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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 [W]

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Cytoplasmic male sterility (CMS) and nucleus-controlled fertility restoration are widespread plant reproductive features that provide useful tools to exploit heterosis in crops. However, the molecular mechanism underlying this kind of cytoplasmic–nuclear interaction remains unclear. Here, we show in rice (*Oryza sativa*) with Boro II cytoplasm that an abnormal mitochondrial open reading frame, *orf79*, is cotranscribed with a duplicated *atp6* (B-*atp6*) gene and encodes a cytotoxic peptide. Expression of *orf79* in CMS lines and transgenic rice plants caused gametophytic male sterility. Immunoblot analysis showed that the ORF79 protein accumulates specifically in microspores. Two fertility restorer genes, *Rf1a* and *Rf1b*, were identified at the classical locus *Rf-1* as members of a multigene cluster that encode pentatricopeptide repeat proteins. RF1A and RF1B are both targeted to mitochondria and can restore male fertility by blocking ORF79 production via endonucleolytic cleavage (RF1A) or degradation (RF1B) of dicistronic B-*atp6*/*orf79* mRNA. In the presence of both restorers, RF1A was epistatic over RF1B in the mRNA processing. We have also shown that RF1A plays an additional role in promoting the editing of *atp6* mRNAs, independent of its cleavage function.

INTRODUCTION

Mitochondrial genomes encode only a fraction of the genetic information required for their biogenesis and function. Consequently, a large number of genetic and biochemical features present in plant mitochondria arose in the context of coevolution and coordinated gene functions between the mitochondrial and nuclear genomes (Mackenzie and McIntosh, 1999). Cytoplasmic male sterility (CMS) is a widespread phenomenon observed in >150 flowering plant species (Laser and Lersten, 1972). CMS is a maternally inherited trait and is often associated with unusual open reading frames (ORFs) found in mitochondrial genomes, and in many instances, male fertility can be restored specifically by nuclear-encoded, fertility restorer (*Rf*) genes (Schnable and Wise, 1998). Therefore, CMS/*Rf* systems are ideal models for studying the genetic interaction and cooperative function of mitochondrial and nuclear genomes in plants.

CMS/*Rf* systems have long been exploited for hybrid breeding to enhance the productivity of certain crops. In rice (*Oryza sativa*), several CMS/*Rf* systems defined by the different CMS cytoplasm with distinct genetic features have been identified. These include CMS-BT (Boro II), CMS-WA (wild abortive), and CMS-HL (Honglian) (Shinjyo, 1969; Lin and Yuan, 1980; Rao, 1988). These systems have been widely used for hybrid rice breeding in China and other Asian countries as hybrid rice crops that often produce higher yields than inbred varieties (Li and Yuan, 2000; Virmani, 2003).

To date, a number of genetic loci for CMS and fertility restoration have been mapped in various plant species. Recently, *Rf* genes have been cloned from maize (*Zea mays*), petunia (*Petunia hybrida*), radish (*Raphanus sativus*), and rice (Cui et al., 1996; Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003; Komori et al., 2004), but very few CMS candidate genes have been functionally tested, and the molecular mechanisms of the CMS/*Rf* systems generally remain unclear (Schnable and Wise, 1998; Wise and Pring, 2002; Hanson and Bentolila, 2004). Of the cloned *Rf* genes, maize *Rf2* encodes an aldehyde dehydrogenase (Liu et al., 2001), and the others are members of a recently defined large gene family encoding pentatricopeptide repeat (PPR)–containing proteins (Small and Peeters, 2000). Many PPR genes are considered to encode RNA

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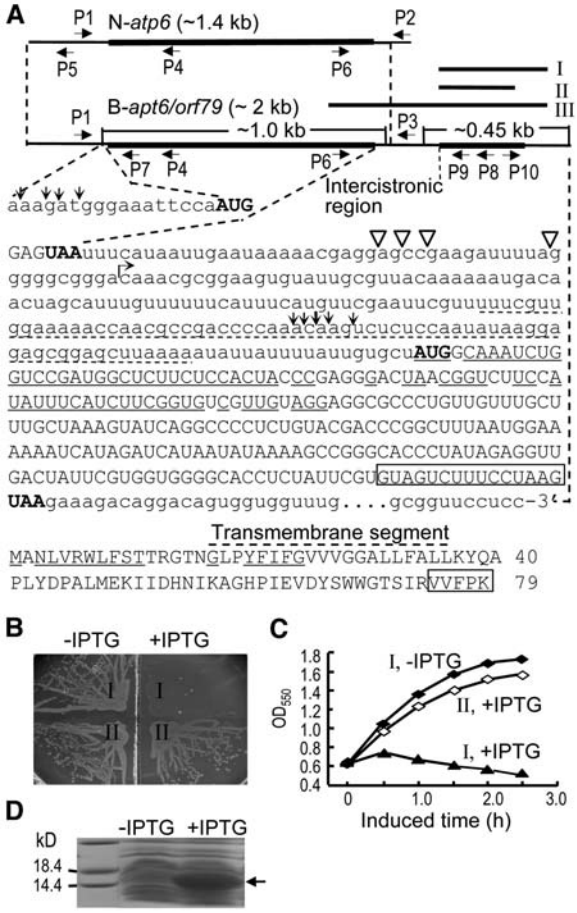

Figure 1. Characterization of the CMS-Associated Gene in Rice with BT-Cytoplasm.

(A) The structures of N-*atp6* and B-*atp6*/*orf79* transcripts, the sequence downstream of B-*atp6*, and the ORF79 peptide sequence. The primers P1, P2, and P3 were used for RT-PCR to examine the editing of N-*atp6* and B-*atp6* mRNAs. To determine the 5′ and 3′ ends of the primary (N-*atp6*) and processed RNA fragments containing B-*atp6* or *orf79* by a CR-RT-PCR method (Kuhn and Binder, 2002) (see Figure 7B), the primers P4 and P8 were used for the reverse transcription of the circularly ligated RNAs, and the reverse-directed primer pairs P5/P6, P7/P6, and P9/P10 for the PCR. The 5′ and 3′ termini of the processed B-*atp6* and *orf79* RNA fragments (~1 and 0.45 kb) are indicated by vertical arrows and open triangles, respectively. The primary 5′ and 3′ termini of N-*atp6* and B-*atp6*/*orf79* mRNAs and their different downstream sequences (starting from the bent arrow) have been described (Iwabuchi et al., 1993). The nucleotides identical to *cox1* and the encoding amino acids are underlined. The dotted underline indicates a segment identical to the 5′ UTR of a predicted mitochondrial gene *orf91* (Itadani et al., 1994). Rectangles indicate the deleted nucleotides in fragment II and the amino acids.

(B) and **(C)** Effect of *orf79* expression with fragments I and II (Figure 1A), indicated by I and II, respectively, on the growth of *E. coli* cells on an agar plate **(B)** and in liquid cultures **(C)** with or without isopropylthio-β-D-galactoside (IPTG). In the liquid cultures **(C),** IPTG was added when the cell growth reached $OD_{550} = 0.6$.

(D) The expressed recombinant ORF79 protein (arrowed) with fragment II.

binding proteins and potentially play important roles in organelle biogenesis. So far, only a few PPR genes of various organisms have been studied in detail, and little is known about their molecular functions (Lurin et al., 2004; Schmitz-Linneweber et al., 2005).

The BT-cytoplasm of rice has been primarily identified in an *indica* variety (*O. sativa* subsp *indica*), Chinsurash Boro II (Shinjyo, 1969), and then transferred into a number of *japonica* and a few *indica* varieties by recurrent backcrossing. It is known that male fertility restoration in the CMS-BT/*Rf* system is controlled by the locus *Rf-1* on chromosome 10 (Shinjyo, 1975; Yu et al., 1995; Akagi et al., 1996). The CMS-BT/*Rf* system is gametophytic in nature (Shinjyo, 1975) (i.e., the male-sterility phenotype appears in male gametophytes). The fertility restoration occurs only in those carrying the *Rf-1* allele. The restoring allele *Rf-1*, also called the dominant one in some documents, is present in some *indica* lines, but typical *japonica* lines (*O. sativa* subsp *japonica*) carry only the nonrestoring allele *rf-1* (Shinjyo, 1975; Zhu, 2000).

