Suppressed expression of *RETROGRADE-REGULATED MALE STERILITY* restores pollen fertility in cytoplasmic male sterile rice plants

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Conflict/reconciliation between mitochondria and nuclei in plants is manifested by the fate of pollen (viable or nonviable) in the cytoplasmic male sterility (CMS)/fertility restoration (Rf) system. Through positional cloning, we identified a nuclear candidate gene, *RETROGRADE-REGULATED MALE STERILITY* (*RMS*) for *Rf17*, a fertility restorer gene for Chinese wild rice (CW)-type CMS in rice (*Oryza sativa* L.). RNA interference-mediated gene silencing of *RMS* restored fertility to a CMS plant, whereas its overexpression in the fertility restorer line induced pollen abortion. The mRNA expression level of *RMS* in mature anthers depended on cytoplasmic genotype, suggesting that *RMS* is a candidate gene to be regulated via retrograde signaling. We found that a reduced-expression allele of the *RMS* gene restored fertility in haploid pollen, whereas a normal-expression allele caused pollen to die in the CW-type CMS. *RMS* encodes a mitochondrial protein, 178 aa in length, of unknown function, unlike the majority of other *Rf* genes cloned thus far, which encode pentatricopeptide repeat proteins. The unique features of *RMS* provide novel insights into retrograde signaling and CMS.

cytoplasmic male sterility | fertility restoration | mitochondria | *Oryza sativa* | retrograde signaling

Cytoplasmic male sterility (CMS), which is a maternally inherited male sterility trait, is observed in more than 150 higher plant species. CMS is a useful system for commercial F_1 hybrid breeding programs. CMS also is a focus of nuclear–mitochondrial research in plants, because aberrant mitochondrial genomic organization causes dysfunction in pollen development (1, 2). Fertility restoration (Rf) often is governed by a nuclear-encoded *Rf* gene. The first *Rf* gene cloned was *Rf2a* from maize, encoding a protein with aldehyde dehydrogenase activity (3, 4). Except for maize *Rf2a*, *Rf* genes in various plant species recently have been found to encode pentatricopeptide repeat (PPR) proteins (5–9). It has been proposed that a common function of PPR proteins is organelle posttranscriptional regulation, exemplified by RNA processing and editing (10, 11). The first PPR-encoding *Rf* gene reported was the petunia *Rf-PPR592*, which contains 11 continuous PPR motifs (5). *Rf-PPR592* eliminates the CMS mitochondrial-specific protein PCF and resides within a high-molecular-weight protein complex associated with the mitochondrial membrane (12). PPRs also are responsible for fertility restoration in *Brassica napus* (6–8); in this case, a PPR protein encoded by *Rfo* reduces the abundance of CMS-associated ORF138 protein (6). In rice, *Rf1a* (13–16) and *Rf1b* (16) for Boro-Taichung (BT)-type CMS also have been identified as encoding PPR proteins. *Rf1a* promotes the processing of the BT-CMS-specific mitochondrial operon B-*atp6-orf79* transcripts (13, 16), whereas *Rf1b* decreases the abundance of dicistronic transcripts of B-*atp6-orf79* (16). Because *Rf1a* and *Rf1b* are located close together on the same chromosome and have highly conserved amino acid sequences, these 2 *Rf*s may be recently duplicated homologous genes. At least 9 *Rf1a* homologous sequences have been found around the *Rf1a* locus (16), and such *Rf* homologous sequences also are present around the respective *Rf* loci in petunia and *B. napus* CMS (5, 7, 8). These findings suggest the complex co-evolution of the *Rf* locus and mitochondrial CMS-associated gene. Other modes of *Rf*s within the same species must be identified to understand fully the co-evolution of these 2 genes. Currently, however, there are no reports in which 2 or more *Rf*s of independent origin have been cloned from the same plant species. In addition, the PPR-encoding *Rf* genes cloned to date all function dominantly against their nonfunctional *rf* allele. Their common working scheme is that the PPR proteins are carried into the mitochondria, preventing the accumulation of CMS-associated gene products. Further, no loss-of-function type *Rf* has been cloned, although CMS is caused by nuclear–mitochondrial incompatibility.

