Fertility restoration of Chinese wild rice-type cytoplasmic male sterility by CRISPR/Cas9-mediated genome editing of nuclear-encoded *RETROGRADE-REGULATED MALE STERILITY*

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Abstract Cytoplasmic male sterility (CMS) is a trait that produces nonfunctional pollen caused by the interaction between mitochondrial and nuclear genes. In Chinese-wild (CW) type CMS, CWA, in rice (*Oryza sativa* L.), its mitochondria enhance the expression of the nuclear gene *RETROGRADE-REGULATED MALE STERILITY* (*RMS*), which causes pollen abortion. Fertility is recovered when its expression decreases in a restorer line, CWR. The expression of *RMS* is controlled by the single nucleotide polymorphism (SNP) located in the promoter region 2,286bp upstream of the start codon of *RMS*. However, another gene, *PPR2*, which encodes pentatricopeptide repeat-domain containing protein, is predicted in the reverse strand of this region and a premature stop codon is created in CWR by the SNP. To prove *RMS* is directly involved in restoring fertility of CW-CMS, we introduced mutations into *RMS* and *PPR2* using CRISPR/Cas9. Fertility was recovered in the genome-edited CMS plants with reduced expression of *RMS* and unaltered expression of *PPR2*, when the mutation was introduced in the promoter regions of *RMS* within or outside the coding sequence (CDS) of *PPR2*. Fertility restoration was not obtained when the mutation was introduced within the CDS of *RMS*. Our results demonstrated that *PPR2* is not responsible for fertility restoration, and fertility was recovered by reduced expression of *RMS*, providing us with a new artificial fertility restorer line for agronomical use.

Key words: CRISPR-Cas9, cytoplasmic male sterility, fertility restoration, genome editing, *Oryza sativa*.

Introduction

Cytoplasmic male sterility (CMS), which is a maternally inherited trait resulting in the failure to produce functional pollen, is caused by the interaction between mitochondrial and nuclear genomes. Expression of a certain mitochondrial chimeric open reading frame (*orf*) gene, which is created through the recombination between different mitochondrial coding regions or between a mitochondrial gene and an unknown sequence, is known to have the ability to cause dysfunctions in pollen development and these *orfs* are termed as CMS-associated genes (Hanson and Bentolila 2004 for a review). In contrast, the expression of the CMS-associated *orf* is often suppressed by the expression of a particular nuclear-encoded gene, called the *RESTORER OF FERTILITY* (*RF*) gene that rescues pollen development resulting in the recovery of fertility.

A CMS line is, in general, bred as a result of nuclear substitution to remove the functional *RF* gene and allow the expression of a CMS-associated mitochondrial gene. A CMS and fertility restoration system, therefore, is an ideal trait to investigate the interaction between mitochondrial and nuclear genes.

Molecular cloning of *RF* genes has been reported for maize (*Zea mays* L.), petunia (*Petunia hybrida* L.), rape (*Brassica napus* L.), sugar beet (*Beta vulgaris* L.), sorghum (*Sorghum bicolor* L.), and rice (*Oryza sativa* L.) (Bohra et al. 2016 for a review). The majority of *RF* genes reported so far encode pentatricopeptide repeat (PPR) family proteins, which are known to be RNA-binding factors involved in processing or translation, or both, of organellar mRNAs (Schmitz-Linneweber and Small 2008). For example, in rice, *Rf1a* and *Rf1b* in Boro-Taichung (BT)-type CMS, *Rf5* and *Rf6* in Honglian (HL)-type CMS, and *Rf4* in wild-abortive (WA)-type

Abbreviations: Cas9, CRISPR-associated endonuclease 9; CMS, cytoplasmic male sterility; CRISPR, clustered regularly interspaced short palindromic repeat; CDS, coding sequence; HPT, hygromycin phosphotransferase; PAM, protospacer-adjacent motif; PPR, pentatricopeptide repeat; RF, restorer of fertility; RT-PCR, reverse transcriptase-PCR; sgRNA, single-guide RNA; SNP, single nucleotide polymorphism; T65, Taichung 65.

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CMS have been reported to encode PPR proteins and to function post-transcriptionally leading to the cleavage or degradation of mitochondrial transcripts of a CMS-associated gene (Akagi et al. 2004; Hu et al. 2012; Huang et al. 2015; Kazama et al. 2008; Kazama and Toriyama 2003, 2014; Komori et al. 2004; Tang et al. 2014; Wang et al. 2006). Some other *RF* genes, such as *Rfo* in Ogura-CMS *rape* and *Rfk1* in Kosen-CMS radish have been reported to regulate translation of mRNA of a CMS-associated gene (Koizuka et al. 2003; Uyttewaal et al. 2008).

