Suppression mechanism of mitochondrial ORF79 accumulation by Rf1 protein in BT-type cytoplasmic male sterile rice

Tomohiko Kazama1,2,3, Takahiro Nakamura4,5,6, Masao Watanabe2,3, Mamoru Sugita6 and Kinya Toriyama1,*

1Laboratory of Environmental Biotechnology, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan,
2The 21st Century Center of Excellence Program, Iwate University, Morioka 020-8550, Japan,
3Laboratory of Plant Reproductive Genetics, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, 980-8577, Japan,
4Department of Research Star Program, Organization for the Promotion of Advanced Research, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka, 812-8581, Japan,
5PREST, Japanese Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan, and
6Center for Gene Research, Nagoya University, Nagoya 464-8601, Japan

Received 27 March 2008; accepted 17 April 2008; published online 4 June 2008.
*For correspondence (fax +81-22-717-8834; e-mail torikin@bios.tohoku.ac.jp).

Summary

In BT-type cytoplasmic male sterile rice (Oryza sativa L.) with Chinsurah Boro II cytoplasm, cytoplasmic male sterility (CMS) is caused by an accumulation of the cytotoxic peptide ORF79. The ORF79 protein is expressed from a dicistronic gene atp6-orf79, which exists in addition to the normal atp6 gene in the BT-type mitochondrial genome. The CMS is restored by a PPR (pentatricopeptide-repeat) gene, Rf1, via RNA processing. However, it has not yet been elucidated how the accumulation of ORF79 is reduced by the action of the Rf1 protein. Here, we report that the level of processed orf79 transcripts in the restorer line was reduced to 50% of the unprocessed atp6-orf79 transcripts in the CMS line. Ninety percent of the processed orf79 transcripts, which remained after degradation, were not associated with the ribosome for translation. Our data suggests that the processing of atp6-orf79 transcripts diminishes the expression of orf79 by the translational reduction and degradation of the processed orf79 transcripts.

Keywords: cytoplasmic male sterility, fertility restorer gene, mitochondrial gene expression, post-transcriptional regulation, translational regulation.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in the inability to produce functional pollen. Recent studies have identified that an aberrant chimeric gene in mitochondria possibly induces CMS in various plant species (Hanson and Bentolila, 2004). In these cases, certain nuclear genes play important roles as fertility restorer genes. The most widely studied CMS/Rf system in rice (Oryza sativa L.) consists of a BT-type CMS cytoplast and a fertility restorer gene, Rf1. The mitochondrial genome of the BT-type cytoplasm contains two duplicated copies of the atp6 gene, encoding a subunit of the ATPase complex. It has been reported that a unique sequence (orf79) located downstream from one of the atp6 genes causes male sterility (Akagi et al., 1994; Iwabuchi et al., 1993; Kadowaki et al., 1990). The orf79 gene encodes a predicted transmembrane protein, with a C-terminal region of unknown function and with the N-terminus showing similarity to rice mitochondrial cytochrome oxidase subunit I (coxI). In the BT-type CMS line, the abnormal atp6 is co-transcribed as 2.0-kb transcripts consisting of normal atp6 and the unique orf79 sequence. Two discontinuous transcripts of 1.5 and 0.5 kb are generated from the 2.0-kb transcripts by RNA processing in the presence of Rf1.

Molecular cloning of the Rf1 has revealed that Rf1 encodes pentatricopeptide repeat (PPR)-containing proteins (Kazama and Toriyama, 2003). PPR protein genes belong to
one of the largest families in plants, and half of them are speculated to function in organelles, especially in mitochondria. A typical PPR motif, consisting of 35 amino acids, is a macromolecular binding motif (Lurin et al., 2004; Small and Peeters, 2000). Recent genetic and biochemical analysis has shown that PPR proteins bind to RNA or DNA with their PPR motif in a sequence-specific manner (Ikeda and Gray, 1999; Lahnby et al., 2000; Nakamura et al., 2003; Schmitz-Linneweber et al., 2005). To date, several fertility restorer genes have been cloned from different species, and have also been shown to encode PPR proteins (Akagi et al., 2004; Bentolila et al., 2002; Brown et al., 2003; Kazama and Toriyama, 2003; Koizuka et al., 2003; Komori et al., 2004). One of them, the Rf1 protein in rice, has been found to cancel the expression of the chimeric gene derived from the aberrant transcripts (Kazama and Toriyama, 2003; Wang et al., 2006). A CMS trait is widely used for F1 hybrid breeding. Besides its commercial use, the CMS and fertility restoration system is a good model for the study of the regulation of mitochondrial gene expression.

