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Identifying the genes around *Rf5* and *Rf6* loci for the fertility restoration of WA-type cytoplasmic male sterile *japonica* rice (*Oryza sativa*) lines

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Abstract Wild abortive (WA)-type, Honglian (HL)-type, and Chinsurah Boro II-type cytoplasm are three typical sterile cytoplasms used to generate three-line hybrid rice, and the fertility restorer (*Rf*) genes are considered to have specificity for fertility restoration of cytoplasmic male sterility (CMS) lines. '93-11', an HL-type *indica* restorer line used widely in China, shows a weak ability to restore the fertility of WA-type CMS lines. *Rf5* and *Rf6*, the fertility restorer genes for HL-type CMS, are members of a multigene cluster that encodes pentatricopeptide repeat proteins in '93-11'. In the present study, we studied the function of *Rf* genes around *Rf5* and *Rf6* loci on fertility restoration to WA-type CMS lines. We generated plants carrying WA-type cytoplasm and different genotypes at the *Rf5* and *Rf6* loci. All plants exhibited no seed setting on bagged panicles but had different anther and pollen grain morphologies. Plants with the genotypes of *Rf5rf5rf6rf6*, *Rf5Rf5rf6rf6*, *rf5rf5Rf6rf6*, *rf5rf5Rf6Rf6*, and *Rf5rf5Rf6rf6* exhibited degraded anthers and typical abortive pollen grains, which were same as those of WA-NipA plants (*rf5rf5rf6rf6*); however, plants with the genotypes *Rf5rf5Rf6Rf6*, *Rf5Rf5Rf6rf6*, and *Rf5Rf5Rf6Rf6* displayed restored anthers and pollen grains. These results indicated that *Rf* genes around the *Rf5* and *Rf6* loci had minor effects on the fertility restoration of WA-type CMS lines, which were mediated by dosage effects. Furthermore, these *Rf* genes functioned to decrease the *WA352* (the mitochondrial gene conferring CMS-WA) transcript levels. Our findings will promote the development of three-line hybrids.

Keywords Rice · Cytoplasmic male sterility · Fertility restorer · Gene effect

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Introduction

Rice (*Oryza sativa*) is the most widely consumed staple food, feeding more than half of the global population. Compared with inbred varieties, hybrid rice lines produce 10–20% higher yields, and hybrid

rice is cultivated on 57% of the rice planting areas in China (Yuan 2014). Hybrid rice lines include three- and two-line hybrids, which are developed by applying cytoplasmic male sterility (CMS) and environmentally sensitive genic male sterility, respectively (Cheng et al. 2007; Huang et al. 2014). A CMS line, a maintainer line, and a restorer line, which carries a fertility restorer gene (*Rf*), are required to develop three-line hybrids. Three representative CMS types, including wild abortive (WA), Honglian (HL), and Chinsurah Boro II (BT), are commonly used to generate three-line hybrid rice. The WA-type CMS belongs to the sporophytic CMS/*Rf* system, and the HL- and BT-type CMS belong to the gametophytic CMS/*Rf* system, and these three CMS types are considered to have different maintainer–restorer relationships (Chen and Liu 2014; Huang et al. 2014; Li et al. 2007). However, there are potential problems regarding the relationships among the three CMS/*Rf* systems. For example, WA-type restorers can restore the fertility of BT- and HL-type CMS lines, but not vice versa (Zhu et al. 2010). Additionally, BT- and HL-type CMS lines exhibit similar maintainer–restorer relationships (Tan et al. 2008; Zhang et al. 2016). Therefore, additional studies should be conducted to determine the roles of mapped and cloned *Rf* genes during the fertility restoration of different types of CMS lines, which may help to determine the relationships among the three CMS/*Rf* systems.