In contrast, several *RF* genes have been reported to encode non-PPR proteins. For example, *Rf2* in maize encodes an aldehyde dehydrogenase (Liu and Schnable 2002), *Rf2* in rice encodes a glycine-rich protein (Itabashi et al. 2011), *bvORF20* in sugar beets encodes a protein resembling yeast OMA1, a peptidase involved in quality control of mitochondrial membrane proteins (Kitazaki et al. 2015), *Rf4* in maize encodes a basic helix-loop-helix (bHLH) transcription factor (Jaqueth et al. 2020), and *Rf17*, which is the *RF* gene we investigated in the current study, for Chinese wild rice (CW)-type CMS in rice encodes an unknown protein with a segment similar to that of a part of an acyl carrier protein synthase-like domain (Fujii and Toriyama 2009).

The CW-type CMS is derived from *Oryza rufipogon* Griff. accession W1 (Fujii and Toriyama 2005). A CMS line, CWA, carries the cytoplasm of W1 and a nuclear genome of a Japonica Group cultivar Taichung 65 (T65) (*Oryza sativa* L.). A CMS-associated gene in CW-CMS has been reported to be a unique *orf* gene called CW-*orf307* (Fujii et al. 2010). The pollen grains of CWA are filled with starch and morphologically normal but lack germination ability, resulting in no seed setting. Its fertility is fully restored in the isonuclear restorer line CWR, which carries a single nuclear gene, *Rf17* (Fujii and Toriyama 2005). Because all the F₂ plants derived from the F₁ hybrid between CWA×CWR are fertile, *Rf17* behaves in a gametophytic manner. Molecular cloning of the *Rf17* gene has revealed that *Rf17* has reduced expression allele of *RETROGRADE-REGULATED MALE STERILITY* (*RMS*) (Fujii and Toriyama 2009; DDBJ accession number AB481199). The expression of the *RMS* allele (= *rf17* allele) in the nucleus of the CMS line CWA is considered to be highly enhanced by a certain retrograde signal from the CW mitochondria, whereas the *rms* allele (= *Rf17* allele) in the fertility restorer line CWR might not perceive the retrograde signal resulting in down-regulation of *RMS* expression and thus, restore fertility. The fertility restoration mechanism is quite distinct from that governed by PPR proteins. To the best of our knowledge, this type of fertility restoration has not been reported elsewhere.

The expression of *RMS* is known to be controlled by the single nucleotide polymorphism (SNP) located in the

promoter region 2,286bp upstream of the start codon of *RMS*, namely *Rf17*, which carries thymine, whereas *rf17* carries adenine (Fujii and Toriyama 2009). The other sequences, including the promoter region and coding sequence (CDS) are completely identical. However, we must note the SNP in CWR creates a premature stop codon in another gene (LOC_Os04g40010) encoding a PPR-domain containing protein, which was predicted in the reverse strand by the Michigan State University (MSU) Rice Genome Annotation Project Release 7. During map-based cloning of our previous study (Fujii and Toriyama 2009), we employed an RNAi approach and demonstrated that down-regulation of *RMS* (named *ORF11* in the previous study) recovered fertility, whereas down-regulation of the PPR gene (named *PPR2* in the previous and current studies) did not, leading to our proposal that *PPR2* is not involved in fertility restoration. However, the RNAi-mediated suppression of *RMS* did not achieve the full restoration of seed setting. We were unable to find any mutants of *PPR2* or *RMS* to date to rule out the possibility of involvement of *PPR2* for fertility restoration and to prove the direct involvement of *RMS* in fertility restoration of CW-CMS.

In the present study, we employed a genome-editing technique, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9), to knock out *RMS* and *PPR2*. The CRISPR/Cas9 system was developed in 2013 (Cong et al. 2013; Jiang et al. 2013; Shan et al. 2013) and effectively applied to plants to generate targeted mutations (Li et al. 2013; Nekrasov et al. 2013). Mutations were introduced in the promoter of *RMS* within the CDS of *PPR2*, in the promoter of *RMS* outside the CDS of *PPR2*, and inside the CDS of *RMS*. Investigation of fertility restoration and expression analysis of *RMS* and *PPR2* revealed that *PPR2* was not related to fertility restoration of CW-CMS and reduced expression level of *RMS* was responsible for fertility restoration of CW-CMS.

Materials and methods

Plant materials

A CW-CMS line CWA and a restorer line CWR are isogenic lines and carry cytoplasm derived from *Oryza rufipogon* Griff. W1 strain, and nucleus of the Japonica Group cultivar Taichung 65 (T65). CWA carries *rf17rf17*, whereas CWR carries *Rf17Rf17* (Fujii and Toriyama 2005, 2009).