The mitochondrial genome of the BT-cytoplasm contains two duplicated copies of the *atp6* gene encoding a subunit of the ATPase complex (Kadowaki et al., 1990; Iwabuchi et al., 1993). These copies, N-*atp6* and B-*atp6*, are transcribed constitutively to produce mRNAs of different lengths due to the B-*atp6* mRNA having an additional downstream sequence containing a predicted ORF called *orf79* (Akagi et al., 1994, 1995). The roles that B-*atp6* and *orf79* play in CMS remain unclear, although it was proposed that RNA editing of B-*atp6* or abnormal transcripts corresponding to the B-*atp6*/*orf79* region may be involved

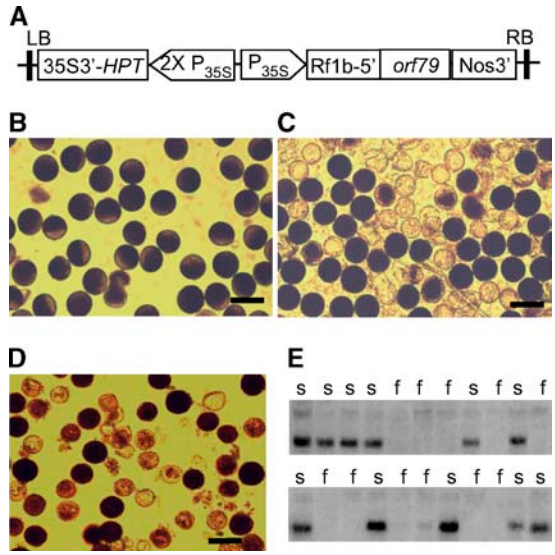

Figure 2. Functional Test of *orf79* in Male Sterility.

(A) The binary construct for expression of recombinant *orf79* in rice. P_{35S}, the CaMV35S promoter; Rf1b-5′, the 5′ segment of *Rf1b* encoding mitochondrion transit signal.

(B) to **(D)** Pollen grains of a normal fertile rice line **(B)** and *orf79* transgenic T0 rice plants with single **(C)** or two **(D)** T-DNA insertions. The darkly stained pollens were fertile and the lightly stained were sterile. Bars = 50 μm.

(E) Cosegregation of the *orf79* transgene and the semi-male-sterility (s) in a T1 family with single T-DNA insertion as assayed by RNA gel blot analysis of young panicle RNA using *orf79* as a probe. f, full male fertility.

(Iwabuchi et al., 1993; Akagi et al., 1994). Recently, a gene has been identified as the *Rf-1* restorer (Komori et al., 2004). However, the complete nature of the chromosomal region carrying *Rf-1* and the molecular mechanism underlying its fertility restoration function have not been clarified.

In this study, we demonstrate that *orf79*, when expressed in *Escherichia coli*, is toxic and that its expression in CMS lines and transgenic rice plants causes gametophytic male sterility. We cloned the genes in the *Rf-1* locus and demonstrated it to be a cluster of duplicated genes of the PPR gene family. Two members of this gene cluster can restore male fertility by silencing *orf79* mRNA via different mechanisms. Furthermore, we show that one of the *Rf* genes plays another role in RNA editing of *atp6*. Thus, our results demonstrate the mechanistic link between the molecular functions of the CMS and *Rf* genes. More generally, it also provides new insights into the molecular interaction between the mitochondrial and nuclear genomes and the function of PPR proteins.

RESULTS

The Chimeric Gene *orf79* Encodes a Cytotoxic Peptide and Confers Gametophytic CMS

Since our results showed that the editing level of *atp6* is not likely correlated with the male fertility status (see below), we examined the possible role of *orf79* in CMS. The 5′ region of *orf79* is similar to the rice mitochondrial *cox1* gene encoding cytochrome

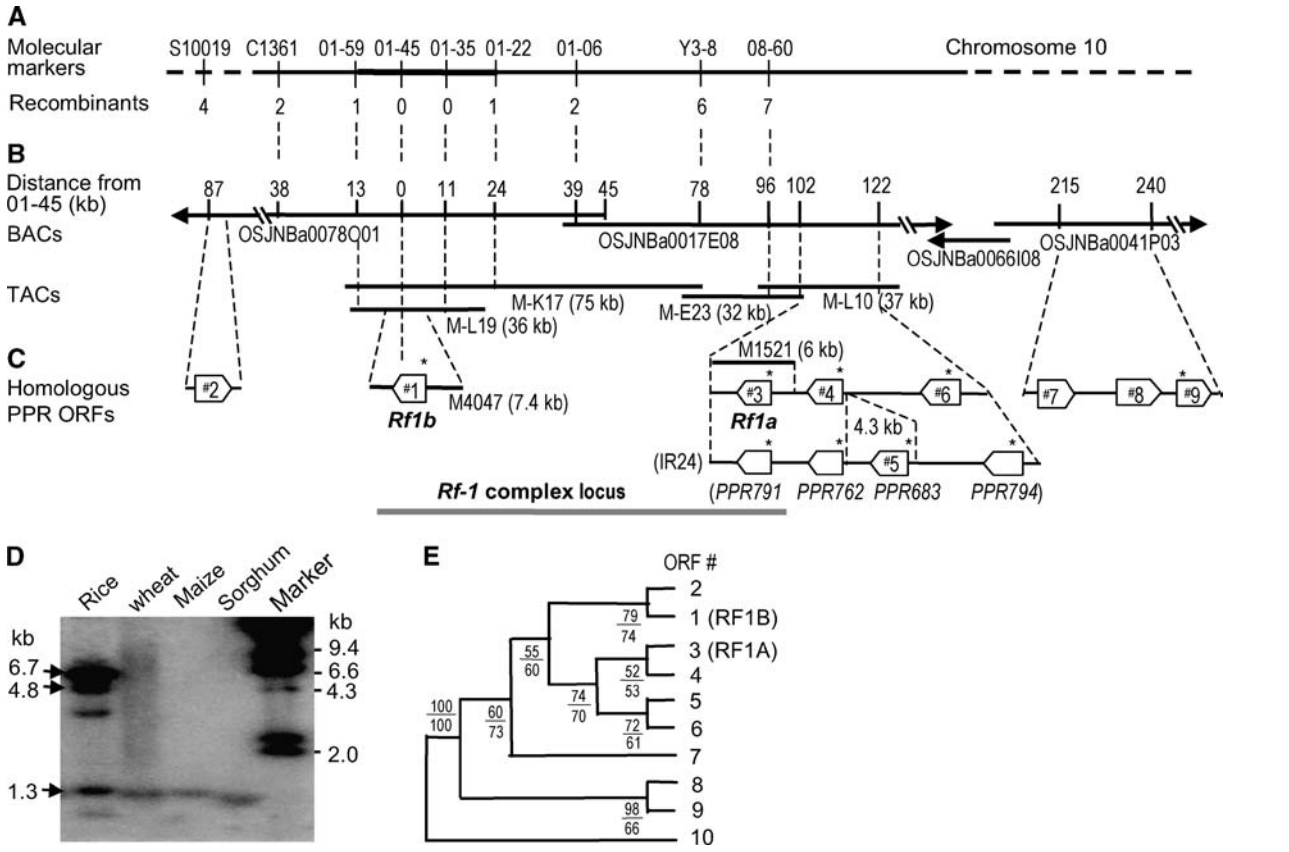

Figure 3. Mapping and Cloning of the *Rf* Genes and Characterization of the PPR Subfamily.

(A) Molecular mapping of an *Rf* gene (*Rf1b*) for the *Rf-1* locus using an F2 population generated from a cross between a CMS line 731A and a restorer line C9083, in which *Rf1b* is functional. The numbers of recombinants between the markers and *Rf1b* are shown.

(B) Physical maps based on BACs (Rice Chromosome 10 Sequencing Consortium, 2003) and TACs from a restorer line MH63 that contains two functional *Rf* genes (*Rf1a* and *Rf1b*). *Rf1b* was located to a 37-kb region by the molecular mapping. The pCAMBIA1300-based subclones M4047 and M1521 were able to restore the male fertility of a CMS-BT line by transformation.