To date, *Rf3* and *Rf4* for WA-CMS lines, *Rf5* and *Rf6* for HL-CMS lines, and *Rf1a*/*Rf1b* for BT-CMS lines have been mapped, and except for *Rf3*, all of them have been cloned (Hu et al. 2012; Huang et al. 2012, 2015; Komori et al. 2004; Tang et al. 2014; Wang et al. 2006; Zhang et al. 1997). Cloning of these genes revealed that *Rf5* is the same as *Rf1a*, and *Rf6* can restore the fertility of BT-CMS lines (Hu et al. 2012; Huang et al. 2015; Komori et al. 2004; Wang et al. 2006; Zhang et al. 2016). All the cloned *Rf* genes encode pentatricopeptide repeat (PPR) proteins, and the amino acid sequence encoded by *Rf4* is highly similar to that of RF1A (Hu et al. 2012; Huang et al. 2015; Komori et al. 2004; Tang et al. 2014; Wang et al. 2006). Previously, we determined that *Rf1a*, a BT-type fertility restorer gene, can partially restore the fertility of HL-type *japonica* CMS lines, and that *Rf5* and *Rf6* can restore the fertility of BT-type CMS lines (Zhang et al. 2016, 2017). Above all, *Rf5* (*Rf1a*) and *Rf6* are involved in the fertility restoration of BT-type and HL-type CMS lines, which are useful to characterize the similar maintainer–restorer relationships among the BT- and HL-type CMS lines. *Rf5* and *Rf6* were identified from '93-11', an elite restorer line for HL-type and two-line hybrid rice, which exhibits high yield and superior quality. In breeding practice, '93-11' is not a restorer for WA-type CMS lines, but was found to carry some *Rf* genes with minor effects for the fertility restoration of WA-type CMS (Tang et al. 2014). The improved '93-11' without minor *Rf* genes for WA\CMS would be termed as a maintainer to develop WA-type hybrids. Therefore, identifying minor *Rf* genes for WA-type CMS in '93-11' is of great interest. Until now, there have been few studies of minor *Rf* genes for WA-type CMS. Considering that *Rf5* and *Rf6* are located in the multigene clusters that encode PPR proteins, these targeted *Rf* genes for WA-type CMS lines located around the *Rf5* and/or *Rf6* loci should be studied as a priority.

In the present study, we used a marker-assisted selection (MAS) strategy to develop near-isogeneic lines (NILs) for *Rf5* and *Rf6*, the polygene pyramid lines that combine *Rf5* with *Rf6*, and bred 'WA-NipponbareA' (WA-NipA), a WA-type *japonica* CMS line using 'Nipponbare' as the maintainer. Then, we constructed several populations to obtain plants carrying WA-type cytoplasm and the different genotypes at the *Rf5* and *Rf6* loci, and the morphologies of the pollen grains and anthers of these plants were observed. We provided information regarding whether the *Rf* genes located around the *Rf5* and *Rf6* loci are involved in the fertility restoration of WA-type CMS lines, and whether these genes function to reduce the *WA352* (the mitochondrial gene conferring CMS-WA) transcript levels. The results will promote the breeding of WA-type CMS lines to develop three-line hybrids.

Materials and methods

Plant materials

'Nipponbare' (Nip), a genome sequenced *japonica* cultivar and is the maintainer for BT-CMS, HL-type CMS, and WA-CMS lines. '93-11', a genome sequenced typical *indica* cultivar, is the restorer for BT-type CMS and HL-type CMS lines, but is not a restorer for WA-type CMS lines. In our previous study, a set chromosome segment substitution lines

(CSSLs) from the cross between '93-11'(donor parent) and Nip (recipient parent) was developed, and these lines were genotyped using resequencing (Zhang et al. 2017). BT-NipA, which has the same nuclear background as Nip, but has a BT-type sterile cytoplasm, was used as the female parent to cross with the CSSLs, and two *Rf* genes for BT-type CMS, *Rf5* and *Rf6* from '93-11', were identified and finely mapped (Zhang et al. 2017). Four markers closely linked with *Rf5* (i.e., STS10-27 and STS10-16) and *Rf6* (i.e., STS8-4 and STS8-32) were developed. Using MAS, NILs for *Rf5* and *Rf6* were developed and designed as $NIL^{Rf5}$and NIL^{Rf6}, respectively. Polygene pyramid lines (PPLs) $PPL^{Rf5+Rf6}$ that combined *Rf5* with *Rf6* were then developed using MAS. For breeding WA-NipA, Nip was used as the male parent in a cross with WA-LiuqianxinA (a WA-type *japonica* CMS line), which was followed by six backcrosses with Nip from 2011 to 2015. In the rice-growing season of 2015, WA-NipA plants were crossed with NIL^{Rf5}, NIL^{Rf6}, and $PPL^{Rf5+Rf6}$, and the F_1 hybrids were sterile. Thus, the corresponding F_1 plants were further crossed with NIL^{Rf5}, NIL^{Rf6}, and $PPL^{Rf5+Rf6}$, respectively, to generate the WA-NipA/NIL^{Rf5}//NIL^{Rf5}, WA-NipA/NIL^{Rf6}//NIL^{Rf6}, and WA-NipA/$PPL^{Rf5+Rf6}$//$PPL^{Rf5+Rf6}$ populations to obtain plants harboring different genotypes at the *Rf5* and *Rf6* loci. In the rice-growing season of 2016, the above three backcross populations were planted, and the target plants were identified using MAS.