Generation of genome-edited lines by CRISPR/Cas9

The Cas9 plant expression vector (pZH_gYSA_MM Cas9) and sgRNA expression vector (pU6gRNA) were described by Mikami et al. (2015). According to the design principles of the target sequences in the CRISPR/Cas9 system, 20 nt upstream of the PAM motif were selected as candidate target sequences

using CRISPR direct (<https://crispr.dbcls.jp/>). Two different target sequences in the promoter and the coding region of *RMS* are listed in Supplementary Table S1. Double stranded target sequences, which were made by annealing the paired single oligonucleotides at 95°C for 5 min, were cloned into the pU6gRNA vector, which was linearized by *Bbs* I. OsU3-gYSA in pZH_gYSA_MMCas9 was replaced by sgRNA expression constructs using *Asc* I and *Pac* I sites. The derived construct was introduced into the calli of CWA and T65 by *Agrobacterium*-mediated transformation. The presence of the introduced sgRNA expression constructs was confirmed by detecting the gene for hygromycin phosphotransferase (HPT) using PCR. Primers are listed in Supplementary Table S2.

Analysis of edited sequences

To analyze the nucleotide sequences of the resultant mutants, genomic DNA was extracted from leaves of transgenic plants. PCR amplifications were conducted using primer pairs flanking the designed target sites, and are listed in Supplementary Table S3. IndelCheck™ CRISPR/TALEN Insertion or Deletion Detection System (GeneCopia, Rockville, MD, USA) were conducted according to the manufacturer's instructions. The nucleotide sequences were determined by direct sequencing of the PCR products or by sequencing of plasmids from at least two colonies after cloning of the PCR products into the pGEM-T-Easy-vector (Promega, WI, USA) using the CEQ8000 genetic analysis system (Beckman Coulter, <https://www.beckmancoulter.com/>). Genome-edited lines were selected and used in the following phenotypic analysis.

Phenotypic analysis

Plants were grown in a greenhouse at 30/25°C (day/night) under natural photoperiodic conditions or in a biotron (LPH-410SPC; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) at 30/25°C (10 h day/14 h night), in which illumination was set at an intensity of 150 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a CO₂ level was set at 500 ppm (Ohnishi et al. 2011). Seed setting rates were determined by counting filled grains and total grains for at least three bagged panicles.

qRT-PCR

Total RNA from anthers at the tricellular pollen stages were extracted using RNeasy (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA contamination was eliminated by treatment with RNase-free DNase I (TaKaRa Bio Inc., Ohtsu, Japan). cDNA synthesis was accomplished using the ReverTraAce (TOYOBO, Osaka, Japan). PCR was performed for four biological replicates using TB Green Premix Ex Taq II (TaKaRa Bio Inc.) for quantitative RT-PCR analysis. Primers used for quantitative RT-PCR of *Tubulin*, *RMS*, and *PPR2* are listed in Supplementary Table S4. Tukey's test was applied to test for differences among treatments at $p < 0.05$ using the R package "multcomp" (Hothorn et al. 2008).

Figure 1. Target sequences on *RMS* and *PPR2*. Blue and red arrowheads indicate target sites on the promoter region of *RMS* and on the CDS of *RMS*, respectively. The start codon of *RMS* is designated as +1.

Results

Selection of CRISPR/Cas9 target sequences

To exclude the possibility that *PPR2* is involved in the fertility restoration of CW-CMS, the target site G1 was designed within the *PPR2* CDS and the target site G2 was in the promoter region, which resided between the *RMS* CDS and the *PPR2* CDS (Figure 1). The target sites, G6 and G7, were selected within *RMS* CDS, and our goal was to directly prove the involvement of *RMS* in fertility restoration of CW-CMS by knocking out the *RMS* (Figure 1). A synthetic 20 bp long nucleotide corresponding to each target sequence was inserted into the binary vector cassette expressing a single guide RNA (sgRNA) and Cas9 endonuclease. We named these constructs G1, G2, G6, and G7, respectively. Each expression vector was introduced into the calli of the CMS line, CWA, and a maintainer line, Taichung T65 (T65) using the *Agrobacterium*-mediated transformation method.

Sequence analysis of transgenic plants with G1 and G2 constructs

We generated 14 independent transgenic CWA plants with the G1 construct and seven with the G2 construct. The presence of the introduced foreign gene was confirmed by detection of the *HPT* gene using PCR (Supplementary Figure S1). The regenerated plants (T₀ plants) were screened for mutations using IndelCheck™ CRISPR/TALEN Insertion or Deletion Detection System. Four plants, named CWA_G1_a, CWA_G1_b, CWA_G1_c, and CWA_G1_d, were predicted to have mutations in the target site G1. Subsequently, sequencing analysis revealed that all four plants held heterozygous mutated alleles with the identical 1 bp insertion in the G1 target site (Figure 2A; chromatograms are shown in Supplementary Figure S2), even though they were regenerated from different calli. Because the CDS of *PPR2* was predicted on the reverse strand of the G1 target site, we investigated the number of PPR motifs using TPRPred (<https://toolkit.tuebingen.mpg.de/tools/tprpred>; Supplementary Figure S3). The *PPR2* gene of CWA contained 13 PPR motifs, whereas a premature stop codon was created in the 4th PPR motif in the

