Precise Genetic Mapping of *Rf18(t)*, A New Fertility Restorer Gene From 'Nipponbare' for Wild Abortive Cytoplasmic Male Sterility in Rice (*Oryza Sativa* L.)

honggen zhang (✉ zhg@yzu.edu.cn)

Yangzhou University <https://orcid.org/0000-0002-9125-2603>

Xixu Li

Yangzhou University

Zuopeng Xu

Yangzhou University

Ruixuan Wang

Yangzhou University

Xiangqiang Zhao

Nantong University

Geliang Tu

Yangzhou University

Guohua Liang

Yangzhou University

Minghong Gu

Yangzhou University

Shuzhu Tang

Yangzhou University

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Abstract

Three-line hybrid rice obtained through cytoplasmic male sterility (CMS) has helped increase the yield of rice globally, and the wild abortive (WA)-type cytoplasm from wild rice (*Oryza rufipogon* Griff.) is used widely in three-line *indica* hybrids. The identification and mapping of the *Restorer-of-fertility* (*Rf*) genes in maintainer lines aided in uncovering the genetic basis of fertility restoration of WA-type CMS and the development of WA-type hybrids. In this study we identified a new *Rf* gene, *Rf18(t)*, for WA-type CMS from the *japonica* maintainer line 'Nipponbare' using a chromosome segment substitution line (CSSL) population derived from a cross between the *indica* line 9311 and 'Nipponbare'. Using a substitution mapping strategy, *Rf18(t)* was delimited to a 48-kb chromosomal region flanked by molecular marker loci ID01M28791 and ID01M28845 on chromosome 1. By comparative sequence analyses, we propose that *LOC_Os01g71320* is the most likely candidate gene for *Rf18(t)*, and it is predicted to encode hexokinase. Furthermore, *Rf18(t)* was found to function in fertility restoration probably by a posttranscriptional mechanism and its function is dependent on the genetic background of 9311. These results broaden our knowledge on the mechanism of fertility restoration of WA-type CMS lines and will facilitate the development of WA-type rice hybrids.

Key Message

We mapped *Rf18(t)*, a *Restorer-of-fertility* gene for wild abortive cytoplasmic male sterility from the *japonica* maintainer 'Nipponbare', to chromosome 1. The best candidate gene, *LOC_Os01g71320*, is predicted to encode hexokinase.

Introduction

Rice is an important staple crop that feeds more than half of the world's population. Hybrid rice, including three-line and two-line hybrid rice, accounts for 57% of the total rice planting area and constitutes 65% of the total rice yield in China (Yuan 2014). Most commercial rice hybrids are based on the three-line system (Chen and Liu 2014; Huang et al. 2014; Kim and Zhang, 2018). Three-line hybrid rice is developed using cytoplasmic male sterility (CMS), a trait controlled by a maternally inherited mitochondrial sterility gene that results in the inability to produce fertile pollen in CMS plants (Chen and Liu 2014; Huang et al. 2014; Luo et al. 2013; Kim and Zhang, 2018). The combination of CMS with maintainer and restorer lines is necessary for the production of hybrid seeds in three-line hybrid systems. In breeding practice, the CMS line is developed by continuous backcrossing with the maintainer as the donor parent. For maintaining the complete sterility of CMS lines, it is generally accepted that no *Restorer-of-fertility* (*Rf*) genes should be present in the genome of the maintainer lines. The *Rf* gene(s) usually encode pentatricopeptide repeat-containing (PPR) proteins and are considered to be present in the restorer lines and will restore male sterility in the F₁ plants (Hanson and Bentolila 2004; Huang et al. 2015; Wang et al. 2006; Melonek et al. 2021).