In rice, more than 20 independent CMS cytoplasms have been described. Accordingly, this plant species is ideal for identifying novel aspects of CMS. Although the CW-type cytoplasm was the first CMS system discovered in rice, the molecular components involved in the induction of male sterility and fertility restoration have not been described. Male fertility of rice plants carrying CW-type male sterility-inducing cytoplasm is restored gametophytically in haploid pollen by a single nuclear gene, *Rf17* (17). Although plants carrying the CW-type CMS cytoplasm develop morphologically normal pollen, the pollen fails to germinate on the stigma after anthesis and lacks germination ability (17). In this study, we performed positional cloning of *Rf17* and found that the reduced expression of a gene designated *RETROGRADE-REGULATED MALE STERILITY* (*RMS*) is sufficient to explain fertility restoration. The identification of *RMS* provided more information about functionally recessive *Rf*s. *RMS* was found not to encode a protein containing a pentatricopeptide repeat but to encode a protein of unknown function with a segment partially similar to acyl-carrier protein synthase (ACPS). The relationship between mitochondrial retrograde signaling and CMS is discussed also.

Results

Suppression of *ORF11* Restored Fertility to a CMS Plant. We previously described the determination of the coarse position of *Rf17* (17). Using 4032 BC_1F_1 individuals and 9184 F_2 individuals derived from a cross of the CW fertility restorer line (CWR) and the nonrestoring Kasalath/Koshihikari chromosome segment substitution line CSSL209, we identified *Rf17* on the long arm of

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The sequence reported in this paper has been deposited in the DNA Data Bank of Japan (accession no. AB481199).

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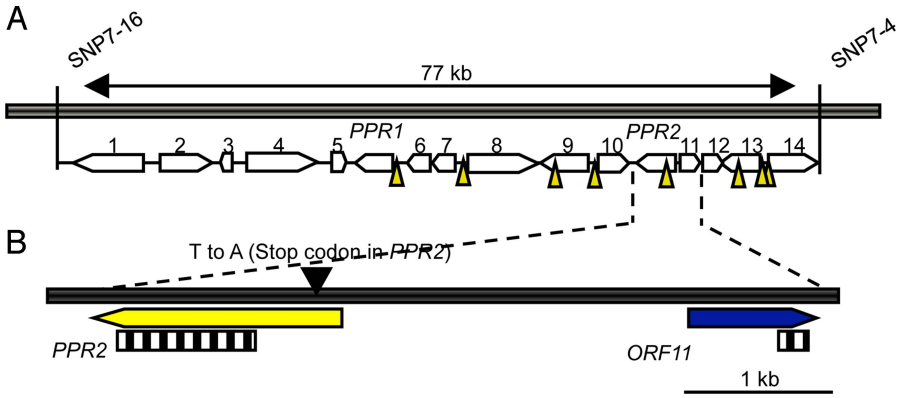

Fig. 1. Mapping of the *Rf17* locus. (*A*) The *Rf17* locus on chromosome 4 mapped between DNA markers SNP7–16 and SNP7–4. Locations of 16 predicted genes are indicated by numbers 1 through 14. Genes encoding the PPR motif were designated *PPR1* and *PPR2*. Arrowheads indicate the positions of 25 SNPs and InDels. SNPs or Indels with duplicate positions were omitted for clarity. (*B*) Detailed view of the 5.0-kb region spanning *PPR2* and *ORF11*. Striped bars in each gene indicate the sequences chosen as the RNAi trigger region.

chromosome 4, in the 77-kb region within 2 cleaved amplified polymorphic sequence (CAPS) markers, SNP 7–16 and SNP7–4, and on a BAC clone, OsJNBa0022H21. The region contained 16 predicted genes, including 2 PPR genes (designated as *PPR1* and *PPR2* in Fig. 1*A*), a retrotransposon (*ORF8* in Fig. 1*A*), and a transposase gene (*ORF9* in Fig. 1*A*). By sequencing the BAC clone of the corresponding region screened from the CWR BAC library, a total of 25 SNPs and insertion/deletions (InDels) were found in the 77-kb region compared with the nonrestoring Nipponbare allele (Fig. 1*A*). In our previous study, we found that fertility restoration of CW-type CMS is controlled in a gametophytic manner (17), but whether *Rf* functions dominantly or recessively was unknown. The only nonsynonymous mutation identified was in the PPR gene Os04g0475800 (*PPR2*) of CWR, in which a T-to-A transition resulted in a premature stop codon (Fig. 1*B*). To gain more information about the predicted genes in this region, we monitored the expression of these 14 genes (excluding transposable elements) by RT-PCR. *ORF11* (Os04g0475900) was the only gene determined to be differentially regulated in the CWR and the CW-CMS line (CWA) in mature anthers [Fig. 2*A*, supporting information (SI) Fig. S1] and was down-regulated in CWR. *ORF11* down-regulation was thought to be caused by the SNP in *PPR2*, which could have been included in a promoter region of *ORF11*. *PPR2* showed similar expression levels in these 2 lines. The result was confirmed by quantitative real-time RT-PCR, and the calculated mRNA abundance of *ORF11* in CWA was ≈10-fold more than that in CWR (Fig. 2*B*). Thus, we considered that either a premature stop codon in a PPR gene or down-regulation of an unknown gene, *ORF11*, was involved in restoring fertility.