Field experiment

All plant materials used in this study were sown in May 20, and the 30-days-old seedlings were transplanted into the field at the experimental farm of Yangzhou University (32°24′N, 119°26′E) in Jiangsu province. Each plot consisted of 2–20 rows separated by 25 cm, with each row consisting of 10 plants, separated by 20 cm, and the management of the field experiments was conducted according to the normal procedures for rice.

Fertility scoring

Mature anthers of WA-NipA plants and plants with different genotypes in the WA-NipA/NIL^{Rf5}//NIL^{Rf5}, WA-NipA/NIL^{Rf6}//NIL^{Rf6}, and WA-NipA/$PPL^{Rf5+Rf6}$//$PPL^{Rf5+Rf6}$ populations were observed under an optical microscope. Pollen grains from mature anthers were stained with a 1% iodine–potassium iodide (I_2–KI) solution and observed using an optical microscope. At the flowering stage, two plants for each genotype and two panicles from each plant were bagged. Natural and bagged spikelet fertility levels of one plant were measured as the average seed-setting rates, which were calculated by counting the filled and unfilled grains of two panicles harvested 20 days after flowering from one plant.

DNA extraction and PCR amplification

Genomic DNA was isolated from the fresh leaves using the cetyltrimethylammonium bromide method (Rogers and Bendich 1985). The markers closely linked with *Rf5* and *Rf6* were developed in our previous study (Zhang et al. 2017), and primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The molecular marker analysis was carried out in a 20-μL reaction containing 2.0 μL of reaction buffer, 0.1 mmol/L of each dNTP, 1.0 U *Taq* polymerase, 0.2 μmol/L primer, 20 ng of template DNA, and ultra-pure water to a final volume of 20 μL. The amplification reaction consisted of one cycle at 94 °C for 4 min; followed by 30 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 50 s; with a final extension step at 72 °C for 5 min. The amplification products were separated by electrophoresis through a 3.0% (w/v) agarose gel containing ethidium bromide, and visualized using the GEL DOC 1000 system (Bio-Rad Laboratories, Hercules, CA, USA).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from leaves, culms, and panicles using a Plant RNA Kit (Tiangen, Beijing, China). The Perfect Real Time Prime Script RT reagent (Takara, Dalian, China) was used for first-strand cDNA synthesis in a 20-μL reaction containing 5 μg of total RNA. The quantitative real-time PCR (qPCR) was conducted in triplicate (i.e., with RNA from three independent extractions) using a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The mitochondrial *atp6* gene was used as an internal reference to determine the relative *WA352* expression

levels. The primers used in this study are listed in Supplementary Table S1.