There are three representative CMS types in rice; wild abortive (WA), Honglian (HL), and Chinsurah Boro II (BT), of which the WA-type CMS has been extensively used in the commercial production of *indica* hybrid rice seeds in China (Chen and Liu 2014; Huang et al. 2014; Kim and Zhang, 2018). The WA-type CMS is a sporophytic male sterility system where the pollen grains of the WA-type CMS lines abort at the uninucleate stage. Sporophytic CMS is more stable than CMS lines derived from a gametophytic system in which pollen grains abort at the binucleate and trinucleate stages. To date, the inheritance of fertility restoration in the WA-CMS system has been extensively investigated. Different genetic models, such as the monogenic (Anandakumar and Subramaniam 1992; Shen et al. 1996), digenic (Bharaj et al. 1991; Yao et al. 1997), digenic with different types of interactions (Govinda and Virmani 1988; Mehrajuddin et al. 2013; Waghmode and Mehta 2011), polygenic (Yang and Chen 1990), and fertility restoration affected by *Rf* QTLs of minor effect (Li et al. 2014) have been suggested for the regulation of WA-CMS restoration. With the advent of molecular mapping, two genes, *Rf3* and *Rf4* that are present in many restorer lines, have been mapped on chromosomes 1 and 10, respectively; these two genes have major effects on fertility restoration in WA-CMS lines (Jing et al. 2001; Ngangkham et al. 2010; Sheeba et al. 2009; Suresh et al. 2012; Tan et al. 1998; Yao et al. 1997; Zhang et al. 1997), and *Rf4* has been cloned (Kazama and Toriyama 2014; Tang et al. 2014). Using molecular markers and a quantitative trait loci (QTL) analysis, several other loci or QTLs have been identified on chromosomes 2, 3, 4, 5, 7, 11, and 12 from different restorer lines (Bazrkar et al. 2008; Zhu et al. 1996; Zhuang et al. 2001). Thus, the underlying genetic basis of fertility restoration in WA-CMS lines is complicated, and the gene numbers, gene positions, and genetic effects of these *Rf* genes are inconsistent based on the literature. Therefore, it is necessary to further explore the genetic basis of fertility restoration in WA-type rice.

In the above-mentioned studies, the genetic populations were usually derived from WA-type CMS lines and restorer lines, and *Rf* genes were identified from the restorer lines, but not in the maintainer/CMS lines. However, some breeders think that there are some minor *Rf* genes present in the *indica* maintainer lines (Lei 1983; You et al. 2003), and *Rf* genes in different maintainer lines may complement each other for the partial fertility restoration of WA-CMS lines (Shalini et al. 2015). There is little doubt that the *Rf* genes in maintainer lines can cause variation in fertility restoration, which may result in a more complicated genetic basis for the fertility restoration of WA-type CMS lines. Thus, the identification of *Rf* genes in maintainer lines is an important step to further explore the genetic basis of fertility restoration in WA-type CMS rice. Although many efforts have made previously to identify the *Rf* genes in maintainer lines, the complex genetic basis and less effective methods hindered the research. At present, there are few studies that have reported the mapping of *Rf* genes in the WA-CMS maintainer lines, and none have been clarified.

Here, we report the identification of *Rf18(t)*, a new *Rf* gene for fertility restoration in WA-type CMS lines from the *japonica* maintainer 'Nipponbare'. *Rf18(t)* was delimited to a small 48-kb region on chromosome 1 using a substitution mapping approach. The most likely candidate genes for *Rf18(t)* and the function of *Rf18(t)* in fertility restoration of WA-type CMS lines were further analyzed. Our results provide new insights into the fertility restoration of WA-type CMS and the breeding of WA-type hybrid rice.

Materials And Methods

Plant materials

In the present study, a set of chromosome segment substitution lines (CSSLs; C1-C135), which were developed from a cross between the *indica* variety 9311 as the recipient and *japonica* variety 'Nipponbare' (NIP) as the donor and were re-sequenced (Xu et al. 2010), were used as paternal parents. Three WA-type *indica* CMS lines, including WufengA (WFA), TianfengA (TFA), and Guang8A, were used as the maternal parents.