To examine these possibilities further, we introduced RNAi constructs carrying specific sequences of each gene as a trigger region into a CWA plant to down-regulate *PPR2* or *ORF11* (Fig. 1*B*). From the 15 independent transgenic lines for each construct, we obtained 7 plants with *ORF11* expression reduced to 30% to 77% of the untransformed CWA, and 6 plants with *PPR2* expression reduced to 27% to 75% (Fig. 2*B*). CWA plants exhibited complete seed-setting sterility, without any defects in pollen development until maturation that could be seen by light microscopy (17). Pollen dysfunction in the CWA line was observed first ≈ 2 h before anthesis after maturation. The CMS plants showed no starch digestion, as seen in wild-type plants. Starch digestion occurs when flowers are ready to open, possibly providing a fusible carbohydrate energy source for pollen germination on the stigma (18) Thus, we counted the starch-digested pollen of transgenic plants 2 to 3 h before anthesis (Fig. 3*A* and *B*). In the male-fertile CWR line, 68% of the pollen exhibited a starch-digested phenotype before anthesis, and 27% of the pollen exhibited a starch-filled, engorged phenotype (Fig. 3*B*), whereas pollen in the CWA showed no starch digestion, and all of the pollen remained engorged with starch (Fig. 3*B*). We also examined the pollen of RNAi-mediated *ORF11* or *PPR2* knockdown transgenic plants and found that *ORF11*-knockdown plants produced starch-digested pollen ranging from 2% to 37.5% (Fig. 3*B*). On the other hand, the *PPR2*-knockdown plant phenotypes were unchanged from CWA, showing no starch-digested pollen at all. Thus, we concluded that suppression of *ORF11* is sufficient for restoring starch digestion in pollen of a CMS plant.

The effects of *ORF11* mRNA suppression on pollen germination were evaluated by counting the germinating pollen on stigmas 6 h after anthesis (Fig. 3*C* and Table S1). Stigmas in CWR and Taichung 65 (T65) plants showed penetration of more than 100 pollen tubes 6 h after anthesis (Fig. 3*C* and Table S1). The frequency of germinating pollen in each stigma was 77% and 81%, respectively, and pollen tube germination was observed in all the florets examined (Table S1). In contrast, pollen germination never was observed in the stigmas of CWA plants after anthesis, although pollen grains without tube emission occasionally were found stuck in the stigmas (Fig. 3*C* and Table S1). The mature pollen of CWA was morphologically normal and was able to bind to stigmas under normal growth conditions. Few pollen grains remained at the time of the pollen tube observation, however, because the aniline-blue staining procedure required washing of the stigmas with alkaline buffer, and pollen grains not emitting tubes were wiped off. In the stigmas of RNAi *ORF11-2* and *-3*, approximately 1/4 of the florets observed held pollen germination events (Table S1). Zero to 5 pollen grains emitted a tube within a floret, which accounted for 0.4 and 0.5 germinating pollen grains per stigma, on average (Fig. 3*C* and Table S1). As expected from the absence of the pollen starch degradation (Fig. 3), stigmas of RNAi *PPR2-1* and *-3* lines lacked pollen tubes (Table S1).

Although the seed-setting rate of CWR plants was greater than 74%, the seed-setting percentages of RNAi *ORF11* lines Nos. 2, 3, 4, and 6 were only 2% to 5% (Fig. 3*D*), possibly because of incomplete suppression of *ORF11* as compared with that of CWR (Fig. 2 *A* and *B*). Nevertheless, we confirmed that the suppression of *ORF11* restored pollen fertility, because 4 independent lines with 37% to 47% suppression of *ORF11* mRNA retained the seed-setting ability.

Overexpression of ORF11 Induces Pollen Lethality in CWR. To investigate further, we overexpressed the 602-bp region including the