Figure 2. Mutated nucleotide and predicted amino acid sequences of genome-edited CWA plant, CWA_G1. (A) Nucleotide sequences around the G1 target sites in CWA_G1 plants in the T₀ generation. The G1 target sequence is underlined and red lowercase letter indicates the insertion. The PAM sequence is shown in blue. WT, untransformed wild-type. Nucleotide positions from the start codon of *RMS* are indicated. (B) The comparison of amino acid sequences of *PPR2* among untransformed CWA_WT, CWR_WT, and CWA_G1. PPR motif is indicated. * Indicates a premature stop codon.

Figure 3. Mutated nucleotide sequences of the genome-edited CWA plant, CWA_G2. The nucleotide sequences around the G2 target site in CWA_G2_a in the T₀ generation. The G2 target sequence in underlined and dashes and red letters indicate deletion and the number of deleted nucleotides, respectively. The PAM sequence is shown in blue. WT, untransformed wild-type. Nucleotide positions from the start codon of *RMS* are indicated.

fertility restorer line CWR because of the SNP. The 1bp insertion in the G1 target site created a premature stop codon in the 6th PPR motif in the mutated allele of CWA_G1_a to CWA_G1_d (Figure 2B).

We also analyzed transgenic plants with the G2 construct and obtained one plant containing a homozygous mutated allele with a 37bp deletion on the G2 target site, which we named CWA_G2_a (Figure 3; chromatograms are shown in Supplementary Figure S4).

Seed setting rates of CWA_G1 and CWA_G2 plants

Seed setting rates of CWA, CWR, and the transgenic

Figure 4. Fertility restoration in genome-edited CWA plants, CWA_G1 and CWA_G2. (A) Seed setting rates of untransformed CWA_WT, CWR_WT and CWA_G1 and CWA_G2 plants in the T₀ and T₁ generations. An error bar indicates SE obtained from the values of three to five panicles per plant. Striped and solid bars indicate that plants carried the heterozygous and homozygous mutated alleles, respectively. The T₀ plant of CWA_G1_a was grown in a greenhouse, while all other plants were grown in a biotron. (B and C) Panicles of the CWA_G1_a and CWA_G2_a in the T₁ generation. Scale bar = 1.0 cm.

plants with the mutated alleles (CWA_G1_a, CWA_G1_c, CWA_G1_d, and CWA_G2_a) were examined (Figure 4A). All the transgenic plants exhibited restored fertility and set seeds upon self-pollination. The resulting T₁ seeds germinated normally and the T₁ plants showed normal vegetative growth without any morphological defects. The T₀ plant of CWA_G1_a was grown in a greenhouse, which might cause the lower seed setting rate of 13.1%, while all other plants were grown in a biotron for further investigation of the seed setting rates. The highest seed setting rates among the CWA_G1 in each generation was 83.7% in T₀ and 83.0% in T₁, and the values were almost identical to that of CWR. Likewise, in CWA_G2_a, the values were 15.9% in T₀ and 12.5% in T₁. The fertility of CWA_G2 was relatively lower compared to those of CWA_G1 (Figure 4B, C). Because the wild-type CWA is a completely sterile CMS line with no seed setting, these results indicated that we successfully created two new alleles that restored CW-CMS.

Seed setting rates were also examined in three plants in the T₂ generation derived from CWA_G1_a. They were 79.3% and 62.4% for the plants with the

Figure 5. Fertility restoration in null-segregants of the genome-edited plants. Seed setting rates (%; Mean $\pm$ SE) of CWA_G1_a plants carrying homozygous or heterozygous mutated alleles in the T₂ generation and PCR detection of the *HPT* gene for verification of the absence of the introduced sgRNA/Cas9-expression construct.

homozygous mutated allele, and 63.0% for the plant with the heterozygous mutated allele (Figure 5). In the two homozygous plants, the nucleotide sequence at the G1 target sites was confirmed to retain the insertion of thymine identical to that of the T₀ generation by direct sequencing (Supplementary Figure S5). As shown in Figure 5, the foreign gene, *HPT* of the sgRNA/Cas9-expression construct, was not detected by PCR analysis in these two plants carrying the homozygous mutated allele and one plant carrying heterozygous mutated alleles in the T₂ generation; thus, they were null-segregants for the introduced gene.