(C) The PPR gene cluster consisting of *Rf1a* and *Rf1b* and their homologs. The ORFs *RRR791*, *PPR762*, and *PPR794* of a restorer line IR24 (Komori et al., 2004) are allelic to ORFs #3, #4, and #6 of MH63, respectively. In the ORF#1 and #3-#6 regions, the locations of the PPR ORFs in the genome of a *japonica* cultivar Nipponbare are the same as MH63. ORFs with predicted or experimentally confirmed mitochondrion transit signals are marked by an asterisk.

(D) DNA gel blot analysis of *Hin*dIII-digested genomic DNA of rice (MH63) and other plant species using *Rf1b* as a probe. The fragments of 6.7, 4.8, and 1.3 kb correspond to fragments containing whole or parts of the ORFs #1, #6, and #3/#4 (overlapped), respectively, according to the sequence of M-L19 and M-L10.

(E) Phylogenetic analysis of the PPR protein subfamily. ORF#10 is a gene located on chromosome 8. Numbers below the branches indicate the bootstrap proportions (%) for maximum parsimony (above) and maximum likelihood (below) analyses.

oxidase subunit I, while the 3′ region is of unknown origin (Akagi et al., 1994). This gene was predicted to encode a transmembrane protein with a molecular mass of 8.9 kD (Figure 1A). To test the function of *orf79*, a DNA fragment of the coding sequence (fragment I, Figure 1A) was cloned in frame into the expression unit of a bacterial expression vector. It was observed that expression of this protein was lethal to the host *E. coli* cells, with cell lysis leading to a rapid decrease in cell density (Figures 1B and 1C). When a 15-bp segment was deleted from the 3′ end of *orf79* (fragment II), cell lethality was not observed (Figures 1B and 1C) even though the protein was expressed at a high level (Figure 1D). These observations indicate that *orf79* encodes a cytotoxic peptide and its C terminus is necessary for the cytotoxicity.

To test whether the expression of *orf79* causes male sterility in rice, a plasmid based on the binary vector was prepared, which carries a fusion gene of the mitochondrion transit signal from an *Rf* gene (*Rf1b*; see below) and the *orf79* coding sequence controlled by the 35S promoter of *Cauliflower mosaic virus* (CaMV35S) (Figure 2A). This plasmid was transferred into a fertile rice line with normal cytoplasm. Eighteen transgenic T0 generation plants were obtained. These plants, containing single or multiple T-DNA insertions (data not shown), exhibited a semi-male-sterility phenotype wherein ~50% or more of the pollen grains were aborted (Figures 2C and 2D). The spikelet fertility (seed setting rate) of the T0 plants ranged from 80 to 90%, indicating that female fertility was unaffected in the transgenic lines. Analysis of the T1 progeny of a T0 plant with a single T-DNA insertion showed that the transgene was present in 36 and absent in 32 plants, fitting a 1:1 segregation ratio (rather than 3:1). Furthermore, the semi-male-sterility phenotype in this population cosegregated with the presence of the transgene (Figure 2E). We determined the T-DNA insertion site in this transgenic line by thermal asymmetric interlaced PCR (Liu and Whittier, 1995) amplification and sequencing of the flanking sequences and found no gene to be tagged (data not shown). This eliminated the possibility of T-DNA insertion in a nuclear gametophytic gene.

It is expected that if the *orf79* transgene was not correlated to pollen sterility, or it caused partial male sterility with a sporophytic effect based on the diploid genotype of the gene, it would segregate at a 3:1 rather than a 1:1 ratio in the progeny. These results indicate that the *orf79* transgene was transmitted normally through the female germ line but poorly or not at all through pollen.

The *Rf-1* Locus Comprises Two *Rf* Genes for the CMS-BT System

To clone *Rf-1*, we mapped the locus to a 37-kb region (Figure 3A) using molecular markers (see Supplemental Table 1 online) and an F2 population. A contig covering the *Rf-1* locus region (Figure 3B) was constructed with clones from a genomic library of transformation-competent artificial chromosomes (TACs) (Liu et al., 1999) of the elite restorer line Minghui63 (MH63) (Liu et al., 2002), which contains functional *Rf* loci for the CMS-BT and CMS-WA systems. A TAC clone (M-L19) covering the 37-kb region was sequenced. In this sequence, an ORF called ORF#1 was predicted to encode a mitochondrion-targeting protein, and this prediction was confirmed using fusion to green fluorescent protein (GFP) (Figure 4A). A 7.4-kb fragment containing ORF#1

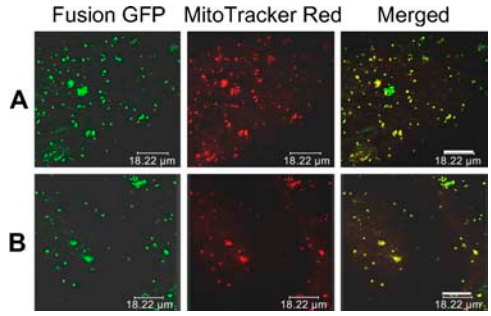

Figure 4. Products of the *Rf* Genes Are Directed to Mitochondria.

Green fluorescence spots in bombarded onion epidermal cells show the expression of GFP fused with the N-terminal sequences of ORF#1 **(A)** or ORF#3 **(B)** containing putative mitochondrion transit signals. The images observed by confocal scanning microscopy show parts of single cells. The GFP fusion proteins are targeted to the mitochondria, according to the colocalization of GFP images with those stained with mitochondrion-specific dye (MitoTracker Red) in the same cells. Bars = 18 μm.

from M-L19 (Figure 3C) was transferred into a CMS-BT line to test for fertility restoration, and 21 transgenic T0 plants were obtained. Expression of the introduced candidate gene was confirmed by RT-PCR with marker 01-45 (see Supplemental Figure 5 online). Approximately 50% of pollen grains within individual flowers of the T0 plants showed restored viability (Figure 5B). The seed-setting rates in the T0 plants ranged up to ~70% (Figure 5C), demonstrating varying degrees of fertility restoration. These results show that ORF#1 is an *Rf* gene for the CMS-BT system.

In a parallel study to isolate another rice *Rf* gene for the CMS-WA system, we identified and sequenced a TAC clone, M-L10, which is located close to M-L19 (Figures 3B and 3C). Three adjacent ORFs with homology to ORF#1 were predicted in this clone (Figure 3C). These ORFs also encode proteins with predicted mitochondrion transit signals. The presence of the mitochondrion transit signal in ORF#3 was further confirmed by the GFP assay (Figure 4B). Functional complementation tests of DNA fragments from M-L10 of MH63 showed that a clone (M1521) containing ORF#3 could restore male fertility to a CMS-BT line (Figure 5D) and process the B-*atp6*/*orf79* transcript in transgenic plants (see below), while other clones containing ORF#4 or ORF#6 could not (data not shown). Sequence analysis identified that, except for a single nucleotide variation, ORF#3 is identical to the recently reported gene *PPR791*/*PPR8-1*/*Rf-1A* for *Rf-1* (Kazama and Toriyama, 2003; Akagi et al., 2004; Komori et al., 2004).

The fertility restorer of CMS-BT has long been described as the single locus *Rf-1* (Shinjyo, 1975; Akagi et al., 1996). From our data it is evident that *Rf-1* is actually a complex locus comprising at least two *Rf* genes within an ~105-kb region, and either of these genes is sufficient to restore fertility to BT rice. To distinguish the two genes, we have renamed them as *Rf1a* for ORF#3 and *Rf1b* for ORF#1 (Figure 3C).

Sequence Analysis of the *Rf* Genes

Rf1a and *Rf1b* are intronless genes that encode PPR proteins of 791 amino acids (87.6 kD) and 506 amino acids (55.4 kD) (Figure

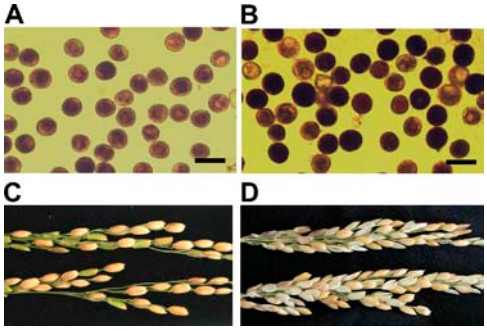

Figure 5. Functional Complementation Test of the *Rf* Gene Candidates.