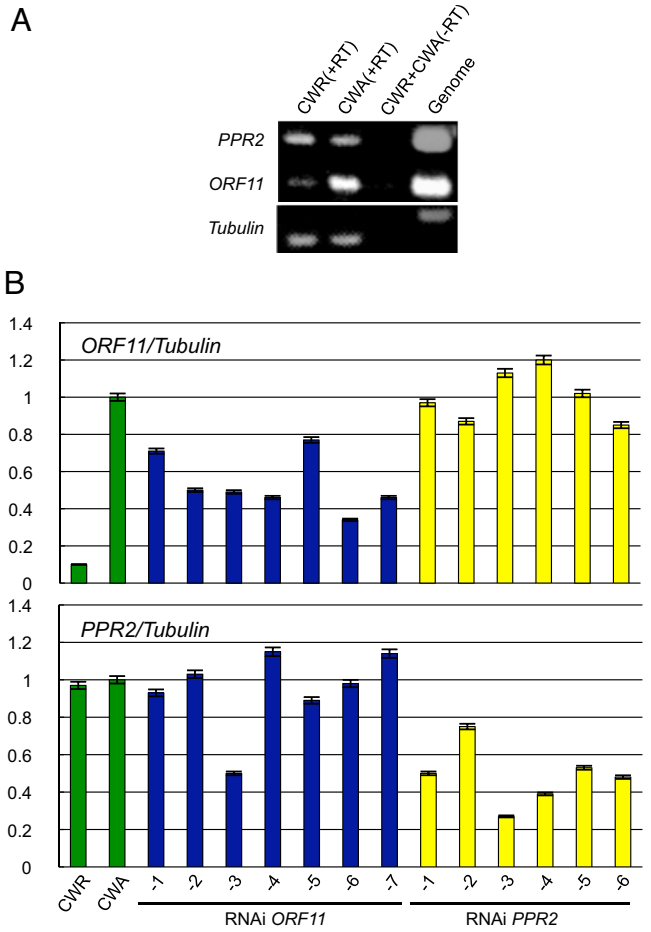

Fig. 2. mRNA expression of *PPR2* and *ORF11*. (*A*) Expression of *PPR2* and *ORF11* in the mature anthers of CW-type fertility-restorer line (CWR) and CW-type CMS line (CWA), as determined by RT-PCR. A mixture of CWR and CWA RNA without reverse transcription was used as the negative control (*Third Lane*). Transcript levels of tubulin were used as the internal control. (*B*) Transcript abundance of *PPR2* and *ORF11* in mature anthers of CWR and CWA *ORF11* RNAi transgenic lines and *PPR2* RNAi transgenic lines, as determined by quantitative real-time PCR. Relative transcript abundance was normalized to the levels of *tubulin alpha-1 chain* is shown as relative mRNA expression values against untransformed CWA (untransformed CWA = 1, *Second Lane*).

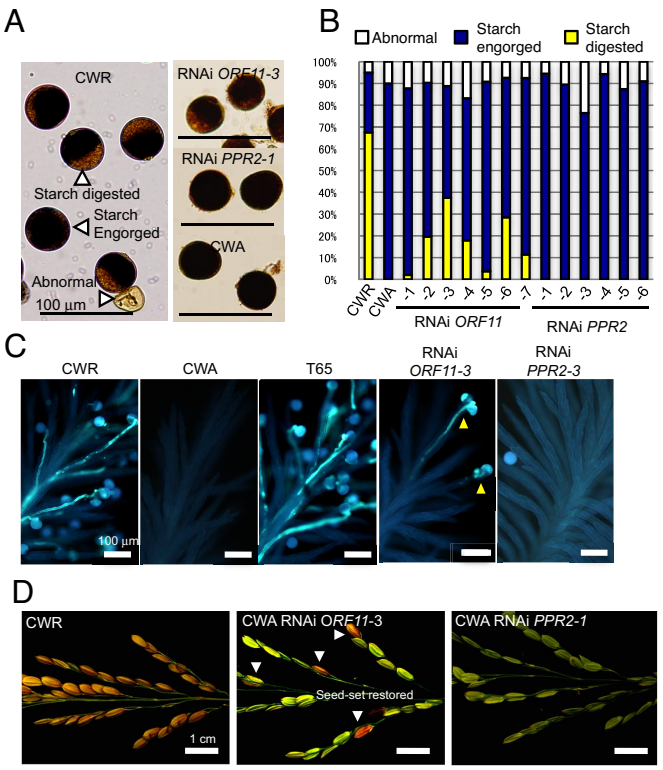

Fig. 3. The effects of *ORF11* suppression on pollen germination and seed setting. (*A*) Pollen was collected 2 h before anthesis and categorized into 3 groups: starch-digested, starch-engorged, and abnormal pollen (CWR, *Left*). (*B*) Ratio of starch-digested, starch-engorged, and abnormal pollen in CWR and CWA *ORF11* RNAi transgenic lines and *PPR2* RNAi transgenic lines. (*C*) Stigmas 6 h after anthesis stained with aniline blue. Arrowheads in RNAi *ORF11-3* panel indicate the traces of pollen germinations. (*D*) Seed setting observed on RNAi *ORF11-3*.

full-length 537-bp ORF of *ORF11* in a CWR background under the control of the *maize ubiquitin* promoter (Fig. S2 and Fig. 4). Of 10 independent transgenic lines, 2 lines carrying 1 copy of *Ubi*::*ORF11* were chosen for further analysis. Approximately half of the pollen in CWR transgenic plants carrying 1 copy of *Ubi*::*ORF11* hemizygously was shrunken and had reduced starch accumulation (Fig. 4 *A* and *B*); this phenotype was not observed in T65 transgenic plants carrying *Ubi*::*ORF11*. Thus, we concluded that the overexpression of *ORF11* caused defects only in a CW-cytoplasm background. The pollen phenotypes of *ORF11* overexpressing CWR lines seemed to be more deleterious than those of CWA lines (Fig. 3*A*), possibly because of the pleiotropic effect of *ORF11* overexpression.

