \(F_1\) 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 \(F_2\) populations were quantified. For pollen fertility, mature anthers were harvested, smeared in 1% iodine-potassium iodide (I\(_2\)-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/µl with TE buffer (pH 8.0) for subsequent analyses. Marker loci closely linked to \(R_f5\) and \(R_f6\) 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 \(R_f5\) and \(R_f6\) 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’ (\(R_f5, R_f6\)). 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<sup>R5</sup>, NIL<sup>R6</sup>, and PPL<sup>R5+R6</sup>), and the testcross F₁ plants were analyzed using I<sub>2</sub>-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 I<sub>2</sub>-KI, and the sterile and fertile pollens were morphologically indistinguishable (Fig. 1I–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 Rf<sup>5</sup> and Rf<sup>6</sup> in the HL-YTA background.

For analyzing the ability of Rf<sup>5</sup> and Rf<sup>6</sup> to restore fertility in HL-type *indica* CMS lines, NILs for Rf<sup>5</sup> and Rf<sup>6</sup> were developed in the HL-YTA background by MAS. In 2015, we first crossed HL-YTA with 93–11, a restorer line carrying Rf<sup>5</sup> and Rf<sup>6</sup>. The

![Fig. 1](image-url) Pollen grains from the parental lines and F₁ hybrids stained with I<sub>2</sub>-KI and corresponding genotypes. (A) HL-YTA (*indica*). (B) 93–11 (*indica*). (C–H) Pollen grains from BT-NIPA, HL-NIPA, BT-LQXA, HL-LQXA, BT-LFA, and HL-LFA, respectively. (I–N) Pollen grains from the F₁ hybrids BT-NIPA/NIL<sup>R5</sup>, HL-NIPA/NIL<sup>R5</sup>, BT-NIPA/NIL<sup>R6</sup>, HL-NIPA/NIL<sup>R6</sup>, BT-NIPA/PPL<sup>R5+R6</sup>, and HL-NIPA/PPL<sup>R5+R6</sup>, respectively. Scale bars = 50 µm
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, and can be considered to be the near isogenic lines 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 restore 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, 2015).

In the testcross population, natural spikelet fertility levels of the testcross F₁ hybrids between BT-type japonica CMS lines and NILRf5, NILRf6, and PPLRf5+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 NILRf5 showed natural spikelet fertility levels of ~30% (ranging from 27.92 to 31.31%), and the natural spikelet fertility levels of the testcross F₁ hybrids between HL-type CMS japonica lines and NILRf6 were ~50% (ranging from 47.77 to 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

![Fig. 2](image_url)  
**Fig. 2** Breeding scheme and gross plant morphology of NILs (near isogenic lines) with HL-YTA genetic background. (A) Breeding scheme showing the development of the YTA-Rf5 and YTA-Rf6. (B) Mature plants of HL-YTA, YTA-Rf5, and YTA-Rf6. Scale bar = 15 cm.
japonica lines and PPL<sup>Rf5+Rf6</sup> were >60%, which is considerably higher than the crosses with NIL<sup>Rf5</sup> and NIL<sup>Rf6</sup>, 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 F<sub>1</sub> 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<sup>Rf5</sup>, HL-NIPA/NIL<sup>Rf6</sup>, and HL-NIPA/PPL<sup>Rf5+Rf6</sup> F<sub>2</sub> populations, respectively (Table 1). Due to outcrossing, the HL-NIPA plants exhibited very low natural spikelet fertility levels (<2%) under natural conditions without isolation. Plants in the three F<sub>2</sub> populations exhibited natural spikelet fertility levels of ≥20.43% at the same time, indicating that all of these F<sub>2</sub> plants were fertile. Also, we screened the plants in the three F<sub>2</sub> 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<sup>Rf5</sup> and HL-NIPA/NIL<sup>Rf6</sup> F<sub>2</sub> populations, respectively, and the segregation ratios of the homozygous and heterozygous genotypes were 1:1 (χ<sup>2</sup> = 0.34 and 0.11 in the HL-NIPA/NIL<sup>Rf5</sup> and HL-NIPA/NIL<sup>Rf6</sup> F<sub>2</sub> populations, respectively, which were <χ<sup>2</sup> = 3.84). Moreover, we failed to detect plants with the genotypes rf5rf5, rf6rf6, and rf5rf5rf6rf6 in the HL-NIPA/NIL<sup>Rf5</sup>, HL-NIPA/NIL<sup>Rf6</sup>, and HL-NIPA/PPL<sup>Rf5+Rf6</sup> F<sub>2</sub> populations, respectively. These results indicate

**Table 1** Number of plants with different genotypes at the Rf5 and Rf6 loci in the three F<sub>2</sub> populations

| Population         | Genotype Description | Number of plants |
|--------------------|----------------------|------------------|
| HL-NIPA/NIL<sup>Rf5</sup> F<sub>2</sub> | Rf5Rf5               | 17               |
|                    | Rf5rf5               | 23               |
| HL-NIPA/NIL<sup>Rf6</sup> F<sub>2</sub> | Rf6Rf6               | 25               |
|                    | Rf6rf6               | 15               |
| HL-NIPA/PPL<sup>Rf5+Rf6</sup> F<sub>2</sub> | Rf5Rf5Rf6Rf6         | 12               |
|                    | Rf5Rf5Rf6rf6         | 23               |
|                    | Rf5rf5Rf6Rf6         | 17               |
|                    | Rf5rf5Rf6rf6         | 21               |
|                    | rf5rf5Rf6Rf6         | 10               |
|                    | rf5rf5rf6rf6         | 7                |
|                    | Rf5Rf5rf6rf6         | 4                |
|                    | Rf5rf5rf6rf6         | 6                |
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, 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 et al., 2012; Huang et al., 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 OsHXK6. 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.

**Author contribution** 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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Data availability All data supporting the conclusions of this article are provided within the article (and its additional files).

Declarations

Ethics approval and consent to participate Not applicable.

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

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