In breeding practice, 9311 is an elite *indica* restorer line that is used for two-line hybrid rice and HL-type hybrid rice, but not for WA-type hybrid rice, and two *Rf* genes, *Rf5* and *Rf6* for HL-type CMS, have been identified in 9311 (Hu et al, 2012; Huang et al, 2015). NIP is a *japonica* maintainer line for BT-, HL- and WA-type CMS (Zhang et al, 2017; Zhang et al, 2021). Initially, a testcross population was generated by crossing three CMS lines with the paternal parents, including 9311, and six CSSLs with the *rf5rf5* genotype (numbers C31, C34, C47, C115, C116, and C119), to check whether *Rf5* functions in fertility restoration of WA-type CMS. Based on the spikelet fertility levels of the testcross F₁ progenies, new *Rf* genes for fertility restoration of WA-type CMS were identified in C119. For the linkage analysis, two testcross populations, including one from 16 CSSLs carrying the overlapping introgressed chromosomal segments with C119 and another from 43 plants in the 9311/C119 F₂ population, were developed using the *indica* CMS lines WFA and TFA, and a new *Rf* gene, *Rf18(t)*, from the donor parent NIP, was identified. For primary mapping of *Rf18(t)*, the testcross populations were developed by crossing 15 CSSLs carrying the introgressed segments surrounding *Rf18(t)* with both WFA and TFA. For fine mapping *Rf18(t)*, the recombinant chromosome substitution lines (RCSLs), each carrying an introgressed NIP fragment surrounding *Rf18(t)*, were developed from the 9311/C119 F₃₋₅ populations, and the testcross population was constructed by crossing the RCSLs with WFA and TFA concurrently. In order to analyze the way in which *Rf18(t)* functions in the fertility restoration of WA-type CMS, a WFA/C119 population consisting of 958 F₂ individuals was used. These materials were planted from 2015 to 2021 with two cropping seasons per year. The CSSLs, RCSLs, and 9311/C119 F₂ population were planted in the experimental field at Lingshui, Hainan Province, and each line was planted in four rows, with 10 plants per row. The testcross populations and the WFA/C119 F₂ population were planted in the experimental field at Yangzhou University in Yangzhou, Jiangsu Province. There were two replicates for each testcross F₁ hybrid including one planted in four rows, with five plants per row under natural conditions, and another planted in a single row, with 10 plants per row and isolated with plastic film (Fig. S1a, b). All of these rice materials were managed with the proper masters.

Fertility scoring and genetic analysis

The pollen fertility, natural spikelet fertility, and bagged spikelet fertility of five plants in each testcross F₁ line from the 9311/C119 F₂ plants, two plants in each testcross F₁ line from the CSSLs and RCSLs, and each plant in the WFA/C119 F₂ population were assessed. For pollen fertility, mature anthers were

harvested and pollen grains were stained with 1% I₂–KI solution. The number of normal, dark-blue (stained), clear (unstained) and typical abortive pollen grains for each individual were observed and counted using an optical microscope (Zhu, 1979). The spikelet fertility was measured as the average seed-setting rate by counting the filled and unfilled grains of two panicles from one plant harvested 30 days after flowering. At the flowering stage, two panicles from two plants of each line grown under natural conditions were bagged. The average values of the two bagged panicles and unbagged panicles on one plant were defined as bagged spikelet fertility and natural spikelet fertility, respectively. The fertility levels of each testcross F₁ line from the CSSLs and RSSLs were observed for at least two years.

For genetic analysis, plants with <1% bagged spikelet fertility were categorized in the sterile class, and plants with >5% bagged spikelet fertility or the isolated plants with >30% natural spikelet fertility were regarded as fertile in the testcross population. The testcross F₁ lines that contained some fertile plants were recorded as fertile lines.

DNA extraction and molecular marker analysis

Genomic DNA was isolated from fresh leaves of field-grown plants using the CTAB method (Rogers and Bendich 1985). Simple sequence repeat (SSR) markers were identified from the Gramene database (<http://www.gramene.org/>). Newly developed insertion-deletion (InDel) markers were based on rice genome sequences (<http://www.ncbi.nlm.nih.gov/>) that were used as queries in BLAST searches against the genome sequences of 'Nipponbare' and 9311. Primers were synthesized by the Shanghai Generay Biotech Co., Ltd. (Shanghai, China). The SSR and InDel markers are given in Supplemental Table 1.

Molecular marker amplifications were performed by PCR in 20 µl reactions containing 0.1 mmol/l of each dNTP, 1.0 U *Taq* DNA polymerase, 0.2 µmol/L primer, and 20 ng template DNA. The amplification conditions consisted of one cycle of 94°C for 4 min, followed by 32 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 50 s, with a final extension step at 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, Hercules, CA USA). DNA fragments corresponding to candidate genes in the mapped region were amplified from NIP and 9311 genomic DNA using KOD Plus high fidelity DNA polymerase (Toyobo, Osaka, Japan) and sequenced. DNA sequence analysis and alignments were performed using DNAMAN 8.

Gene expression assays by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from young panicles using the Plant RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Removal of contaminating genomic DNA and cDNA synthesis were performed using the QuantiTect Reverse Transcription kit (Vazyme, Nanjing, China). The primers were designed using QuantPrime (<http://www.quantprime.de/>). qRT-PCR reactions were performed as previously described (Zhou et al. 2018). The rice *ATP6* and *Actin* (*Os03g0718100*) genes were used as internal controls for normalization of gene expression. Quantitative real-time PCR (qRT-PCR) assays for

WA352 and candidate genes were performed with two biological replicates using SYBR Premix Ex Taq (Vazyme, Nanjing, China) on a CFX96 Real-Time PCR instrument (Bio-Rad, Hercules, CA, USA). Relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The names and sequences of the gene-specific primers are given in Supplemental Table 1.