Results

Construction of NIL^{Rf5}, NIL^{Rf6}, and PPL$^{Rf5+Rf6}$

During 2004–2013, a population comprising 127 CSSLs (N1–N127) was developed using crossing and back crossing, assisted by 352 molecular markers, and the genotypes of these CSSLs were identified using a high-throughput resequencing strategy (Zhang et al. 2011, 2017). BT-NipA was crossed with these CSSLs, and two *Rf* genes, *Rf5* and *Rf6*, were identified and finely mapped (Zhang et al. 2017). Based on resequencing data, N91 (Fig. 1a) harboring an *Rf6*-containing region between STS 8-4 and STS 8-32 (IRGSP-1.0 position 0.38 Mb and 0.56 Mb) was selected to cross with BT-NipA and N114 (Fig. 1b) harboring an *Rf5*-containing region between STS 10-27 and STS 10-16 (IRGSP-1.0 position 18.67 Mb and 19.17 Mb), and the testcross F_1s from N91 and N114 produced offspring by self-pollination. Using MAS, the plants carrying the genotypes of *Rf6Rf6* were selected from the BT-NipA/N91 F_2 self-crossed progeny, and were designed as NIL^{Rf6}. Except for the introgression segment covering the *Rf5* locus, N114 carried another introgression segment (IRGSP-1.0 position 2.12 Mb and 10.02 Mb) on Chr.10, and two markers (RM25222 and RM25384) were selected to detect this introgression segment. Using MAS, plants specific for the *Rf5*-containing region and carrying the genotypes of *Rf5Rf5* were selected from the BT-NipA/N114 F_2 population, and were designed as NIL^{Rf5}. In 2014, BT-NipA/N91F_1 plants were crossed with BT-NipA/N114 F_1 plants, and MAS experiments were conducted to obtain plants harboring the genotype *Rf5rf5Rf6rf6* and only the introgression segments covering the *Rf5* and *Rf6* loci in the BT-NipA/N91// BT-NipA/N114 population. In 2015, an F_2 population comprising 200 plants was generated, and plants

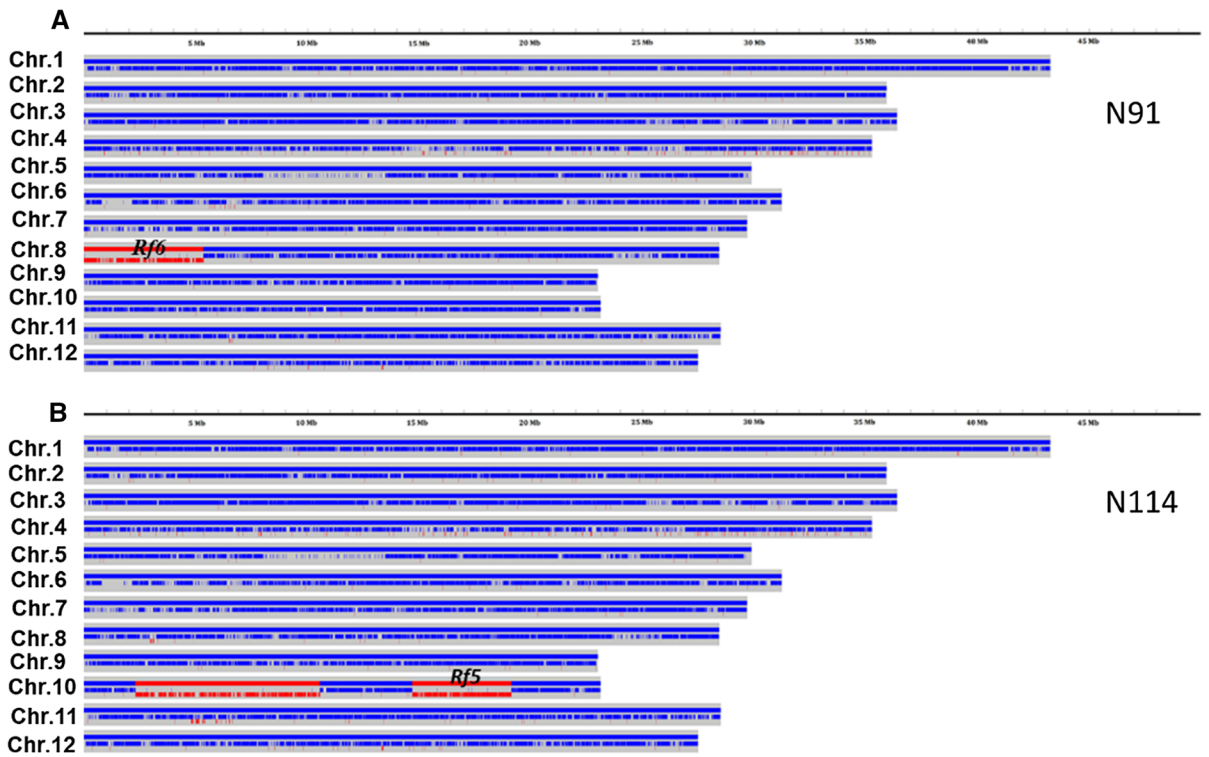


Fig. 1 Genome constitution of CSSLs based on resequencing data analyses. **a** Genome constitution of N91 (carrying *Rf6*). Each blue line and red line represent single nucleotide polymorphisms of 'Nipponbare' and '93-11', respectively. The red box in Chr.8 indicates a substituted fragment derived from 93-11. **b** Genome constitution of N114 (carrying *Rf5*). The red boxes in Chr.10 indicate substituted fragments derived from 93-11. (Color figure online)

harboring the genotype *Rf5Rf5Rf6Rf6* in the F_2 population were obtained and designed as $PPL^{Rf5+Rf6}$. All these materials had a similar genetic background to Nip, and exhibited similar agronomic traits to Nip.