(A) Completely sterile pollen of a CMS-BT line KFA.
(B) Fertility-restored pollen in dark staining (~50%) of an ORF#1 transgenic T0 plant of KFA. Bars in **(A)** and **(B)** = 50 μm.
(C) and **(D)** Panicles of the transgenic T0 plants with ORF#1 (*Rf1b*) and ORF#3 (*Rf1a*) transgenes, respectively, showing restored spikelet fertility.

6A; see Supplemental Figures 1 to 3 online), respectively, and share 70% identity between their protein sequences. RF1A and RF1B contain mitochondrion transit signals at the N termini and contiguous arrays of 18 and 11 PPR repeats, respectively. No other known motifs were found in these proteins.

To identify functional variations between the restoring *Rf1b* and nonrestoring *rf1b* alleles, we sequenced the gene of six restorer lines and six nonrestorer (CMS or maintainer) lines. A total of nine amino acid substitutions were detected; among these, a common alteration of Asn^{412}-to-Ser, caused by an A^{1235}-to-G variation, was found between the *Rf1b* and *rf1b* allele groups (Table 1). This mutation could account for the loss of the restoration function. Four *rf1a* alleles from *japonica* lines were identical to each other and encode a truncated putative protein of 266 amino acids due to a frame-shift mutation, which is consistent with previous reports (Kazama and Toriyama, 2003; Komori et al., 2004). In addition, we found another *rf1a* allele in an *indica* line encoding a full-length protein with 55 substituted amino acids (see Supplemental Figure 2 online).

A PPR Subfamily Involving the *Rf* Genes

Genomic DNA gel blot analysis using *Rf1b* as a probe detected several fragments of different signal intensities in rice and one weak hybridizing signal in wheat (*Triticum aestivum*), maize, and sorghum (*Sorghum bicolor*) (Figure 3D). No hybridization signal was detected in several dicotyledon plants (*Arabidopsis thaliana*, radish, and *Brassica*; data not shown). The sequence homology between the rice restorer proteins and those from petunia and radish (Bentolila et al., 2002; Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003) are low (<30%). Through database searching, seven or eight homologs of *Rf1a* and *Rf1b* were found in the genomes of different rice varieties. These homologs share 61 to 93% amino acid identity and a PPR consensus sequence (Figure 6B), thus forming a rice-specific PPR gene subfamily. Apart from ORF#10 on chromosome 8, all homologs are clustered within an ~330-kb region on chromosome 10 (Figure 3C). The genome of restorer line IR24 contains an additional homolog *PPR683* (Komori et al., 2004) located adjacent to *Rf1a*, suggesting a recent duplication event. With one exception (ORF#2), these putative PPR proteins contain two long PPR motifs (Figure 6A; see Supplemental Figure 4 online), which have also been identified in proposed PPR proteins of land plants (Lurin et al., 2004). Phylogenetic analysis showed that the homology relationship between these members is consistent with their chromosomal locations, with those grouped into the same or closely related clades more tightly linked (Figure 3E).

RF1A and RF1B Mediate Destruction of B-*atp6/orf79* mRNA with Different Mechanisms

RT-PCR analysis showed that the *Rf* genes were expressed in all organs tested, including young panicles, leaves, and roots (see

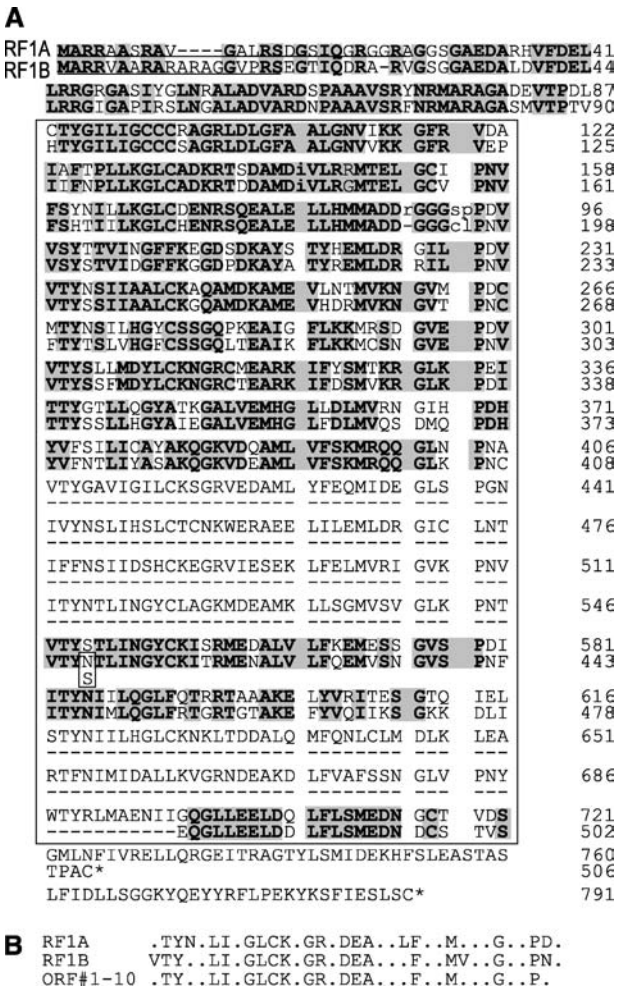

Figure 6. The Rice Restorer Proteins and PPR Consensus Sequences of the Rice PPR Subfamily.

(A) Alignment of RF1A and RF1B sequences from MH63. The PPR repeats are inside the rectangle, of which the second and third are PPR-like L motif. The functional alteration of Asn^{412}-to-Ser between proteins encoded by *Rf1b* and *rf1b* is shown. The underlined sequences correspond to mitochondrion transit signals predicted with probabilities of 0.95 and 0.97, respectively.
(B) PPR consensus sequences for RF1A, RF1B, and all the members of the rice PPR subfamily.

Table 1. Nucleotide and Amino Acid Variations among the *Rf1b* and *rf1b* Alleles in Restorer and Nonrestorer Lines

		Nucleotide Position[a]																		
Rice Line	Genotype	12	17	24	26	97	102	153	231	393	421	450	466	704	847	979	1125	1203	1235	1379
C9083(*j*)[b]	*Rf1b*	c/R[c]	c/A	c/R	c/A	g/A	c/G	c/A	t/R	g/L	a/T	c/R	g/G	t/I	c/Q	g/D	c/V	g/Q	a/N	c/A
MH63(*i*)[b]	*Rf1b*	c/R	c/A	c/R	c/A	g/A	c/G	c/A	c/R	g/L	a/T	c/R	g/G	c/T	c/Q	t/Y	t/V	c/Q	a/N	g/G
C418(*j*)	*Rf1b*	c/R	c/A	a/R	g/G	g/A	c/G	c/A	c/R	g/L	a/T	c/R	g/G	t/I	c/Q	g/D	c/V	g/Q	a/N	c/A
Xiangqing(*j*)	*Rf1b*	c/R	a/D	c/R	c/A	g/A	a/G	c/A	c/R	g/L	a/T	c/R	g/G	t/I	c/Q	g/D	c/V	g/Q	a/N	c/A
Teqing(*i*)	*Rf1b*	t/R	c/A	c/R	c/A	g/A	a/G	t/A	c/R	g/L	a/T	c/R	g/G	c/T	c/Q	g/D	c/V	g/Q	a/N	c/A
C98(*j*)	*Rf1b*	c/R	c/A	c/R	c/A	g/A	c/G	c/A	c/R	g/L	a/T	c/R	g/G	t/I	c/Q	g/D	c/V	g/Q	a/N	c/A
731A(*j*)	*rf1b*	c/R	c/A	c/R	c/A	g/A	a/G	c/A	c/R	g/L	a/T	t/R	a/S	t/I	c/Q	g/D	c/V	g/Q	g/S	c/A
KFA(*j*)	*rf1b*	c/R	c/A	c/R	c/A	g/A	a/G	t/A	c/R	t/L	g/A	t/R	a/S	c/T	c/Q	g/D	c/V	g/Q	g/S	c/A
ZD88A(*j*)	*rf1b*	c/R	c/A	c/R	c/A	g/A	a/G	t/A	c/R	g/L	a/T	t/R	a/S	t/I	c/Q	g/D	c/V	g/Q	g/S	c/A
WYJ8A(*j*)	*rf1b*	c/R	c/A	c/R	c/A	t/F	a/G	c/A	c/R	g/L	a/T	c/R	g/G	t/I	a/K	g/D	c/V	g/Q	g/S	c/A
Nipponbare(*j*)	*rf1b*	c/R	c/A	c/R	c/A	g/A	c/G	c/A	c/R	g/L	a/T	t/R	a/S	t/I	c/Q	g/D	c/V	g/Q	g/S	c/A
Fuyu1A(*i*)	*rf1b*	c/R	c/A	c/R	c/A	g/A	c/G	c/A	c/R	t/L	a/T	c/R	g/G	c/T	c/Q	t/Y	t/V	g/Q	g/S	c/A

[a] Nucleotide positions in the coding regions are shown.