Data analysis

The analysis of variance procedure package in SPSS15.0 was used for the statistical analysis of the fertility of the materials and populations used in this study.

Results

Identification of *Rf* genes in C119

In the breeding of three-line hybrid rice, 9311 cannot be used as the maintainer for WA-type CMS because it carries minor *Rf* genes (Li et al. 2016; Li et al. 2020). Because *Rf5* and *Rf6* were identified in 9311 we chose to prioritize these two *Rf* genes for initial analysis. Based on the genomic re-sequencing results, C31, C34, C47, C115, C116, and C119 were found to carry the introgressed DNA segments spanning the *Rf5* locus on chromosome 10 (Fig. 2a, Fig. S2), but none was found to carry introgressed segments encompassing the *Rf6* locus in the CSSL population. Based on this information, we generated seven pairs of testcross F_1 hybrids by crossing three WA-type CMS lines with 9311 and the six CSSLs carrying the *rf5rf5* genotype. We performed an I_2 -KI staining assay to analyze the pollen fertility of plants in each testcross F_1 line. The WA-type CMS plants were sterile, and the pollen grains produced by these plants were typically aborted (Fig. 1a, e). All the testcross F_1 hybrids had 90% stainable pollen grains (Fig. 1f-k), indicating that 9311 carries *Rf* genes for fertility restoration of WA-type CMS. The testcross F_1 hybrids using 9311 exhibited low natural spikelet fertility (Fig. 1b-d) and no bagged spikelet fertility when grown under natural field conditions, and complete sterility when grown in isolation (Fig. S1c). The testcross F_1 hybrids in which the CSSLs were paternal parents, except for C119, showed similar spikelet fertility levels with those from 9311 (data not shown). For the C119 testcross F_1 hybrids, the bagged spikelet fertility levels ranged from 8.58–27.82%, and the natural spikelet fertility levels were >50% under natural field conditions or >30% when grown in isolation (Fig. 1b-d, Fig. S1d). These observations indicated that C119 carries additional *Rf* genes, compared with 9311, for fertility restoration of WA-type CMS. We hypothesized that the additional *Rf* genes in C119 most likely originated from the donor parent NIP and were located on the introgressed segments.

Linkage analysis of candidate *Rf* loci

Intrigued by these findings, we sought to determine the candidate chromosomal regions carrying the target *Rf* genes in C119. From the re-sequencing results, we identified five introgressed segments on chromosomes 1, 4, 10, and 12 in C119 (Fig. 2a). Based on this information, 17 CSSLs sharing the overlapping introgressed chromosomal segments with C119 were chosen from the CSSL population and

were then crossed with WFA and TFA, resulting in two groups of testcross F_1 hybrids (Table 1). In this testcross population, only the testcross F_1 hybrids from C57 exhibited the fertile phenotype, similar to those from C119, while the other testcross F_1 hybrids were sterile (Table 1). C57 carries only a single substitution segment on chromosome 1 and shares an overlapping region with C119 (Fig. 2b). These results confirmed that C119 harbors an additional *Rf* gene that is from the donor parent NIP and is located on the long arm of chromosome 1. In addition, C54 and C122 were found to carry shorter substitution segments in the target region, from which we can infer that the target *Rf* gene is located at the distal end of the long arm of chromosome 1 (Fig. 2c, d).

Table 1

Phenotypes of the testcross F_1 hybrids between 9311 and the CSSLs and the genotypes of the four introgressed chromosomal segments from 'Nipponbare'

Male Line	Chr. 1	Chr. 4	Chr. 10	Chr. 12	Phenotype of the testcross F_1 hybrid
C119	+	+	+	+	F
9311	□	□	□	□	S
C11	□	□	□	+	S
C24	□	□	+	□	S
C26	□	+	□	□	S
C27	□	+	□	□	S
C31	□	□	+	□	S
C37	□	+	□	□	S
C54	+	□	□	□	S
C47	□	□	+	□	S
C49	□	□	+	□	S
C57	+	□	□	□	F
C61	□	+	□	□	S
C65	□	□	□	+	S
C67	□	□	□	+	S
C78	□	+	□	□	S
C80	□	□	□	+	S
C122	+	□	+	□	S
C129	□	+	□	□	S
C130	□	+	□	□	S
"+" and "-" indicate the presence or absence of introgressed chromosomal segments within the target regions. "F" and "S" indicate fertile and sterile testcross F_1 hybrids, respectively.					