Generation of plants carrying different genotypes at the *Rf5* and *Rf6* loci

During 2010–2015, WA-NipA was bred using Nip as the maintainer with continuous backcrossing. In 2015, NIL^{Rf5}, NIL^{Rf6}, and $PPL^{Rf5+Rf6}$ were used as the male parents to cross with WA-NipA. The resulting F_1 plants were backcrossed with NIL^{Rf5}, NIL^{Rf6}, and $PPL^{Rf5+Rf6}$ plants, respectively, to produce three BC_1F_1 populations, including WA-NipA/NIL^{Rf5}//NIL^{Rf5}, WA-NipA/NIL^{Rf6}//NIL^{Rf6}, and WA-NipA/$PPL^{Rf5+Rf6}$//$PPL^{Rf5+Rf6}$. There were 47, 59, and 58 plants in these three populations, respectively. Plants harboring one of eight genotypes (i.e., *Rf5rf5rf6rf6*, *Rf5Rf5rf6rf6*, *rf5rf5Rf6rf6*, *rf5rf5Rf6Rf6*, *Rf5rf5Rf6rf6*, *Rf5rf5Rf6Rf6*, *Rf5Rf5Rf6rf6*, and *Rf5Rf5Rf6Rf6*) were detected from the BC_1F_1 populations using MAS, and there were more than three plants for each genotype (Table 1). The plants with different genotypes exhibited similar agronomic traits to those of WA-NipA (Fig. 2a).

Rf genes around the *Rf5* and *Rf6* loci are involved in fertility restoration of WA-type CMS lines

In 2016, we analyzed the anther and pollen grain morphologies, as well as the seed-setting rates, of all plants in WA-NipA/NIL^{Rf5}//NIL^{Rf5}, WA-NipA/NIL^{Rf6}//NIL^{Rf6}, and WA-NipA/$PPL^{Rf5+Rf6}$//$PPL^{Rf5+Rf6}$ populations. Plants carrying the genotypes of *Rf5rf5rf6rf6*, *Rf5Rf5rf6rf6*, *rf5rf5Rf6rf6*, *rf5rf5Rf6Rf6*, and *Rf5rf5Rf6rf6* produced milky white, slender, and stunted anthers that contained shrunken pollen grains. In contrast, the anthers of WA-NipA-*Rf5Rf5Rf6rf6*, WA-NipA-*Rf5rf5Rf6Rf6*, and WA-NipA-*Rf5Rf5Rf6Rf6* plants were yellow and engorged, with pollen grains that could be stained with 1% potassium iodide (Fig. 2b, c). None of these plants were able to set seeds on the bagged panicles, and had extremely low seed-setting rates ($\leq$ 1.1%) on the unbagged panicles. These results suggested that *Rf* genes around the *Rf5* and *Rf6* loci are involved in the fertility restoration of WA-type CMS and are influenced by a gene dosage effect during the recovery of normal anther and pollen grain morphologies in the WA-CMS lines. Therefore, we concluded that *Rf* genes around both the *Rf5* and *Rf6* loci are responsible for the weak capability of '93-11' to restore the fertility of WA-type CMS lines. Thus, it would be necessary to breed WA-type maintainers with the genetic background of '93-11' by eliminating the *Rf* genes around the *Rf5* and *Rf6* loci.