[b] (*j*) and (*i*) indicate the nuclear backgrounds of *japonica* and *indica*, respectively. The restorer lines with *japonica* nuclear background (*j*) are restorer near-isogenic lines bred by recurrent backcrosses with *indica* restorer lines as the donor parents.

[c] Nucleotide/amino acid.

Supplemental Figure 5 online). However, RNA gel blot hybridizations detected no signal, even on rice poly(A)$^+$ mRNA samples (see Supplemental Figure 5 online), indicating a low level of constitutive expression.

To gain insights into the molecular mechanism of the CMS restoration interaction, we analyzed the mRNA levels of B-*atp6*/*orf79* in the presence of *Rf1a* and/or *Rf1b*. In *Rf1a* transgenic plants, the steady state level of B-*atp6*/*orf79* mRNA was greatly reduced, while two new transcripts of ~1 and 0.45 kb were detected (Figure 7A) when fragment III (see Figure 1A) was used as a probe. This altered expression profile is similar but not identical to the previous observations in cytoplasmic hybrid plants (Iwabuchi et al., 1993; Akagi et al., 1994). Two possible mechanisms by which RF1A affects the transcript profile are post-transcriptional processing or alterative de novo transcription. To address this issue, we characterized the 5′ and 3′ termini of the altered transcripts using a circularized RNA (CR)-RT-PCR method (Kuhn and Binder, 2002). With this method, the 5′ termini of cleavage-processed RNA molecules can be ligated to their 3′ ends for inverse RT-PCR, whereas 5′ termini of primary transcripts carrying triphosphates must be treated with tobacco acid pyrophosphatase (TAP) to remove the 5′ pyrophosphate to allow ligation to occur (Kuhn et al., 2005).

The results showed that, without the TAP treatment, CR-RT-PCR products could only be obtained from the newly generated transcripts containing a B-*atp6* or *orf79* region in an *Rf1a* transgenic plant (Figure 7B). Sequence analysis found that the ~1-kb RNA molecules contained different 5′ termini located 11 to 16 bases upstream of the start codon of B-*atp6* and different 3′ ends at the region just downstream of the stop codon, while the 0.45-kb *orf79*-containing RNAs had different 5′ termini in the intercistronic region (Figure 1A). Moreover, the feature of the multiple 5′ termini at adjacent sites of these RNAs is obviously distinct from that of primary mitochondrial mRNAs generated from different transcription initiation sites by multiple promoters, which are characterized by their scattered distribution along a certain chromosomal region (Nakazono et al., 1996; Lupold et al., 1999; Q. Zhang and Y.-G. Liu, unpublished data). Taken together, it is evident that RF1A functioned to mediate endonucleolytic cleavage of B-*atp6*/*orf79* mRNA at three major regions, each with multiple cleaving sites. Analysis of the termini of N-*atp6* transcripts from CMS-BT and *Rf1a*-restored plants found no cleavage processing of the molecules.

In *Rf1b* transgenic plants and fertility-restored hybrid plants carrying *Rf1b* but lacking *Rf1a* allele, the abundance of the B-*atp6*/*orf79* transcript was greatly decreased, but no processed intermediate product was detected (Figure 7C). The N-*atp6* transcript level was not affected by *Rf1b* (Figure 7C), although both *atp6* copies shared identical promoter/regulatory sequences. Therefore, the reduction of the transcript could be attributed to posttranscriptional degradation mediated specifically by RF1B. The processing patterns of the mRNA by the two *Rf* genes in other tissues, such as leaves and *Rf* gene transgenic calli, were consistent with those of young panicles as described earlier (data not shown).

RF1A Is Epistatic to RF1B in mRNA Processing

Since *Rf1a* and *Rf1b* alleles, when existing alone, function independently to process the same mRNA with different mechanisms, it would be of interest to know how the mRNA is processed in the presence of both restoring alleles of the genes. It was observed that in hybrid plants carrying *Rf1a* and *Rf1b*, the pattern and abundance of the processed B-*atp6*/*orf79* mRNA were the same as that of *Rf1a* alone (Figure 7D). This indicates that B-*atp6*/*orf79* was normally transcribed and the mRNA was preferentially cleaved by the action of RF1A, exhibiting an epistatic effect over RF1B. This observation also supports the conclusion that RF1B functions to mediate the degradation of B-*atp6*/*orf79* mRNA rather than to suppress its transcription. The mRNA fragments cleaved by RF1A were resistant to this specific degradation.

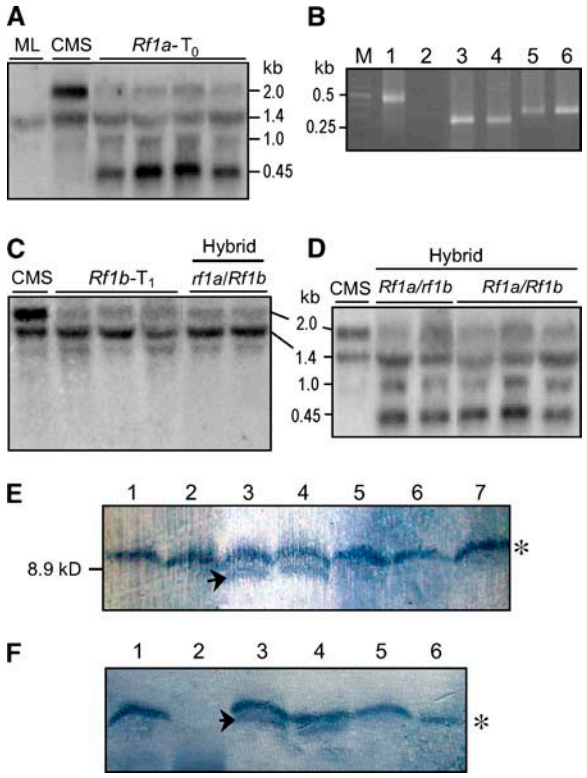

Figure 7. Processing of B-*atp6*/*orf79* mRNA and Suppression of the ORF79 Production by the *Rf* Genes.

(A) Transcript profile of B-*atp6*/*orf79* in transgenic plants with *Rf1a* assayed by RNA gel blot analysis. The relatively low signal intensity of the 1.4- and 1.0-kb bands was due to the relatively smaller portion of the DNA fragment III probe (see Figure 1A) hybridizing to *atp6*. The 1.4-kb band (N-*atp6*) also served as a loading control. ML, maintainer line.

(B) CR-RT-PCR analysis of the 5′ and 3′ termini of the primary N-*atp6* transcript from a maintainer line (lanes 1 and 2), the processed B-*atp6* (lanes 3 and 4), and *orf79* (lanes 5 and 6) mRNA fragments from an *Rf1a* transgenic plant using the primer pairs P5/P6, P7/P6, and P9/P10, respectively (see Figure 1A). The RNA samples were treated with (lanes 1, 3, and 5) and without (lanes 2, 4, and 6) TAP before the RNA ligation reaction. M, molecular weight marker.

(C) Transcript profile of B-*atp6*/*orf79* in transgenic and fertility-restored hybrid plants with *Rf1b*.

(D) Transcript profile of B-*atp6*/*orf79* in fertility-restored hybrid plants with *Rf1a* alone or both of *Rf1a* and *Rf1b*.

(E) Immunoblot analysis probed with an antibody to ORF79. Proteins were prepared from anthers containing microspores of a CMS-BT line (lanes 3 and 4), fertility-restored transgenic plants with *Rf1a* (lane 1) or *Rf1b* (lane 2), fertility-restored hybrid plants carrying *Rf1a* and *Rf1b* (lanes 5 and 6), and a maintainer line (lane 7). The specific band (lanes 3 and 4) for ORF79 is indicated with an arrow, and the top one marked with an asterisk is a cross-reacting unknown protein.