At the same time, we also generated an F_2 population derived from the cross of 9311 and C119, and grew a total of 400 9311/C119 F_2 plants. All of the F_2 plants were genotyped with four polymorphic markers, including RM3482 (Chr. 1), RM252 (Chr. 4), RM1375 (Chr. 10), and RM7018 (Chr. 12), and 49 plants were selected to make controlled crosses with WFA and TFA, resulting in 49 pairs of testcross F_1 hybrids. In this testcross population, fertile plants were present in most of the testcross lines (30/33 pairs) derived

from male plants carrying the introgressed segments at the RM3482 locus. In contrast, most testcross F₁ hybrids (14/16 pairs) derived from male plants without the introgressed chromosomal segments at the RM3482 locus were sterile (Table 2). These results imply that the target *Rf* gene is located on the introduced DNA segment that originated from NIP on chromosome 1, which is consistent with the results from the CSSL-derived testcross population. Taken together, our results show that we located the target *Rf* gene on the long arm of chromosome 1, and we named the *Rf* gene *Rf18(t)*.

Table 2
Phenotypes of the testcross F₁ hybrids from the 9311/ C119 F₂ plants

Markers				No. of F ₂ plants	No. of fertile testcross F ₁ plants	No. of sterile testcross F ₁ plants
RM3482	RM252	RM1375	RM7018			
–	–	–	+	1	0	1
–	–	+	+	3	0	3
–	+	–	–	5	1	4
–	+	–	+	3	1	2
–	+	+	+	4	0	4
+	–	–	–	4	4	0
+	+	–	–	6	5	1
+	+	–	+	2	2	0
+	+	+	–	5	4	1
+	–	–	+	4	4	0
+	–	+	–	6	6	0
+	–	+	+	3	2	1
+	+	+	+	3	3	0
“+” and “–” indicate the presence or absence, respectively, of the introgressed chromosomal segments at the target loci.						

Primary mapping of the *Rf18(t)* locus

In order to determine the general position of the *Rf18(t)* locus, a total of 15 CSSLs that share the overlapping introgressed segments with C119 on chromosome 1 were identified in the CSSL population, and a testcross population was developed. In addition, we obtained 10 polymorphic markers that map to loci within the target region, and 11 markers (including RM3482) were used to genotype the 16 CSSLs; of these, 10 lines were found to carry the introduced segments based on the marker alleles (Fig. 3). In the testcross population, the testcross F₁ hybrids from C14, C56, C57, and C119 were fertile, and the testcross

F₁ hybrids from the other 11 CSSLs were sterile. Based on the genotypes of these CSSLs and the phenotypes of their derived testcross F₁ hybrids, *Rf18(t)* was located within a region of 900.29 kb between the RM5310 and STS1-170.4 loci on chromosome 1 (Fig. 3).

Fine mapping the *Rf18(t)* locus

To fine-map *Rf18(t)*, a large population of 5,000 individual plants was developed from three 9311/C119 F₂ individuals containing a single introgressed NIP segment that were heterozygous at the *Rf18(t)* region. Among the F₃ plants, 57 recombinants were detected using the markers RM5310 and STS1-170.4. Using an additional six polymorphic markers that map within the target interval, a total of nine types of homozygous recombination lines (Types Ⅰ-Ⅸ) were characterized (Fig. 4a). The phenotypes of the F₁ testcross hybrids from the 57 newly developed recombination lines were scored, and the location of *Rf18(t)* was reduced to a region of ~187 kb based on the reference sequence of the NIP genome, between the markers RM5310 and RM12812 (Fig. 4a). Another 20 markers were developed within the mapped region, four of which were polymorphism. CSSL number C54 and seven RCSLs (Types Ⅰ and Ⅱ), called CZ1-CZ7, were genotyped with these markers, and *Rf18(t)* was finally localized to a 48 kb region flanked by marker loci ID01M28826 and ID01M28865 (Fig. 4b).