Rf genes function to reduce the *WA352* transcript level

To examine the mechanism underlying the fertility restoration of WA-CMS lines by *Rf* genes around the *Rf5* and *Rf6* loci, we first used qPCR to examine the expression patterns of *WA352*, which is a mitochondrial gene responsible for the male sterility of WA-

Table 1 Numbers of plants harboring different genotypes in the BC1F1 populations

Population	Genotype	Number of plants	Total number
WA-NipA/NIL^{Rf5}//NIL^{Rf5}	*Rf5rf5rf6rf6*	28	47
	Rf5Rf5rf6rf6	19	
WA-NipA/NIL^{Rf6}//NIL^{Rf6}	*rf5rf5Rf6rf6*	27	59
	rf5rf5Rf6Rf6	32	
WA-NipA/$PPL^{Rf5+Rf6}$//$PPL^{Rf5+Rf6}$	*Rf5rf5rf6rf6*	4	58
	Rf5Rf5rf6rf6	5	
	rf5rf5Rf6rf6	7	
	rf5rf5Rf6Rf6	5	
	Rf5rf5Rf6rf6	15	
	Rf5rf5Rf6Rf6	10	
	Rf5Rf5Rf6rf6	9	
	Rf5Rf5Rf6Rf6	3	

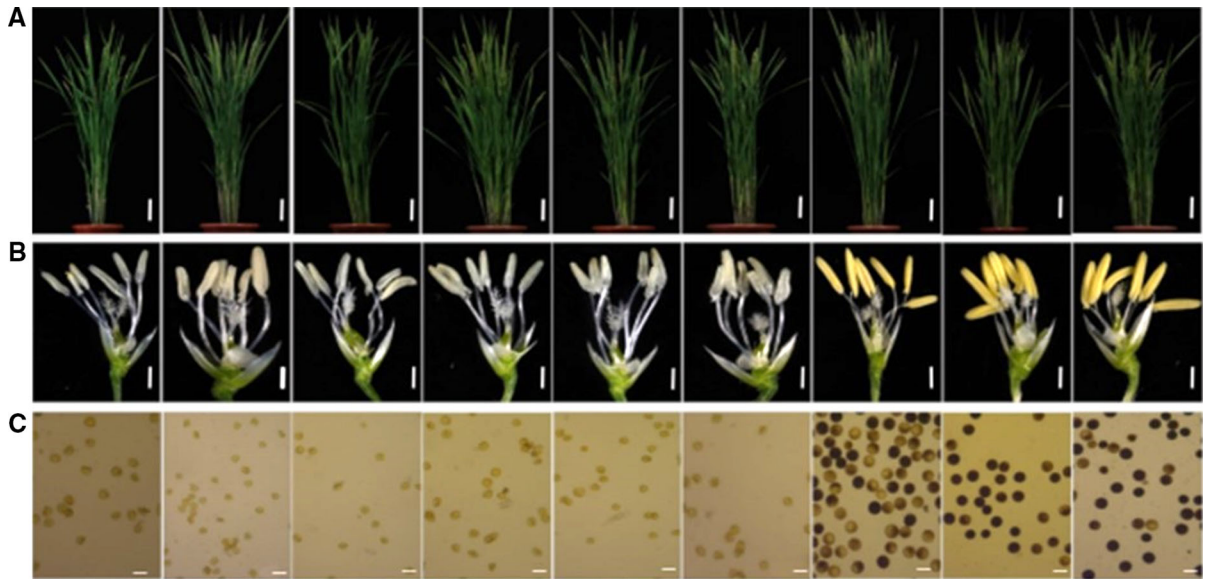
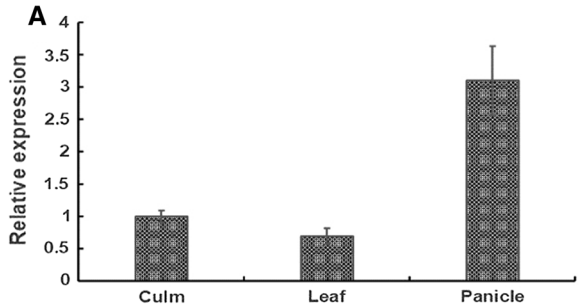