***ORF11* Encodes an ACPS-Like Domain Containing Protein.** *ORF11* was predicted to encode a protein 178 aa long with an ACPS-like domain in amino acids 139–156 (Fig. 5*A*). ACPSs are directly involved in the elongation of acetyl-CoA to 18:0-ACP (19). Although the conserved part of the ACPS sequence was present in ORF11, it is not likely that ORF11 functions as a general ACPS, because the ACPS-like domain is shorter than ACPS catalytic domains, which normally are 70–90 aa long; furthermore, the conserved lysine residue among species is replaced by alanine (amino acid 140 in Fig. S3). BLASTP and BLASTN searches revealed no similar proteins in other organisms. Although we ran 4 different protein-targeting prediction programs (iPSORT, Mitoprot, Predotar, and TargetP v1.1), these tools did not produce a consistent prediction regarding the subcellular localization of ORF11. Thus, the GFP was fused to ORF11 and stably introduced into rice. GFP fluorescence in the protoplasts from the transgenic calli co-localized with the fluorescence of

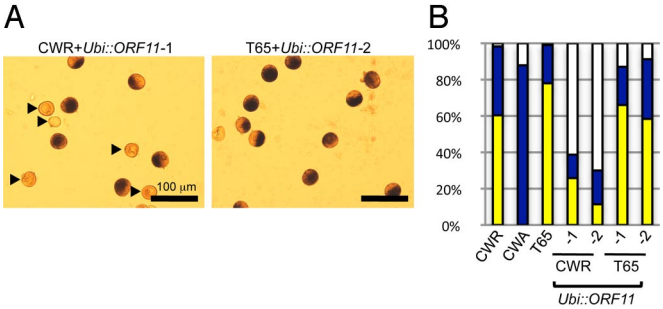

Fig. 4. The effects of *ORF11* overexpression in CWR and Taichung 65 (T65). (*A*) Pollen 2 h before anthesis. Arrowheads indicate the shrunken pollen observed in *CWR*+*Ubi*::*ORF11* lines. (*B*) Pollen was categorized into 3 groups as in Fig. 3. The ratio of starch-digested, starch-engorged, and abnormal pollen in each line is shown.

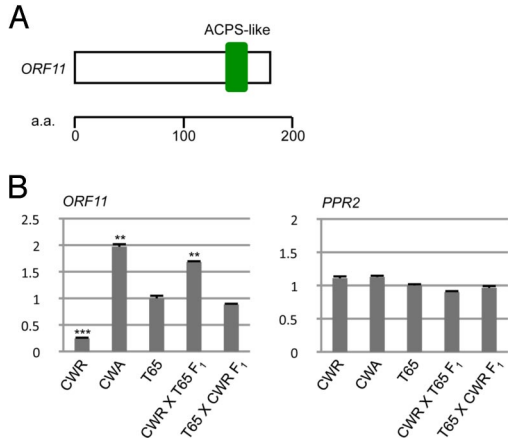

Fig. 5. *ORF11* expression in reciprocal hybrids. (*A*) Schematic structure of *ORF11*. (*B*) Relative expression of *ORF11* and *PPR2* in mature anthers of CWR and CWA, Taichung 65 (T65), and reciprocal F_1 hybrids of CWR (female) X T65 (male), and T65 (female) X CWR (male). Relative transcript abundance was normalized to the levels of *tubulin* alpha-1 chain and is shown as relative mRNA expression values against untransformed T65 (T65 = 1).

Mito Tracker Red, which indicated that ORF11 was targeted to mitochondria (Fig. S4).