Candidate gene prediction

Based on the available sequence annotation (<http://rice.plantbiology.msu.edu/>), only two open reading frames, *LOC_Os01g71310* and *LOC_Os01g71320*, were predicted to be located within the mapped interval. These ORFs encode a cytokinin dehydrogenase precursor and hexokinase (type B), a mitochondria-associated enzyme, respectively. To identify the candidate genes for *Rf18(t)*, we used PCR and DNA sequencing to examine the genomic sequence variation of *LOC_Os01g71310* and *LOC_Os01g71320* between the parental lines 9311 and NIP. Sequence comparison of *LOC_Os01g71310* showed only three base substitutions and four insertions in the promoter region between 9311 and NIP. Sequence analysis of *LOC_Os01g71320* identified six variations in the promoter as well as three base substitutions in exons between 9311 and NIP, and the variation in exon number 8 is predicted to cause an amino acid change from arginine to cysteine (Fig. 4c). To verify the expression levels of *LOC_Os01g71310* and *LOC_Os01g71320*, qRT-PCR was performed using RNA from young panicles of WFA/C119 (fertile) and WFA/9311 (sterile) F₁ plants. The qRT-PCR results showed that there were no difference in the expression levels of these two ORFs between the fertile and sterile testcross lines (Fig. 4d). Overall, our results indicate that *LOC_Os01g71320* is the most likely candidate gene for the fertility restorer gene *Rf18(t)*.

Rf18(t) does not affect the level of WA352-specific mRNA

In a previous study, *WA352*, a mitochondrial gene, was found to be related to WA-type CMS (Luo et al, 2013). To reveal the mechanism by which *Rf18(t)* restores CMS lines to fertility, we examined the levels of *WA352*-specific mRNAs in four testcross F₁ hybrids, including two fertile lines (WFA/C119 F₁ and

WFA/CZ1 F₁) and two sterile lines (WFA/9311 F₁ and WFA/CZ6 F₁) by qRT-PCR. We found that the WA352 mRNA levels were similar in the fertile and the sterile testcross lines (Fig. 5). Therefore, we speculated that *Rf18(t)* does not function to affect WA352 mRNA levels, and that a posttranscriptional mechanism probably acts to suppress WA352-mediated male sterility.

Rf18(t) restoration of CMS fertility is dependent on the 9311 genetic background

In order to identify the manner in which *Rf18(t)* acts to restore fertility in WA-type CMS plants, we analyzed the pollen grains from 958 WFA/C119 F₂ plants. We found 87 plants exhibiting the typical abortion of all pollen grains, indicating that *Rf18(t)* and the unknown *Rf* genes from 9311 restore the fertility of WA-type CMS in a sporophytic manner. Also, we identified 63 plants with natural spikelet fertility levels of >85%. Two linked marker loci, RM5310 and RM12812, were used to genotype the selected plants. Among the 87 sterile plants tested, the numbers of plants with genotypes *Rf18(t)Rf18(t)*, *Rf18(t)rf18(t)*, and *rf18(t)rf18(t)* were 17, 47, and 23, respectively. Among 63 fertile plants tested, the numbers of plants with the genotypes *Rf18(t)Rf18(t)*, *Rf18(t)rf18(t)*, and *rf18(t)rf18(t)* were 20, 29, and 14, respectively. The allelic segregation of these two markers gave a good fit to a 1:2:1 segregation ratio in the sterile and fertile subpopulations ($\chi^2 = 0.50$ and 0.46 in the sterile and fertile subpopulations, respectively, which were $< \chi^2_{0.05} = 5.99$). These results indicate that *Rf18(t)* does not restore the fertility of WA-type CMS lines independently, but shows a strong reciprocal interaction with unknown restorer gene(s) present in 9311.

Discussion

WA-type CMS was the first type of male sterility used in hybrid rice breeding and has been used extensively in the breeding of hybrid rice since the 1970s. Because of the great impact of WA-type CMS on agriculture, the CMS-causing gene and the fertility restorer genes for WA-type CMS have been studied intensively. In the present study, we identified *Rf18(t)*, a new fertility restoration gene from the *japonica* maintainer line NIP. We employed a substitution mapping strategy combined with testcrossing to localize *Rf18(t)* to a region of 48-kb on the long arm of rice chromosome 1. We succeeded in reducing the number of *Rf18(t)* candidates, to two genes, and *LOC_Os01g71320* is the strongest candidate gene for *Rf18(t)*.