Fig. 2 Plant and anther morphology, and pollen grain stainability, of plants with WA-type cytoplasm and different genotypes at the *Rf5* and *Rf6* loci. **a** Morphology of plants with the WA-type cytoplasm and different genotypes at the *Rf5* and *Rf6* loci. **b** Anthers of plants with WA-type cytoplasm and different genotypes at the *Rf5* and *Rf6* loci. **c** Pollen grains of plants with WA-type cytoplasm and different genotypes at the *Rf5* and *Rf6* loci. Pollen grains were stained with 1% I_2–KI. Scale bars = 10 cm (**a**), 1 mm (**b**), and 50 μm (**c**). Genotypes (left to right): WA-NIPA, WA-NIPA-*Rf5rf5rf6rf6*, WA-NIPA-*Rf5Rf5rf6rf6*, WA-NIPA-*rf5rf5Rf6rf6*, WA-NIPA-*rf5rf5Rf6Rf6*, WA-NIPA-*Rf5rf5Rf6rf6*, WA-NIPA-*Rf5Rf5Rf6rf6*, WA-NIPA-*Rf5rf5Rf6Rf6*, and WA-NIPA-*Rf5Rf5Rf6Rf6*

CMS lines (Luo et al. 2013). The qPCR data for the different WA-NipA plant tissues indicated that *WA352* was highly expressed in young panicles, but expressed at low levels in the other examined tissues (Fig. 3a), which was the same as that described in a former study (Luo et al. 2013; Tang et al. 2014). We then investigated *WA352* expression in the young panicles of plants carrying different genotypes. The *WA352* transcript levels were lower in plants carrying the *Rf5* and/or *Rf6* loci than that in WA-NipA plants. Additionally, many *Rf* genes were associated with lower relative *WA352* expression levels, which was consistent with the morphology of the pollen grains and anthers (Fig. 3b). Therefore, *Rf* genes around *Rf5* and *Rf6* loci are involved in decreasing *WA352* mRNA levels to suppress WA352-mediated male sterility, which is similar to the role of *Rf4* (Tang et al. 2014).



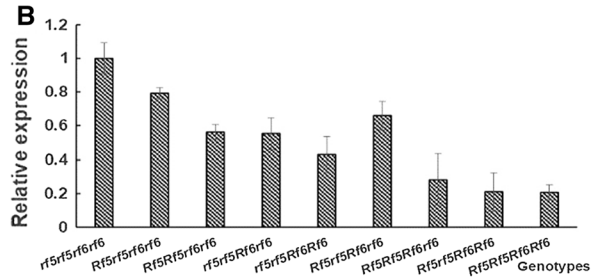


Fig. 3 Functional analysis of *Rf5* and *Rf6* in the fertility restoration of WA-type CMS. **a** Relative *WA352* transcript abundance, as analyzed by qPCR. **b** Relative *WA352* transcript levels in the young panicles of plants with WA-type cytoplasm and different genotypes at the *Rf5* and *Rf6* loci. The *atp6* gene was used as an internal control. The data are shown as the mean ± standard deviation (n = 3)

Discussion

Since the 1970s, three-line hybrid rice varieties have been used widely in China. The application of the three-line hybrid breeding system depends on the identification of CMS lines containing recessive *Rf* alleles and a corresponding *Rf* allele-containing restorer line. To date, WA-, HL-, and BT-type cytoplasms were the typical sterile cytoplasms used for breeding CMS lines, and five *Rf* genes including *Rf4*, *Rf5* and *Rf6*, *Rf1* (*Rf1a/Rf1b*) have been isolated from rice restorer plants (Hu et al. 2012; Huang et al. 2015; Komori et al. 2004; Tang et al. 2014; Wang et al. 2006). In breeding practice, it used to be thought that there is a one-to-one match between each type CMS line and the restorer line, and each *Rf* functions specifically in a particular type of CMS system, which has a unique phenotype caused by the specific type of mitochondrial DNA and corresponding nuclear gene(s) (Chen and Liu 2014; Tang et al. 2017). In *indica*, WA-type CMS plants usually exhibit stunted anthers, typical abortive pollen grains, and no seed setting on bagged panicles. '93-11' is not a restorer for WA-type CMS lines, and the allele of *Rf4*, the major fertility restorer gene for WA-type CMS in '93-11', was identified to be *rf4* (Tang et al. 2014). However, '93-11' was identified to carry some minor *Rf* genes of WA-type CMS, because the F_1 plants from the crosses between WA-type CMS lines and '93-11' exhibited normal anthers and stained pollen grains. In a previous study, only *Rf5* and *Rf6*, the fertility restoration genes for HL-type CMS, were isolated from '93-11'. It was believed that there might be no genes around *Rf5* and *Rf6* loci for the fertility restoration of WA-type CMS in '93-11'. In the present study, we generated plants carrying the background of WA-NipA and different genotypes at the *Rf5* and *Rf6* loci. As WA-NipA is a stable WA male sterile line, exhibiting stunted anthers and typical abortive pollen grains, it presumably does not carry *Rf* genes for WA-type CMS. WA-NipA-*Rf5rf5Rf6Rf6*, WA-NipA-*Rf5rf5Rf6Rf6*, and WA-NipA-*Rf5Rf5Rf6Rf6* plants exhibited normal anthers and stained pollen grains, and these plants carried only the introgression segments covering the *Rf5* and *Rf6* loci from '93-11'. These results indicated that *Rf* genes in '93-11' having minor effects on the fertility restoration of WA-type CMS lines are located around the *Rf5* and *Rf6* loci, which would be very useful to develop pairs of WA male sterile and maintainer lines with the background of '93-11'. These findings also provided new insights into the maintainer–restorer relationships among different types of CMS.