***ORF11* mRNA Expression Is Dependent on the Cytoplasmic Genotype.** The mRNA expression level of *ORF11* in mature anthers in CWA was approximately double that of T65 (Fig. 5*B*), whereas *PPR2* mRNA expression levels were unchanged. The nuclear backgrounds of T65 and CWA were identical on the 157 simple sequence repeat (SSR) markers across chromosomes (see *Materials and Methods* for details), but their mitochondrial sequences were different (Fig. S5). Therefore, we concluded that mRNA expression of *ORF11* depends on the cytoplasmic genotype and, given the nature of CMS, *ORF11* gene expression could depend on the mitochondrial genotype. To confirm the relevance of the cytoplasmic genotype to the mRNA expression level of *ORF11*, we compared the expression level of *ORF11* in the F_1 plants obtained from the reciprocal crosses between CWR and T65 plants. In comparison with T65 plants, significant up-regulation of *ORF11* was observed in the F_1 plants carrying the CW cytoplasm (CWR X T65 F_1), whereas slight down-regulation of *ORF11* was observed in the F_1 plants carrying the T65 cytoplasm (T65 X CWR F_1) (Fig. 5*B*). A wealth of recent studies support the cytoplasmic control of nuclear gene expression and retrograde signaling in broad range of Eukaryote species (20–22). It is likely that the up-regulation of *ORF11* under CW cytoplasm could be controlled by a retrograde signaling pathway. Because the suppression of *ORF11* restored pollen fertility (Fig. 3) and overexpression caused deleterious effects in pollen carrying the CW cytoplasm background (Fig. 4), we designated *ORF11 RETROGRADE-REGULATED MALE STERILITY* (*RMS*).

We also were curious whether the difference in mRNA expression level of *RMS* between CWR and CWA was controlled via a *cis*-element. As we reported in the prior section, the only nucleotide mutation in the *RMS* gene region was the SNP 2,286 bp upstream of the start codon, which could be included in the genic region of *PPR2* (Fig. 1*B*). We constructed a reporter assay experiment using the *monomeric red fluorescent protein* (*mRFP*) gene, fusing *mRFP* downstream of the *RMS* promoter and coding regions of the CWR-type allele (carrying T at the −2,286th nucleotide) or the CWA-type allele (carrying A at the −2,286th nucleotide). *mRFP* reporter constructs were stably introduced into T65 plants, and 3 independent transgenic lines carrying 1 copy of the transgene were chosen for the analysis. As a result, in plants carrying CWA*_RMS* promoter::*mRFP* hemizygously, ≈ 25% to 33% of the pollen emitted the RFP fluorescence (Fig. S6). On the other hand, the frequency of pollen fluorescence of the plants carrying CWR*_RMS* promoter::*mRFP* was equivalent to that of the plants with the empty vector carrying solely *35S*::*HPT* (Fig. S6). Thus, we concluded that the SNP at 2,286-bp upstream in the promoter region of *RMS* might be important for its suppression in the *Rf17* allele.

Discussion

We performed positional cloning of *Rf17* and succeeded in delimiting *Rf17* to 2 candidates, *PPR2* and *ORF11* (*RMS*). By suppressing *RMS*, CW-CMS plants reverted to seed setting, although the molecular function of the putative mitochondrial RMS protein remains unknown. RMS possesses a partial ACPS domain that does not seem to function as general ACPS when compared with other proteins containing an ACPS domain (Fig. S3). In CW-CMS plants with depressed levels of *PPR2* mRNA expression, there was no recovery of either seed setting or pollen fertility. Overexpression of *RMS* apparently was lethal to pollen in the CW cytoplasm background. We conclude that the CW-type CMS system of rice results from *RMS* up-regulation and that CWR retains male fertility because *RMS* expression may be reduced by the SNP in its promoter region, resulting in restored compatibility between the nucleus and mitochondria. We therefore propose that the CWR allele expressing relatively low levels of *RMS* mRNA is *Rf17* and that the CWA allele expressing high levels of *RMS* mRNA is *rf17*.

RMS does not encode a PPR-containing protein, although, except for *Rf2a* for maize T-CMS, all the other *Rf* genes so far reported encode PPR proteins (3–9, 13–16). In addition to *Rf1* for BT-CMS rice, our study cloned a second *Rf* gene of independent origin in the same plant species. Our RNAi experiment does not, however, completely rule out the possibility that *PPR2* is a factor that restores the relationship between the CW cytoplasm and the nucleus. It is possible that RNAi-mediated suppression of *RMS* has epistatic effects on the neighboring gene, *PPR2*. Furthermore, *PPR2* contains an AUG codon downstream of the premature stop codon in the CWR allele, which could produce a protein of 330 aa with 8 PPR motifs that might function as the fertility-restorer protein. Antibodies to PPR2 are required to determine if such a truncated protein is produced. Nevertheless, based on our current results, it is unlikely that *PPR2*, rather than *ORF11*, is the restorer gene. Additional mutants would be necessary to assess further the function of *PPR2* in CW-CMS. Unfortunately, we were unable to find mutants with *Tos17* or T-DNA inserted in *PPR2* or *RMS*.