(F) Immunoblot analysis of ORF79 using proteins prepared from microspores of fertility-restored transgenic plants with *Rf1a* (lane 1), mitochondria of seedling leaves of the CMS-BT line (lane 2), microspores of the CMS-BT line (lanes 3 and 4), and anther wall tissue not including the microspores (lanes 5 and 6). The cross-reacting unknown protein, as well as ORF79, was not detected in the mitochondria of the seedling leaves of the CMS-BT line (lane 2).

ORF79 Accumulates Specifically in the Microspores and Its Production Is Suppressed by the *Rf* Genes

An immunoblot was performed to examine ORF79 in the CMS-BT line and the inhibitory effect of the *Rf* genes on its production. The results showed that the ORF79 protein was detected in anthers, including microspores of a CMS-BT line; however, this protein was undetectable or the level greatly reduced in fertility-restored transgenic and hybrid plants with *Rf1a* and/or *Rf1b* (Figure 7E). We further performed the immunoblot analysis using proteins prepared from isolated microspores, anther wall tissue in which microspores were removed, and mitochondria purified from young seedling leaves. We found that ORF79 was detected in microspores of the CMS-BT line but not in the anther wall tissue and seedling leaves (Figure 7F). These data indicate that expression and silencing of *orf79* at the protein level are correlated with CMS and restoration, respectively. Moreover, specific accumulation of the product in microspores is consistent with the genetic feature of this CMS/*Rf* system.

Rf1a Plays a Role to Promote *atp6* mRNA Editing

We found that the restoring alleles of *Rf1a* and *Rf1b* exist widely in *indica* cultivars and wild rice species, regardless of the presence or absence of *orf79* (X. Li and Y.-G. Liu, unpublished data). This raised a question of whether the *Rf* genes have other important biological functions in addition to their role as fertility restorers. To address this issue, we investigated their possible role in editing of *atp6* mRNAs.

Sequence analysis of *atp6* cDNA from young panicles showed that C-to-U editing occurred at 17 sites of the *atp6* transcripts (see Supplemental Table 2 online). The editing sites were the same for N-*atp6* and B-*atp6* transcripts, indicating that the different 3′ downstream sequences did not affect the specificity of the editing sites. The editing rates of the sites in B-*atp6* and N-*atp6* mRNAs were relatively low and similar among plants lacking *Rf1a*, namely, CMS-BT lines, maintainer lines, and the *Rf1b* transgenic plants, irrespective of the male fertility status (Table 2; see Supplemental Table 2 online). When *Rf1a* was introduced by either transformation or crossing, the editing levels of both N-*atp6* and the trace of uncleaved B-*atp6* RNA molecules were increased significantly by 8 to 19% on average for the sites, with much higher increases seen for site 13 (Table 2). These results demonstrate that RF1A functions to promote the editing of *atp6* mRNA, while *Rf1b* has no such effect. This activity is independent of its cleavage of B-*atp6*/*orf79*. No RNA editing was detected in the *orf79* region.

DISCUSSION

Abnormal Mitochondrial ORFs and CMS

CMS has been found to be associated with abnormal mitochondrial ORFs in a number of plant species (Schnable and Wise, 1998). In this study, we demonstrated that the chimeric rice gene *orf79* encodes a transmenbrane protein that is cytotoxic to *E. coli* and that its recombinant transgene leads to gametophytic male sterility, mimicking the genetic effect of the BT-cytoplasm. This

Table 2. Editing Rates (%) of Site 13 and the Average of the 17 Sites in N-*atp6* and B-*atp6* mRNAs in Plants with or without *Rf1a* or *Rf1b*

	N-*atp6*					B-*atp6*[a]			
Site	ML[b]	CMS[b]	Hyb[c]	*Rf1a*-T1[b]	*Rf1b*-T2[b]	CMS	Hyb[c]	*Rf1a*-T1[b]	*Rf1b*-T2[b]
13	48.5	44.4	82.3	90.3	43.3	43.6	65.2	69.7	45.2
Average	83.4	80.2	97.8	91.2	81.1	71.5	89.1	90.5	76.0
Duncan's test[d]	C	C	A	B	C	B'	A'	A'	B'

[a] B-*atp6* cDNAs of hybrid, *Rf1a*-T1, and *Rf1b*-T2 were generated from the trace of uncleaved or undegraded B-*atp6*/*orf79* RNA molecules.
[b] The maintainer lines (KFB) and CMS-BT (KFA) and the fertility-restored transgenic plants (*Rf1a*-T1 and *Rf1b*-T2) have the same nuclear background.
[c] The hybrid (Hyb) plant carried *Rf1a*.
[d] The statistical analysis was performed for the 17 sites in N-*atp6* or B-*atp6* of the lines (see Supplemental Table 2 for details) at the 5% level. The averages marked with the same letter are not significantly different.

feature is distinct from that of the common bean (*Phaseolus vulgaris*) CMS gene *orf239*, which results in sporophytic male sterility in transgenic tobacco plants (He et al., 1996). It was previously shown that the maize CMS-T gene *urf13* also encodes a cytotoxic peptide (Dewey et al., 1988). In addition, the expression of CMS-associated genes *orf552* from sunflower (*Helianthus annuus*) and *orf138* from radish were also lethal to *E. coli* (Nakai et al., 1995; Duroc et al., 2005; Y.-G. Liu, unpublished data). Therefore, we suggest that many abnormal mitochondrial CMS genes, if not all, may encode cytotoxic proteins that can disrupt the development of male sporophytic and/or gametophytic cells.

The C-terminal sequence of ORF79 is similar to the predicted protein of a CMS candidate locus *orf107* in sorghum (Tang et al., 1996), thus providing an unusual case of sequence similarity between CMS-associated loci in different species. Moreover, we have shown that the C-terminal region of ORF79 is essential for its cytotoxic effect in *E. coli.* These findings strongly suggest that the C-terminal region of the peptide is important for its detrimental effect on male development. Despite the constitutive RNA expression of *orf79*, its product accumulates specifically in the microspores. This provides a tight correlation between the expression of *orf79* and the phenotype of gametophytic male sterility. We propose that there could be a regulatory mechanism, likely at the posttranslational level, for the specific suppression of ORF79 accumulation in the sporophytic tissues, thus restricting its detrimental effect to microspores. Further study into the mechanism behind this cell type–specific protein accumulation/degradation will provide insight into the molecular basis of gametophytic CMS in plants.

Two Distinct RNA Silencing Pathways Are Integrated in the CMS-BT/*Rf* System

CMS/*Rf* systems with two major *Rf* loci have been identified genetically in several plants (Schnable and Wise, 1998), including the CMS-WA (Zhang et al., 1997, 2002) and CMS-HL (Liu et al., 2004) systems in rice. This study revealed that the classical *Rf-1* locus consists of two closely linked *Rf* genes, *Rf1a* and *Rf1b*, as members of the PPR cluster. Different restorer lines for this system may carry distinct genotypes of the two genes, with both or either being functional for restoration. Therefore, the use of different restorer lines for fine mapping has resulted in the precise location of *Rf-1* to *PPR791*/*Rf-1A* (*Rf1a*) in previous reports (Akagi et al., 2004; Komori et al., 2004) and *Rf1b* in this study.

To date, only one other rice PPR gene (*OsPPR1*) has been the subject of functional analysis (Gothandam et al., 2005). Here, we have shown that RF1A is the key factor for the cleavage processing of B-*atp6*/*orf79* mRNA. Despite the fact that two of the three major cleaving positions are located in a region of the B-*atp6* sequence that is identical with N-*atp6*, this RF1A-dependent cleavage does not occur in N-*atp6* mRNA. This indicates that a specific interaction between RF1A or an RF1A-containing complex and the special intercistronic/*orf79* sequence is required for the cleavage activity. It is known that special sequences, other than Shine-Dalgarno–like sites, in 5′ untranslated regions (UTRs) of yeast mitochondrial mRNAs are essential for initiation of translation (Dunstan et al., 1997). Little is known about the translation machinery for plant mitochondrial mRNAs, particularly polycistronic mRNA (Giege and Brennicke, 2001). However, it is noteworthy that a 67-base segment in the intercistronic region covering one of the RF1A-dependent cleaving positions is identical to the 5′ UTR sequence of a predicted rice mitochondrial gene *orf91* (Itadani et al., 1994) (Figure 1A). This sequence may play an important role in the translation initiation; thus, the destruction of this sequence by RF1A would lead to the *orf79*-containing mRNA fragments being untranslatable. Indeed, the production of ORF79 is blocked by the cleavage processing, as well as the degradation, of B-*atp6*/*orf79* mRNA. Since the cleaved B-*atp6* mRNA loses the entire 5′ UTR sequence, it should also be defective for translation. These facts indicate that this duplicated gene is not essential for the biological function of ATP6. However, this gene copy plays a role in the expression of *orf79* through cotranscription and in the processing of the dicistronic mRNA; therefore, it is also an essential component of the CMS-BT/*Rf* system.