RMS and PPR2 expression analysis of CWA_G1 and G2

To investigate the relationship between gene expression of *PPR2* and *RMS* and the fertility recovery of the genome-edited plants of CWA, we analyzed these transcripts in mature anthers of CWA, CWR, and CWA_G1_c, and CWA_G2_a plants with the homozygous mutated alleles by quantitative real-time RT-PCR (qRT-PCR; Figure 6). For *PPR2* expression, no significant differences were observed among the plants. However, the expression level of *RMS* in CWA_G1_c was significantly decreased compared to that of CWA and was identical to that of CWR. The expression level of *RMS* in CWA_G2_a was also decreased compared to untransformed CWA_WT, although the difference was not statistically significant. These results suggested that the G2 target site was less important than the G1 target site for decreasing *RMS* expression. Because the mutation at the G2 site did not affect CDS of *PPR2* and the RNA level of *PPR2* was not changed in CWA_G2_a, we could rule out the possibility that *PPR2* was involved in fertility restoration, and conclude the reduced *RMS* expression was responsible for fertility restoration of CW-CMS.

Disruption of RMS CDS

To investigate whether disruption of the *RMS* CDS

Figure 6. Relative expression level of *RMS* and *PPR2*. qRT-PCR analysis of relative *PPR2* and *RMS* mRNA levels in the mature anthers of the T₁ transgenic plants and CWA and CWR_WT plants. The transcription level of tubulin was used as an internal control of gene expression. Error bars indicate standard deviation obtained from the values of four biological replicates. The different alphabets above the bars represent significant differences among the samples at $p < 0.05$ by Tukey's test. The numbers under the name of plants represent seed setting rates (%; mean $\pm$ SE) of plants, which were used for qRT-PCR analysis.

induced fertility restoration, we introduced the sgRNA/Cas9-expression vectors, G6 and G7, whose target sites were within the CDS of *RMS*, into CWA and T65. Fifteen independent CWA transgenic plants and five independent T65 transgenic plants (T65_G6 and T65_G7) were obtained for each vector. Although we screened mutations at the target regions in these transgenic plants for the T₀ generation, mutations were not detected by the IndelCheck™ CRISPR/TALEN Insertion or Deletion Detection System. We suspected that even if there were no edited alleles in the T₀ plants, we might obtain edited alleles in the next generation. Because those of T₀ plants of the transgenic CWA_G6 and CWA_G7 were sterile, we made a cross between CWA_G6 (female) and the pollen of fertile T65_G6, and between CWA_G7 (female) with the pollen of fertile T65_G7. Finally, we obtained the T₁ generation that harbored G6 or G7 sgRNA/Cas9-expression constructs in the nucleus and the CW mitochondria in the cytoplasm. Then, we screened these T₁ plants using the IndelCheck™ CRISPR/TALEN Insertion or Deletion Detection System and found mutations in six of 21 plants for the G6 sites and three out of 27 plants for the G7 sites. All these nine plants were shown to possess *HPT* in the sgRNA/Cas9-expression vector (Supplementary Figure S6). Deletions and insertions, which caused knockouts of the *RMS* allele, were found by nucleotide sequencing of three independent clones of PCR-amplified products in

A	WT	+105	+124
		CAG	CGACAGCACATGGGCAGGGATG
	CWA_G6_a	CAG	CGACAGCACATGGGCAGGGATG
		CAG	CGACG-----CATGGGCAGGGATG
	CWA_G6_b	CAG	CGACAGCACATGGGCAGGGATG
		CAG	CGACAGCA-----TGGGCAGGGATG
	CWA_G6_c	CAG	CGACAGCACATGGGCAGGGATG
B		+96	+115
	WT	AAG	TGCAGCCACGACAGCACATGGGCAG
	CWA_G7_a	AAG	TGCAGCCACGACAGCA CATGGGCAG
		AAG	TGCAGCCACGACAGCAgCATGGGCAG
	CWA_G7_b	AAG	TGCAGCCACGACAGCACATGGGCAG
		AAG	TGCAGCCACGACA-----TGGGCAG
	CWA_G7_c	AAG	TGCAGCCAC-----ATGGGCAG
		AAG	TGCAGCCACGACAGCAgCATGGGCAG
		AAG	TGCAGCCACGACAGCA+CATGGGCAG

Figure 7. Mutated nucleotide sequences of genome-edited CWA plants, CWA_G6 (A) and CWA_G7 (B). The nucleotide changes (dashes in red for deletion, lowercase letters in green for substitutions and purple for insertions) are indicated in the sequences. The G6 and G7 target sequences are underlined. The PAM sequence is shown in blue. WT, untransformed wild-type. Nucleotide positions from the start codon of *RMS* are indicated.

CWA_G6_a, CWA_G6_b, CWA_G6_c, CWA_G6_d, CWA_G6_e, CWA_G6_f, CWA_G7_a, CWA_G7_b, and CWA_G7_c (Figure 7; Chromatograms are shown in Supplementary Figure S7). CWA_G6_c, CWA_G6_e, and CWA_G7_c were likely to be chimeric mutants.