To the best of our knowledge, the only loss-of-function fertility restorer locus reported to date is *restorer-of-fertility lethal 1* (*rfl1*) for maize S-CMS (23). The dominant *Rfl1* allele is related positively to the accumulation of the mitochondrial A subunit of ATP synthase (ATPA), and ATPA could interact with the mitochondrial *orf355-orf77* gene product, which is associated with CMS. *rfl1* homozygosity is lethal, however, because of the lack of mitochondrial ATPA accumulation (23). A significant aspect of our findings is that a reduced-expression allele of *RMS* restored fertility in haploid pollen, whereas a normal-expression allele caused pollen lethality in the CW-type CMS. Although there were no indications of RMS functions other than the partial ACPS-like domain, we speculate that some metabolic alteration in mitochondria restores pollen fertility, similar to the mechanism in the maize *Rf2* system (3, 4). We do not consider RMS, unlike other PPR-encoding *Rf* genes previously identified (5–9, 13–16), to be the protein responsible for posttranscriptional RNA modification of the mitochondrial CMS-associated gene expression; rather, loss-of-function of the *RMS* gene may provide a bypass to fertility restoration.

Retrograde regulation of nuclear genes by organelle status has been reported recently in animals, yeast (20), and in plants (21, 22, 24). It is assumed that this gene regulation is required for the coordinated expression of nucleus and organelle genes in plants, because protein complexes participating in the respiratory chain in mitochondria are composed of chimeric structures of nuclear and mitochondrial-derived proteins (25). Although virtually nothing is understood about mitochondrial retrograde regulation in plants, a key component in plastid retrograde signaling, *GUN1*, has been cloned and shown to encode a PPR protein (26). *GUN1* mediates plastid signals to ABI4, a transcription factor containing an AP2 (APETALLA2) domain. ABI4 binds to G-box elements in a retrogradely regulated *LHCB* promoter, negatively regulating its expression (26). Our results indicate that up-regulation of *RMS* occurs upon introduction of the CW-type cytoplasm, suggesting that *RMS* is a candidate gene to be regulated via retrograde signaling. Because overexpression of *RMS* results in pollen sterility (Fig. 4), it is possible that retrograde signaling is directly involved in the occurrence of CW-CMS. This issue could be clarified by isolating a factor that promotes the expression of *RMS* and by analyzing its function in relation to mitochondrial signaling. Our present research suggests how CMS occurs and may help reveal the relationship between mitochondria and CMS and mitochondrial–nuclear retrograde signaling.

We also showed that the SNP at 2,286 bp upstream of *RMS* might be involved in the suppression of *RMS* in CWR by reporter assay experiments (Fig. S6). Recently, a 12-kb upstream SNP was shown to be involved in the *cis*-regulatory system of the spatial expression pattern of *OsRPL*, a gene responsible for seed shattering in rice (27). Expression of *RMS* is likely to be regulated by the SNP, although we have not detected any known *cis*-regulatory elements in this region.

CMS has been used widely for hybrid rice breeding in Southeastern Asia. In China, hybrid rice, which has an average 15% to 20% yield advantage over inbred strains, is planted on ≈16 million hectares—more than half of China's total rice area of 28 million hectares (28). The CW-CMS and its restorer gene have not been used for hybrid rice production, however. As discussed by Komori and Imaseki (29) and Sattari *et al.* (30), most commercial rice hybrids are based on a single CMS source, the wild-abortive (WA) cytoplasm. To avoid the potential threat of genetic vulnerability of rice hybrids, the development of several CMS/*Rf* systems is desirable. Introduction of loss-of-function type *Rf* genes to hybrid breeding programs will broaden the combination of F_1 pairs and contribute to improve yields.

Materials and Methods

Plant Materials. The BC_3F_1 CMS line was derived from a cross of *Oryza japonica* cultivar Reimei and *Oryza rufipogon* Griff., W1 strain (31). The T65 nuclear background CMS line, CWA with [*cms-CW*] *rf17rf17*, was obtained by backcrossing T65 6 times with the Reimei CMS line. The genotype of T65 is [*normal*] *rf17rf17*. The fertility-restorer line, CWR, was obtained by backcrossing T65 5 times with a restorer line described in (31) and has a genotype of [*cms-CW*] *Rf17Rf17*. We carried out the marker-assisted selection of CWA and CWR to obtain efficiently lines with nuclear genomes substituted by T65, using 157 SSR markers covering all the chromosomes (Table S2). The nuclear genomic substitutions of CWA and CWR used in this study were assessed by the 157 SSR markers. As a result, we obtained CWA and BTA lines completely isogenic to T65. CWR had almost the same genome except for the *Rf17* region of chromosome 4, the region within SSR markers RM7535 and RM3276. BC_1F_1 and F_2 mapping populations were derived from the cross of CWR and the nonfertility restoring Kasalath/Koshihikari chromosome segment substitution line CSSL209 (32). Most of chromosome 4, except for the 15-cM telomeric region of the long arm, was replaced by Kasalath.