In the absence of RF1A, RF1B can function in the processing of target mRNA. However, when both are present, RF1A seems to interact preferentially with the mRNA, thus exhibiting an epistatic effect over RF1B. The inability of RF1B to destabilize the cleaved RNA fragments suggests that this cleavage also destroys a recognition sequence in the intercistronic region necessary for RF1B-dependent RNA degradation. This form of mitochondial mRNA decay pathway seems to differ from those involving processing signals of homopolymeric tails or secondary structures at the 3′ UTRs (Bellaoui et al., 1997; Dombrowski et al., 1997; Gagliardi and Leaver, 1999; Kuhn et al., 2001).

Although some *Rf* loci are known to affect the transcript profile of CMS-associated loci in several plant species, the action mode of these *Rf* loci and how the altered expressions lead to fertility restoration have not been elucidated (Hanson and Bentolila, 2004). In this regard, two major possibilities exist: the *Rf* genes function to suppress the expression of CMS genes that are detrimental to male development, or they compensate or normalize the impaired expression of mitochondrial genes essential for male fertility. This study demonstrated that *Rf1a* and *Rf1b* function independently to restore male fertility by silencing the CMS gene via different RNA processing pathways. Thus, there are at least two posttranscriptional controlling mechanisms for the CMS-BT/*Rf* system. Similar mechanisms may exist for other CMS/*Rf* systems with two major *Rf* loci, such as the rice CMS-HL and maize CMS-S (Wen et al., 2003) systems.

Functions of PPR Proteins in Organelle Biogenesis

The precise mechanism underlying how PPR proteins interact with target mRNAs remains unclear (Lurin et al., 2004). Since RF1A and RF1B lack obvious domains likely to have catalytic activity, the PPR protein–mediated RNA processing may require the activities of other cofactor(s), as proposed for the general action mode of PPR proteins (Lurin et al., 2004). The mechanism by which the Asn^{412}-to-Ser substitution in RF1B results in a loss of the mRNA processing function is unclear. However, computer analysis detected variations in the secondary structure between the restoring and nonrestoring proteins (data not shown), which may affect the activity of the RNA–protein interaction.

Since the *atp6* transcripts are edited at basal levels by unknown factors in male sterile and fertile rice plants without *Rf1a*, the role RF1A plays in enhancement of the *atp6* editing, which is independent of the cleavage processing, appears to be redundant for the biological function of ATP6 and is not necessary for the fertility restoration. It has been reported recently that a PPR protein, CRR4, is required for editing of the chloroplast *ndhD* mRNA in *Arabidopsis* (Kotera et al., 2005). These facts suggest that some PPR proteins are involved in the editing of specific mRNAs, either as essential factors such as CRR4, or as helper factors like RF1A. Therefore, this role of RF1A is likely to be its normal function, while the activity for fertility restoration might be a new function of the gene. Similar mechanisms likely exist in other CMS/*Rf* systems, since in many instances the sequences of *atp* or other mitochondrial genes are also linked to and are cotranscribed with CMS-associated loci (Schnable and Wise, 1998).

The PPR Subfamily and the Evolutionary Relationships among CMS/*Rf* Systems in Rice

In rice, >600 PPR genes are predicted (Lurin et al., 2004). Many PPR genes in rice are clustered in chromosomal regions (D. Zou and Y.-G. Liu, unpublished data). It is known that PPR-containing *Rf* genes in petunia, radish, and rice are also clustered with their homologs (Bentolila et al., 2002; Desloire et al., 2003; Komori et al., 2004). In this study, we further characterized the unique PPR cluster involving the *Rf* genes in rice. Interestingly, we found that the phylogenetic relationship of members in this PPR cluster is consistent with their chromosomal locations. This and other features, such as varied numbers of members in different varieties and the existence of null alleles, provide a striking example of the rapid and dynamic evolution of multigene clusters through gene amplification, functional divergence, and birth-and-death process (Nei et al., 1997). By contrast, the homologs of these rice PPR genes in wheat, maize, and sorghum retain a single copy.

We have mapped an *Rf* gene for the CMS-WA system on a region corresponding to this PPR cluster (Zhang et al., 2002). In addition, by comparing the molecular maps of *Rf5* and *Rf6*(*t*) for the CMS-HL system (Liu et al., 2004) with our current one, we estimated that these *Rf* loci may also be located in this PPR cluster region. The data suggest that a number of the members of this PPR cluster have been recruited as fertility restorers with diverged molecular functions for the same or different CMS systems. These genetic similarities demonstrate the common origin and coevolution of these systems in rice.

METHODS

Expression of *orf79* in *Escherichia coli* and Rice

The mitochondrial DNA fragments I and II containing *orf79* (Figure 1A) were amplified from the CMS-BT line KFA using the following oligonucleotide primer pairs: 5′-ATTTTUCTCGAGUCTATGGCAAATCTGGTC-3′ and 5′-GTATGTCTAGACCACCACTGTCC-3′, and 5′-ATTTTUCTCGAGUCTATGGCAAATCTGGTC-3′ and 5′-TGTCTTCTAGACTTAACGAATAGAGGAGCCCCA-3′. *Xho*I and *Xba*I sites in the primer sequences are underlined, and the corresponding enzymes were used to clone these fragments in frame into the bacterial expression vector pThioHis (Invitrogen). Expression of *orf79* in *E. coli* TOP10F' cells was induced by adding 1 mM IPTG.

The 5′ sequences of *Rf1b* (Rf1b-5′, 305 bp), encoding the putative mitochondrion transit signal peptide, and the *orf79* fragment were amplified using the following oligonucleotide primer pairs: 5′-CAGGCCGGTCCATGGTCACGCC-3′ and 5′-GCCCGCATCTGCAGTCAGCAG-3′, and 5′-ATTTTATCTGCAGTTATGGCAAATCTGGTC-3′ and 5′-GTATCACGTGGGATCCACCACTGTCC-3′. *Nco*I, *Pst*I. and *Pml*I sites in the primer sequences are underlined. The fragments were each cloned with a TA-cloning vector (pMD18-T, Takara) and then linked in frame using the *Pst*I site. A plant expression construct was prepared by replacing the *Nco*I-*Pml*I fragment (*GUSPlus*) in the binary vector pCAMBIA1305.1 with the fusion gene *Rf1b*-5′:*orf79* (Figure 2A). After checking the fusion gene by sequencing, the construct was transferred into a *japonica* variety with normal cytoplasm by *Agrobacterium tumefaciens*–mediated transformation (Hiei et al., 1994).