Seed setting rates were investigated for these nine plants. None set any seeds. The results are in contrast to our expectation that the plants with a heterozygous knockout allele might set seeds because *Rf17* acts gametophytically in pollen and allows the plant with *Rf17rf17* to set seeds normally. These results indicated that knockout of *RMS* does not restore fertility of CW-CMS.

Discussion

Fertility was restored by a mutation in the promoter of *RMS*

In this study, we created two new alleles that restored CW-CMS. As shown in Figure 4, fertility was fully restored in CWA_G1 and partially restored in CWA_G2 plants. The G1 target site exists at −2,425 to −2,444 bp upstream from the *RMS* start codon, which was near the SNP site at −2,286 bp in the original fertility restorer line CWR (Figures 1–3). It is assumed that the G1 target site was more important for reducing *RMS* expression and leading to fertility restoration than the G2 target site at −912 to −931 bp (Figure 6). Further study of additional mutants is needed to delimit the *cis*-element responsible for *RMS* expression. Examples in which regulatory sequences located distant to the CDS have also been reported in other genes. The expression of *QTL of seed shattering in chromosome 1 (qSH1)* and *FLOWERING LOCUS T* are regulated by 12 kb and 5.7 kb upstream of their start codon (Konishi et al. 2006; Liu et al. 2014). Considering the analysis of the CWA_G2 plant, in which the target site was located in the promoter of *RMS* outside of the CDS of *PPR2*, we concluded that *PPR2* is not linked to CW-CMS. This is in contrast to most studies of *RF* genes that encode *PPR* proteins.

A premature stop codon was created in *PPR2* of CWR_WT and CWA_G1 plants, which indicated that these plants lost the function of *PPR2* protein. Because no defective phenotypes were observed in these plants, the function of *PPR2* is still unknown.

The seed setting rates of the CWA_G1 plants were higher than those of the RNAi-mediated transgenic plants with slightly reduced expression of *RMS* in our previous study (Fujii and Toriyama 2009). The current study clearly demonstrated that the reduced expression allele is responsible for the fertility restoration of CW-CMS.

Hypotheses why fertility was not restored in *RMS* knockout lines

We obtained nine transgenic lines which harbor *RMS* knockout alleles. We expected, at the beginning of this study, that they would restore fertility. All of them, however, showed no restoration of fertility. We would rule out the possibility that it was caused by an off-target effect, because it was unreasonable to think that both of the G6 and G7 mutants suffered from the same off-target effect that cause sterility. The reason why CW-CMS was not restored in the *RMS* knockout lines is still unknown. Because the mRNA expression level of *RMS* in mature anthers of a normal fertile cultivar T65 has been reported to be approximately half that of CWA (Fujii and Toriyama 2009), it is possible that the knockout *RMS* allele might be lethal to pollen development.

The *RMS* encodes a protein 178 aa long, and contain a 139th to 156th aa segment similar to a part of the acyl-carrier protein synthase (ACPS) domain. It is known that ACPS is directly related to the elongation of 18:0 ACP (Nishida 2004). Thus, we speculated that *RMS* has another function related to ACPS and involved in pollen development directly or indirectly. Regarding ACPS in rice, knockout of *OsACOS12*, which encodes a rice fatty acyl-CoA synthetase, has been reported to cause pollen abortion (Yang et al. 2017; Zou et al. 2017). In tobacco, the β -Ketoacyl-[acyl carrier protein] synthase I (*KASI*) mutant has been reported to cause abnormal development of embryos and differentiation of chloroplasts and reduce seed setting rates (Wu and Xue 2010). In addition, there were several reports that revealed the knockout of genes related to acyl-carrier protein caused male sterility, including *Defective Pollen Wall* in rice that encodes a fatty acyl-carrier protein reductase (Shi et al. 2011), *Defective Pollen Wall 2* in rice that encodes an acyl transferase (Xu et al. 2017), and *Male Sterile2* in *Arabidopsis* that encodes a fatty acyl-carrier protein reductase (Chen et al. 2011). Recently, an *RF* gene, which has a function other than fertility restoration in CMS, has been reported. *Rf4* in maize, which has been cloned as an *RF* gene for C-type CMS, has been reported to have another function that regulates the development and specification of the tapetum (Jaqueth et al. 2020; Nan et al. 2017). Considering the above reports, we suggest that *RMS* might not only be related to CW-CMS but also have an essential function for pollen development.

Creation of a new artificial restorer line

CMS is an agronomically important trait often used for F_1 hybrid breeding, as most commercial hybrid crops have been developed based on a three-line system, namely a CMS line, a maintainer line, and a restorer line. F_1 hybrid seeds are produced by crossing a CMS line and a restorer line. Because of hybrid vigor, the F_1 hybrid rice grows stronger and results in 15 to 30% higher yields than conventional inbred lines (Huang et al. 2014). CW-CMS has been demonstrated to be useful for producing CMS lines of many elite Indica Group rice cultivars (Toriyama and Kazama 2016; Toriyama et al. 2019).