It was previously reported that *WA352* is the candidate CMS-associated gene in WA-type CMS (Luo et al. 2013). *Rf4*, the major *Rf* gene for WA-type CMS, encodes a PPR-containing protein that generally binds to RNA and is involved in RNA metabolism in mitochondria or plastids (Lurin et al. 2004). *Rf4* was demonstrated to reduce the level of *WA352* transcripts during fertility restoration of WA-type CMS (Tang et al. 2014). In previous studies, a cluster of PPR genes was observed at the *Rf5* locus on chromosome 10 and the *Rf6* locus on chromosome 8 (Hu et al. 2012; Komori et al. 2004; Tang et al. 2014; Wang et al. 2006). In the present study, qPCR analysis showed that the *WA352* transcript level varied between WA-NIPA and other lines carrying the *Rf5* and/or *Rf6* loci. The *WA352* transcript levels were decreased in plants carrying the *Rf5* and/or *Rf6* loci, indicating that the *Rf* genes around the *Rf5* and *Rf6* loci contributed to the decrease in *WA352* transcript levels. Accordingly, we presumed that *Rf* genes located around the *Rf5* and *Rf6* loci that influenced the fertility restoration of WA-type CMS lines in '93-11' might encode PPR-containing proteins. The cloning of rice fertility restorer genes showed that *Rf5* is the same gene as *Rf1a*. Additionally, *Rf5* and *Rf6*, both of which were identified from HL-type *indica* restorer, and similar to *Rf1a* from CMS-BT, could restore the fertility of BT-type CMS lines (Hu et al. 2012; Huang et al. 2012, 2015; Zhang et al. 2017). These results suggested that the functions of *Rf5* and *Rf6* are not specific to restore the fertility of HL-type CMS lines. Furthermore, the amino acid sequences encoded by *Rf4* and *Rf5* are highly similar, and *Rf6* is only one member of the cluster at *Rf6* locus has the function to restore fertility to HL-type CMS line (Huang et al. 2015; Tang et al. 2014). Therefore, we hypothesized that the *Rf* genes around the *Rf5* and *Rf6* loci that influenced the fertility restoration of WA-type CMS lines might actually be *Rf5* and *Rf6*. More studies are required to test this hypothesis.

In the CMS/*Rf* systems, two or more fertility restorer genes are required to produce viable pollen. For example, the fertility recovery of maize CMS-T was influenced by the cumulative action of *Rf1* and *Rf2a* (Wise et al. 1999), and the fertility was partially recovered in the presence of genes *Rf8* and *Rf** with *Rf2a* (Dill et al. 1997). In rice, for the full recovery of

fertility of WA-CMS lines, *Rf3* on chromosome 1 and *Rf4* on chromosome 10 are required (Ahmadikhah and Karlov 2006; Jing et al. 2001; Luo et al. 2013; Ngangkham et al. 2010; Yao et al. 1997; Zhang et al. 1997). In the present study, we also determined that the functions of *Rf* genes around the *Rf5* and *Rf6* loci are mediated by dosage effects during the fertility restoration of WA-CMS lines. These results may be useful to further characterize the restoration of fertility in WA-type CMS lines, and provide new information for the breeding of maintainers and restorers for WA-type CMS.

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Author contributions HZ analyzed the data and drafted the manuscript. XC and LZ completed the phenotypic evaluations and data analyses. QL and MG were involved in designing the study. ST designed the study and revised the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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