Genetic Mapping Experiments. For positional cloning, recombinants were screened from 4032 BC_1F_1 and 9184 F_2 progeny. The 21 PCR markers used in this study are listed in Table S2. Through mapping, 2 recombinants were obtained for each of the cleaved amplified polymorphic markers, SNP7–16 and SNP7–4, which were ≈77 kb apart. Construction of the BAC library and sequencing were performed by the previously described methods (33).

Production of Transgenic Rice Lines. *ORF11* (accession no. Os04g0475900 in the Rice Annotation Project DataBase, RAP-DB and *PPR2* (accession no. Os04g0475800) RNAi constructs overdriven by the maize *ubiquitin* promoter were constructed using the pANDA vector provided by D. Miki and K. Shimamoto, following their protocols (34). A gene-specific region of *ORF11* was PCR cloned by primer set 5′-CACCAGCGTTGAAGAGTTTGGGGA-3′ and 5′-CGAGCTCCAACATACTGGCT-3′. 5′-CACCTGAAGAGTGCAAACCCTCTG-3′ and 5′-TTCGCAGACTCTCAACAAGG-3′ were used for *PPR2*. Nucleotides CACC were added to the original *ORF11* and *PPR2* sequences for cloning into the pENTER D-TOPO vector (Invitrogen). For overexpressing *ORF11*, the 602-bp region including the full-length 537-bp translational region and the 5′UTR region was PCR-cloned using the primer pairs 5′-CACCGGATCCCTCGCTGAGGTGCTCTCCCTCT-3′ and 5′-GGATCCCTAGGTGGCTAAACTTGGCCAGC-3 and was fused to the maize ubiquitin promoter. The resulting RNAi and overexpressing constructs were introduced into *Agrobacterium tumefaciens* EHA105 and were further introduced into plants by the previously described method (35).

ORF11 (*RMS*) promoter and coding regions from each CWR and CWA were PCR-cloned using primer pairs 5′-GGATCCCCTTCAAGCCTTCATGAAATGCTCC-3′ and 5′-GGATCCGGTGGCTAAACTTGGCCAGC-3′. The 4,603-bp regions were fused in upstream of the *mRFP* gene, as described in our previous study (36). The constructs were stably introduced to T65 plants as described in the previous paragraph.

Subcellular Localization of ORF11-GFP Fusion Protein. The stop codon-deleted *ORF11* was fused to the *N*-terminus of GFP and was driven by the *maize ubiquitin* promoter to produce T65 plants stably expressing the ORF11-GFP fusion protein. Calli from the transgenic lines were treated with 0.05% (wt/vol) pectolyase Y-23 (Kyowa Chemical Products), 0.05% (wt/vol) cellulase RS (Yakult Pharmaceutical Ind. Co.), 0.01% (wt/vol) calcium chloride, and 0.9% (wt/vol) mannitol-containing buffer (pH 5.6) and were subsequently stained with Mito Tracker Red (Invitrogen) according to the manufacturer's protocols. Protoplasts were washed with 0.01% calcium chloride and 0.9% mannitol-containing buffer and were observed under fluorescent microscopy for GFP fluorescence.

mRNA Expression Analysis. Total RNA from mature anthers was extracted using RNeasy (Qiagen) according to the manufacturer's instructions. DNA elimination and cDNA synthesis were performed using the QuantiTect Reverse Transcription kit (Qiagen). RT-PCR for genes predicted in the *Rf* region was performed using *exTaq* DNA polymerase (TaKaRa-Bio). PCR cycles were as follows: 94°C for 3 min, followed by 23, 27, and 30 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and final extension at 72°C for 3 min. Band intensities at a nonsaturating condition were compared. Quantitative RT-PCR for *ORF11* and *PPR2* was performed for 3 biologic replicates, using SYBR Premix Ex Taq (TaKaRa-Bio) and Thermal Cycler Dice Real Time System TP800 (TaKaRa-Bio). The expression level of *tubulin alpha-1 chain* (accession no. Os07g0574800) was used as the internal control. Specific primers used for each gene are listed in Table S3.

Pollen Observation. Spikelets were harvested 2 to 3 h before anthesis and fixed in 3:1 ethanol:acetic acid (vol/vol). After staining with 2% iodine-potassium iodide, pollen was classified into 3 groups: starch-digested, starch-engorged, and abnormal pollen, and grains were counted under light microscopy. At least 400 pollen grains were counted per spikelet, and 5 spikelets were counted for each line. Pollen germination on the stigmas was observed by the method previously described (17).

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