We obtained the null-segregant from the CWA_G1_a in the T_2 generation, which did not harbor a foreign gene, but still carried mutated alleles homozygously and exhibited as high as a 79.3% seed setting rate (Figure 5). The seed setting rates were the same as that of the original CWR and were high enough to use as a new restorer line for practical breeding. We will be able to easily produce artificial restorer lines on demand by introducing the G1 sgRNA/Cas9 expression vector. The creation of artificial restorer lines has not yet been reported for any other crops or vegetables.

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References

- Akagi H, Nakamura A, Yokozeki-Misono Y, Inagaki A, Takahashi H, Mori K, Fujimura T (2004) Positional cloning of the rice *Rf-1* gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. *Theor Appl Genet* 108: 1449–1457
- Bohra A, Jha UC, Adhimoolam P, Bisht D, Singh NP (2016) Cytoplasmic male sterility (CMS) in hybrid breeding in field crops. *Plant Cell Rep* 35: 967–993
- Chen W, Yu XH, Zhang K, Shi J, De Oliveira S, Schreiber L, Shanklin J, Zhang D (2011) *Male Sterile2* encodes a plastid-localized fatty acyl carrier protein reductase required for pollen exine development in *Arabidopsis*. *Plant Physiol* 157: 842–853
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823
- Fujii S, Kazama T, Yamada M, Toriyama K (2010) Discovery of global genomic re-organization based on comparison of two newly sequenced rice mitochondrial genomes with cytoplasmic male sterility-related genes. *BMC Genomics* 11: 209
- Fujii S, Toriyama K (2005) Molecular mapping of the fertility restorer gene for *ms-CW*-type cytoplasmic male sterility of rice. *Theor Appl Genet* 111: 696–701
- Fujii S, Toriyama K (2009) Suppressed expression of *RETROGRADE-REGULATED MALE STERILITY* restores pollen fertility in cytoplasmic male sterile rice plants. *Proc Natl Acad Sci USA* 106: 9513–9518
- Hanson MR, Bentolila S (2004) Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *Plant Cell* 16 (suppl): S154–S169
- Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. *Biom J* 50: 346–363
- Hu J, Wang K, Huang W, Liu G, Gao Y, Wang J, Huang Q, Ji Y, Qin X, Wan L, et al. (2012) The rice pentatricopeptide repeat protein RF5 restores fertility in Hong-Lian cytoplasmic male-sterile lines via a complex with the glycine-rich protein GRP162. *Plant Cell* 24: 109–122
- Huang JZ, Zhi-Guo E, Zhang HL, Shu QY (2014) Workable male sterility systems for hybrid rice: Genetics, biochemistry, molecular biology, and utilization. *Rice (NY)* 7: 13
- Huang W, Yu C, Hu J, Wang L, Dan Z, Zhou W, He C, Zeng Y, Yao G, Qi J, et al. (2015) Pentatricopeptide-repeat family protein RF6 functions with hexokinase 6 to rescue rice cytoplasmic male sterility. *Proc Natl Acad Sci USA* 112: 14984–14989
- Itabashi E, Iwata N, Fujii S, Kazama T, Toriyama K (2011) The fertility restorer gene, *Rf2*, for Lead Rice-type cytoplasmic male sterility of rice encodes a mitochondrial glycine-rich protein. *Plant J* 65: 359–367
- Jaqueth JS, Hou Z, Zheng P, Ren R, Nagel BA, Cutter G, Niu X, Vollbrecht E, Greene TW, Kumpatla SP (2020) Fertility restoration of maize CMS-C altered by a single amino acid substitution within the *Rf4* bHLH transcription factor. *Plant J* 101: 101–111

- Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA (2013) RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol* 31: 233–239
- Kazama T, Nakamura T, Watanabe M, Sugita M, Toriyama K (2008) Suppression mechanism of mitochondrial ORF79 accumulation by Rf1 protein in BT-type cytoplasmic male sterile rice. *Plant J* 55: 619–628
- Kazama T, Toriyama K (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice. *FEBS Lett* 544: 99–102
- Kazama T, Toriyama K (2014) A fertility restorer gene, *Rf4*, widely used for hybrid rice breeding encodes a pentatricopeptide repeat protein. *Rice (NY)* 7: 28
- Kitazaki K, Arakawa T, Matsunaga M, Yui-Kurino R, Matsuhira H, Mikami T, Kubo T (2015) Post-translational mechanisms are associated with fertility restoration of cytoplasmic male sterility in sugar beet (*Beta vulgaris*). *Plant J* 83: 290–299
- Koizuka N, Imai R, Fujimoto H, Hayakawa T, Kimura Y, Kohno-Murase J, Sakai T, Kawasaki S, Imamura J (2003) Genetic characterization of a pentatricopeptide repeat protein gene, *orf687*, that restores fertility in the cytoplasmic male-sterile Kosen radish. *Plant J* 34: 407–415
- Komori T, Ohta S, Murai N, Takakura Y, Kuraya Y, Suzuki S, Hiei Y, Imaseki H, Nitta N (2004) Map-based cloning of a fertility restorer gene, *Rf-1*, in rice (*Oryza sativa* L.). *Plant J* 37: 315–325
- Konishi S, Izawa T, Lin SY, Ebana K, Fukuta Y, Sasaki T, Yano M (2006) An SNP caused loss of seed shattering during rice domestication. *Science* 312: 1392–1396
- Li JF, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat Biotechnol* 31: 688–691
- Liu F, Schnable PS (2002) Functional specialization of maize mitochondrial aldehyde dehydrogenases. *Plant Physiol* 130: 1657–1674
- Liu L, Adrian J, Pankin A, Hu J, Dong X, Von Korff M, Turck F (2014) Induced and natural variation of promoter length modulates the photoperiodic response of *FLOWERING LOCUS T*. *Nat Commun* 5: 4558
- Mikami M, Toki S, Endo M (2015) Comparison of CRISPR/Cas9 expression constructs for efficient targeted mutagenesis in rice. *Plant Mol Biol* 88: 561–572
- Nan GL, Zhai J, Arikis S, Morrow D, Fernandes J, Mai L, Nguyen N, Meyers BC, Walbot V (2017) *MS23*, a master basic helix-loop-helix factor, regulates the specification and development of the tapetum in maize. *Development* 144: 163–172
- Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat Biotechnol* 31: 691–693
- Nishida I (2004) Plastid metabolic pathways for fatty acid metabolism. In: Daniell H, Chase C (eds) *Molecular Biology and Biotechnology of Plant Organelles*. Springer, The Netherlands, pp 543–564
- Ohnishi T, Yoshino M, Yamakawa H, Kinoshita T (2011) The biotron breeding system: A rapid and reliable procedure for genetic studies and breeding in rice. *Plant Cell Physiol* 52: 1249–1257
- Schmitz-Linneweber C, Small I (2008) Pentatricopeptide repeat proteins: A socket set for organelle gene expression. *Trends Plant Sci* 13: 663–670
- Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, et al. (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 31: 686–688
- Shi J, Tan H, Yu XH, Liu Y, Liang W, Ranathunge K, Franke RB, Schreiber L, Wang Y, Kai G, et al. (2011) *Defective pollen wall* is required for anther and microspore development in rice and encodes a fatty acyl carrier protein reductase. *Plant Cell* 23: 2225–2246
- Tang H, Luo D, Zhou D, Zhang Q, Tian D, Zheng X, Chen L, Liu YG (2014) The rice restorer *Rf4* for wild-abortive cytoplasmic male sterility encodes a mitochondrial-localized PPR protein that functions in reduction of *WA352* transcripts. *Mol Plant* 7: 1497–1500
- Toriyama K, Kazama T (2016) Development of cytoplasmic male sterile IR24 and IR64 using CW-CMS/*Rf17* system. *Rice (NY)* 9: 22
- Toriyama K, Kazama T, Sato T, Fukuta Y, Oka M (2019) Development of cytoplasmic male sterile lines and restorer lines of various elite Indica Group rice cultivars using CW-CMS/*Rf17* system. *Rice (NY)* 12: 73
- Uyttewaal M, Arnal N, Quadrado M, Martin-Canadell A, Vrielynck N, Hiard S, Gherbi H, Bendahmane A, Budar F, Mireau H (2008) Characterization of *Raphanus sativus* pentatricopeptide repeat proteins encoded by the fertility restorer locus for Ogura cytoplasmic male sterility. *Plant Cell* 20: 3331–3345
- Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D, et al. (2006) Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. *Plant Cell* 18: 676–687
- Wu GZ, Xue HW (2010) *Arabidopsis* β -ketoacyl-[acyl carrier protein] synthase I is crucial for fatty acid synthesis and plays a role in chloroplast division and embryo development. *Plant Cell* 22: 3726–3744
- Xu D, Shi J, Rautengarten C, Yang L, Qian X, Uzair M, Zhu L, Luo Q, An G, Waßmann F, et al. (2017) *Defective Pollen Wall 2 (DPW2)* encodes an acyl transferase required for rice pollen development. *Plant Physiol* 173: 240–255
- Yang X, Liang W, Chen M, Zhang D, Zhao X, Shi J (2017) Rice fatty acyl-CoA synthetase *OsACOS12* is required for tapetum programmed cell death and male fertility. *Planta* 246: 105–122
- Zou T, He Z, Qu L, Liu M, Zeng J, Liang Y, Wang T, Chen D, Xiao Q, Zhu J, et al. (2017) Knockout of *OsACOS12* caused male sterility in rice. *Mol Breeding* 37: 126