Cloning of *Rf* Genes

An F2 population of 1250 plants was generated from a cross between a CMS line 731A and a restorer line C9083. The F2 plants producing full-fertile pollen grains (602 plants) and semifertile (~50%) pollen grains (648 plants) represented plants homozygous and heterozygous for *Rf-1*, respectively. A total of 603 F2 plants were used for mapping. The restorer locus was mapped using three restriction fragment length polymorphism markers, Y3-8 (Zhang et al., 2002), C1361, and S10019 (MAFF DNA Bank of Japan), and five newly developed markers (see Supplemental Table 1 online). A TAC genomic library of MH63 was screened using the markers O01-35 and 08-59 as probes. For the functional complementation test, the TAC clones M-L19 and M-L10 were partially digested with *Sau*3AI, and DNA fragments of 5 to 8 kb were recovered and subcloned into the *Bam*HI site of the binary vector pCAMBIA1300. Constructs containing the

restorer candidates were selected and transferred into the CMS line KFA by *Agrobacterium*-mediated transformation (Hiei et al., 1994). Male fertility was assayed by potassium iodide (1% I_2-KI) staining of pollen and the ability for seed setting after self-pollination. To select hybrid plants that carried either *Rf1a* or *Rf1b* allele generated from recombination events, a cross between the CMS line KFA and a restorer line C98 (*Rf1aRf1a*/*Rf1bRf1b*) was prepared, and the >3000 F2 plants were genotyped with the polymorphic markers 08-60 and 01-45 (Figure 3; see Supplemental Table 1 online). The sequences of the genes of the selected plants were further confirmed by sequencing. The single nucleotide polymorphic marker 01-45 located within *Rf1b* was designed from the A^{1235}-to-G functional mutation, and the detection of this marker was done as follows. A primary PCR (32 cycles) using oligonucleotide primers 45F (5′-CTTCATGGGTATGCTATCG-3′) and 45R (5′-CAGTCGAAGCTTCAACGG-3′) was performed and then the product (0.5 μL) was subjected to two secondary PCRs (10 cycles) using primer pairs 45A (5′-CCTAATTGTGTTACGTATA**A**-3′, *Rf1b*-specific) and 45R, and 45G (5′-CCTAATTGTGTTACGTATA**G**-3′, *rf1b*-specific) and 45R, respectively.

Subcellular Localization

The 5′ sequences of *Rf1a* (222 bp) and *Rf1b* (231 bp) encoding the putative mitochondial targeting signals were amplified from the cloned genes using the oligonucleotide primers 5′-AGGGTCGACATGGCACGCCGCGTCG-3′ and 5′-GTGCCATGGGGTTGAAGCGGGAC-3′. After digestion with *Sal*I and *Nco*I (underlined in primer sequences), the fragments were fused to and cloned in frame with the *GFP* coding sequence (Cormack et al., 1996) in a pUC18-based vector and placed under the control of the CaMV35S promoter. The constructs were transiently transformed into onion epidermal cells (Scott et al., 1999) on agar plates by a helium-driven accelerator (PDS/1000; Bio-Rad). Bombardment parameters were as follows: 1100 p.s.i. bombardment pressure, 1.0-μm gold particles, a distance of 9 cm from macrocarrier to the samples, and a decompression vacuum of 88,000 Pa. After culture for 1 d, the bombarded epidermal cells were treated with 500 nm of the mitochondrion-selective dye MitoTracker Red CM-H2XRos (Molecular Probes). GFP expression in general and colocalization of GFP fusion proteins to mitochondria were viewed using a confocal scanning microscope system (TCS SP2; Leica) with 488-nm laser light for fluorescence excitation of GFP and 578-nm laser light for excitation of MitoTracker Red.

RT-PCR and RNA Gel Blot Analysis

Total RNA was extracted from rice (*Oryza sativa*) tissue using TRIZOL reagent (Invitrogen), and poly(A)$^+$ RNA was purified using the PolyATract kit (Promega). DNA fragment III (Figure 1A) was PCR amplified from a CMS-BT line using the following primers: 5′-GGCCGGTCATAGTTCAGT-3′and 5′-GTATGTCTAGACCACCACTGTCC-3′. The PCR product was labeled with P^{32}-dCTP using a random-primed kit for RNA gel blot analysis. The 5′ and 3′ mRNA termini of N-*atp6* and RF1A-processed *atp6* and *orf79* mRNA were determined by the CR-RT-PCR method (Kuhn and Binder, 2002). The oligonucleotide primers P4 (5′-TATTGAAACGGATACGCT-3′) and P8 (5′-CTCCTACAACGACACCGT-3′) were used for the initial reverse transcription of the circularly ligated B-*atp6*- and *orf79*-containing RNAs, and primer pairs P7 (5′-CTGTTCAACGAGTTCACGT-3′) and P6 (5′-ACCGGTCTGGAATTAGGTG-3′), and P9 (5′-TGGAAGACCGTTAGTCCCT-3′) and P10 (5′-CCTCTGTACGACCCGGCTT-3′) were used in inverse PCR, respectively. The 5′ and 3′ termini of the primary *atp6* mRNAs were determined by the same CR-RT-PCR method with primer P4 for reverse transcription and primers P5 (5′-GACTGATCTCAACTGGCCT-3′) and P6 (5′-ACCGGTCTGGAATTAGGTG-3′) for inverse PCR, except that the RNA samples were treated with TAP (Epicentre Technologies) before RNA ligation (Kuhn et al., 2005). To investigate the editing frequencies of *atp6*, cDNA sequences were obtained from N-*atp6* and B-*atp6* transcripts from young panicles by RT-PCR using primer pairs P1 (5′-TCTCCCTTTCTAGGAGCAGAG-3′) and P2 (5′-TATGTCGCTTAGACTTGACC-3′), and P1 (5′-TCTCCCTTTCTAGGAGCAGAG-3′) and P3 (5′-TAACGCAATACACTTCCGCG-3′), respectively. The amplified cDNAs were cloned, and 40 to 60 clones for each sample were sequenced. Statistical analysis of the editing rates of the samples was performed by analysis of variance and Duncan's multiple range test using the SAS program.

Immunoblot Analysis

A peptide antigen corresponding to residues 1 to 30 of ORF79 was synthesized and used to immunize rabbits. Total proteins were extracted from various rice tissues, including anthers, isolated microspores, anther wall tissue in which the microspores were removed, and purified mitochondria from young seedling leaves. The anther wall tissue and microspores were isolated as described earlier (Honys and Twell, 2003; Sze et al., 2004), and their purities were checked by light microscopy. The proteins were separated by 20 to 25% SDS-PAGE containing 36% urea and blotted on Immobilon-P^{SQ} transfer membrane (PVDF type; Millipore). The membrane blots were incubated in blocking buffer (1% BSA, 0.05% Tween 20, 20 mM Tris-HCl, and 500 mM NaCl, pH 7.5) for 1 h, washed twice (5 min each) with TBST (0.05% Tween 20, 20 mM Tris-HCl, and 500 mM NaCl, pH 7.5), and incubated with the primary antibody serum (1:100 dilution) for 1 h at room temperature. After two rinses (5 min each) with TBST, the blots were incubated in the secondary antibody solution (affinity-purified antibody phosphatase-labeled goat anti-rabbit IgG [H+L], 1:1000 dilution; Kirkegaard and Perry Laboratories) for 45 min at room temperature, washed twice (5 min each) with TBST, and immediately incubated in the substrate buffer (0.33 mg/mL nitroblue tetrazolium [Sigma-Aldrich], 0.165 mg/mL BCIP [Bio-Basic], 0.1 M Tris, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 9.5) for 2 min, and then the signal was detected.

Sequence Analysis

The ORF79 sequence was analyzed by the software TMHMM server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM). Gene sequences were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). ORF annotation and PPR motif prediction were performed with the RiceGAAS system (http://rgp.dna.affrc.go.jp). The mitochondrion transit peptide was predicted by MITOPROT (http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter/). Protein sequences were aligned using the ClustalX program (Thompson et al., 1997). We manually inspected and modified the alignment output. Phylogenetic analysis was performed with maximum parsimony and maximum likelihood methods (Ln likelihood = −3474.83) implemented in PHYLIP 3.57 (Felsenstein, 1997) and PUZZLE (γ-corrected JTT model) (Strimmer and von Haeseler, 1996). Bootstrap analysis was performed with 1000 replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ311052 to DQ311054.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Genomic Sequence Comprising *Rf1a* and the Deduced Amino Acids.

Supplemental Figure 2. Nucleotide Sequence of an *indica rf1a* Allele and the Deduced Amino Acids.

Supplemental Figure 3. Genomic Sequence Comprising *Rf1b* and the Deduced Amino Acids.

Supplemental Figure 4. Multialignment of the Deduced Amino Acid Sequences of the PPR Subfamily Members in Rice.

Supplemental Figure 5. Expression of the *Rf* Alleles in Rice.

Supplemental Table 1. Molecular Markers Developed in This Study for the Mapping.

Supplemental Table 2. Editing Rates (%) of 17 Sites in N-*atp6* and B-*atp6* RNA Coding Sequences in Plants with or without *Rf1a* or *Rf1b*.

Supplemental Table 3. ANOVA of the Editing Rates of the Lines and Sites for N-*atp6* mRNA.

Supplemental Table 4. ANOVA of the Editing Rates of the Lines and Sites for B-*atp6* mRNA.

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