In rice production, WA-type CMS has only been used for the development of *indica* hybrids, not *japonica* hybrids. *Rf* genes in *indica* maintainer or CMS lines, which would influence the stability of male sterility in WA-type CMS lines, have drawn the attention of rice breeders. Minor *Rf* genes in maintainer lines have been found by observing the percentage of normal pollen grains and low seed-setting rates in advanced backcrossing progeny plants in breeding programs of WA-type CMS lines transformed from maintainer lines (Lei 1983). From a test F₁ population made by crossing V21A, a WA-type CMS line, with 1,228 rice varieties, the phenotypes of both fertile and sterile anthers present in the spikelets were observed, and it was speculated that fertility restoration is controlled by minor *Rf* genes (You et al. 2003; Fu et al. 2010). Some level of partially fertile and fully fertile plants were present in three-way test crosses involving B/B combinations, indicating that the complementation of *Rf* genes with null or minimal effects in maintainer

lines may be responsible for the appearance of fully fertile plants (Shalini et al. 2015). The majority of *japonica* cultivars can be used as maintainers for WA-type CMS, and WA-type *japonica* CMS lines display more stable sterility and poor recoverability than WA-type *indica* CMS lines, which makes it extremely difficult to exploit hybrid vigor through the use of WA-type CMS in *japonica* rice hybrids (Tang et al, 2014). Based on extensive breeding experience, *japonica* cultivars are generally thought to carry no *Rf* genes for WA-type CMS that can be used in rice breeding. In the present study, the fertility restorer gene *Rf18(t)* was identified from 'Nipponbare', a typical *japonica* cultivar, and it can be used as the maintainer for all the types of CMS used in practice. To the best of our knowledge, this is the first study to identify *Rf* genes for WA-type CMS in a *japonica* variety. Our results provide new insights into the genetic basis of fertility restoration for WA-type CMS, which will aid in the development of WA-type hybrids.

It is well known that fertility restoration of WA-type CMS is mainly controlled by two major *Rf* genes, *Rf3* and *Rf4*. However, the mechanism of fertility restoration in WA-type CMS has not been elucidated clearly. In general, pollen and spikelet fertility of plants carrying CMS is vulnerable to environmental conditions, and this is particularly true of fertility restoration in WA-type CMS lines. Pollen and spikelet fertility levels show a continuous distribution in a given genetic population, and the criteria for fertile and sterile plants is somewhat ambiguous. Several genetic hypotheses, ranging from monogenic to digenic with interactions to trigenic with interactions, have been reported for fertility restoration in WA-type CMS in previous studies (Govinda Raj and Virmani 1988; Bharaj et al. 1991; Hossain et al. 2010). In the present study, we found that *Rf18(t)* controls fertility restoration in WA-type CMS in a sporophytic manner, which is similar to the actions of *Rf3* and *Rf4*. Fertility restoration of WA-type CMS by *Rf18(t)* is dependent on the 9311 genetic background, indicating that 9311 carries *Rf* genes that can interact with *Rf18(t)*; thus, a complex regulatory network is involved in the fertility restoration of WA-type CMS. These results have enhanced our understanding of the genetic basis of fertility restoration in WA-type CMS. Further research is warranted to identify and characterize the *Rf* genes for WA-type CMS fertility restoration that are present in 9311, which would make it possible to elucidate the interactive effects of *Rf* genes on fertility restoration in WA-type CMS.

Major advances have been made in understanding the regulation of fertility restoration in WA-type CMS. *Rf3* and *Rf4*, two major *Rf* genes present in restorer lines, have been identified and mapped to loci on chromosomes 1 and 10, respectively (Jing et al. 2001; Ngangkham et al. 2010; Sheeba et al. 2009; Suresh et al. 2012; Tan et al. 1998; Yao et al. 1997; Zhang et al. 1997). *Rf4* is the major locus for fertility restoration of WA-CMS in most cases, and it has been cloned (Kazama and Toriyama 2014; Tang et al. 2014). QTL analyses have been performed to detect minor-effect *Rf* genes in restorer lines, but none have been verified and precisely mapped. In addition, *Rf* genes are usually considered to come from restorer lines, and there are few published studies describing the genetic analysis and mapping of *Rf* genes in maintainer lines. Thus, relatively little is known about the minor *Rf* genes present in restorer or maintainer lines. In this study, a candidate gene for *Rf18(t)* was mapped to a 48-kb region on chromosome 1. Based on the genetic mapping results, we can conclude that *Rf18(t)* is a new fertility restorer gene and may be the only locus conferring fertility restoration for WA-type CMS identified in maintainer lines, especially

japonica maintainers. Our study provides a solid foundation to clone *Rf18(t)* and reveal the regulatory network involved in the fertility restoration of WA-type CMS.

At present, a number of *Rf* genes have been cloned from rice, including *Rf2* in LD-type CMS (Itabashi et al. 2011), *Rf1a* and *Rf1b* in BT-type CMS (Wang et al. 2006), *Rf17* in CW-type CMS (Fujii and Toriyam 2009), *Rf5* and *Rf6* in HL-type CMS (Hu et al. 2012, Huang 2015), and *Rf4* in WA-type CMS (Kazama et al. 2014). Among the cloned *Rf* genes, the majority have been found to encode PPR proteins, except for *Rf2* and *Rf17*, and diverse mechanisms underlying fertility restoration for CMS have been identified. In general, the proteins encoded by *Rf* genes are predicted or have been shown to be localized to mitochondria to suppress CMS defects. In this study, only two ORFs were predicted in the 48 kb chromosomal region containing *Rf18(t)*. *LOC_Os01g71320* has been identified as *OsH XK3*, which encodes a protein belonging to a hexokinase (HXK) family comprising 10 members in rice and was shown to be located to mitochondria in a previous study (Cho et al. 2006). In HL-type rice, *OsH XK6* was identified as an RF6 partner that participates in the processing of a mitochondrial RNA transcript, resulting in fertility restoration in HL-type CMS lines. A sequence comparison between 9311 and NIP revealed several polymorphisms in promoter and coding regions in the *OsH XK3* genes, and the variation in exon 8 led to an amino acid change from arginine to cysteine. Taken together, we suggest that *LOC_Os01g71320* is the most likely candidate gene for *Rf18(t)*. A functional complementation study of this gene is now in progress to determine its contribution to fertility restoration in WA-type CMS rice lines.

Declarations

Author Contributions: HZ conducted the data analysis and drafted the manuscript. XL and ZX performed the phenotypic evaluation and data analysis. RW, XZ, and GT participated in the construction of the testcross populations. GL and MG participated in the design of the study. HZ and ST designed the study and revised the manuscript. All of the authors have read and approved the final manuscript.

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Conflict of interest: The authors declare that they have no conflicts of interest.

Data Availability Statement: All data generated or used during the study appear in the submitted article.

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Figures

Figure 1

Gross morphology of rice plants and pollen viability.

(a) WufengA-WFA. (b-d) F_1 plants of WFA x 9311, WFA x C119, TFA x 9311, TFA x C119, Guang8A x 9311, and Guang8A x C119. (e-k) Pollen grains stained with I_2 -KI from corresponding plants of WFA, WFA x 9311, WFA x C119, TFA x 9311, TFA x C119, Guang8A x 9311, and Guang8A x C119, respectively. Scale bars=20 cm in a-d, 50 μ m in e-k.

Figure 2

Graphical genotypes of the CSSL lines C119 (a), C57 (b), C54 (c), and C122 (d). The position of the introgressed chromosomal segments originating from 'Nipponbare' are shown in blue. The red and yellow lines represent the homozygous 9311 genotype and the heterozygous genotypes, respectively.

Figure 3

Primary mapping of *Rf18(t)*.

Numbers under the partial map of chromosome 1 indicate physical distances. Black and white rectangles represent regions homozygous for NIP and 9311, respectively. “F” and “S” indicate the fertile and sterile testcross F₁ hybrids, respectively.

Figure 4

Fine mapping and candidate gene analysis of *Rf18(t)*.

(a and b) Fine mapping of *Rf18(t)* based on the genotypes (left) and phenotypes (right) of the recombinant substitution individuals selected from a segregating populations derived from 9311 and CSSL line C119. (c) Two open reading frames (ORFs) were found to be present in the 48 kb target region, and comparisons of the genomic sequences of the two ORFs between 9311 and NP. (d) Comparisons of the relative expression of *LOC_Os01g71310* and *LOC_Os01g71320* mRNA in fertile and sterile plants. Black and white rectangles represent regions homozygous for NIP and 9311 genotypes, respectively, in A and B. "F" and "S" indicate the fertile and sterile testcross F₁ hybrids, respectively. ▲ indicates the position of inserted nucleotides in 9311. Gene expression levels were normalized against the expression of the rice *UBQ* gene. Means ± SD were obtained from three technical replicates and three biological replicates.

Figure 5

Relative expression of *WA352* in young panicles of sterile and fertile testcross F₁ plants.

Left to right: WFA x 9311 F₁, WFA x CZ6 F₁, WFA x C119 F₁, and WFA x CZ1 F₁. Expression levels were normalized against the expression of the rice *UBQ* gene. Means ± SD were obtained from three technical replicates and three biological replicates.

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