A recent study has demonstrated that ORF79 is a cytotoxic peptide, and that it causes male sterility in BT-type CMS rice (Wang et al., 2006). The study also identified two PPR protein genes, Rf1a and Rf1b, which restore male fertility. However, it has not yet been elucidated how the action of the Rf1 gene product reduces ORF79 accumulation.

In this report, a detailed analysis on the accumulation of Rf1 and ORF79 protein suggested that the level of ORF79 quantitatively affects male sterility. The expression of orf79 is downregulated in terms of both RNA stability and translation, following the processing of atp6-orf79 transcripts. The Rf1 protein is directly involved in the processing of atp6-orf79 transcripts. We discuss how the products of the fertility restorer genes suppress the expression of aberrant orf in CMS plants.

Results

Rf1 promotes processing of the atp6-orf79 transcripts

We have previously reported that the PPR protein gene Rf1 is a fertility restorer gene of BT-type CMS in rice, and that it promotes the processing of atp6-orf79 transcripts in mitochondria. In this study, we employed two Rf1 transformants, C1 and C2, which were generated through the introduction of the Rf1 cDNA driven by a maize ubiquitin promoter (Figure 1a), because the Rf1 gene was shown to be expressed in all examined tissues, calli, roots, leaves and mature anthers (data not shown for RT-PCR). Restorations of seed fertility were confirmed in both C1 and C2 (data not shown). Processing of atp6-orf79 transcripts was observed in the callus of the Rf1 transformants (C1 and C2), and in the restorer line of [cms-bo]Rf1RF1, but not in the CMS line of [cms-bo]rf1rf1 (Figure 1b). In the CMS line (rf1rf1), an intense signal at 2.0 kb and a weak signal at 1.5 kb were detected. The 2.0- and 1.5-kb signals indicated unprocessed atp6-orf79 transcripts and N- atp6 transcripts, respectively (Figure 2). In the restorer line (Rf1RF1), the 1.5- and 0.5-kb signals, but not the 2.0-kb signal, were detected. The 0.5-kb signal indicated the orf79 transcripts that were derived from the processed atp6-orf79 transcripts. In the Rf1 transformants, there were three signals: unprocessed atp6-orf79 transcripts at 2.0 kb, atp6 transcripts at 1.5 kb and orf79 transcripts at 0.5 kb. The 1.5-kb transcripts possibly contained N-atp6 and processed atp6 transcripts from atp6-orf79 transcripts. The presence of a strong signal at 2.0 kb indicates that the processing of atp6-orf79 was not completely performed in these transgenic lines, although the transgenic lines restored pollen fertility, as described above.

To reveal an accurate processing site of atp6-orf79 transcripts, we determined the 5′ termini of the processed

Figure 1. Processing of atp6-orf79 transcripts in the presence of the Rf1 gene in transgenic lines. (a) The T-DNA region of the vector used for transformation of a cytoplasmic male sterile (CMS) line. The coding region of the Rf1 gene is indicated in bold letters. Abbreviations: Ubi. pro., promoter region including the first intron of maize ubiquitin gene; 2x HA, tandem-duplicated human influenza virus hemagglutinin gene; 6x His, six-repeated histidine; NOS ter., terminator of nopaline synthase gene; NOS pro., promoter of nopaline synthase gene; NPT II, neomycin phosphotransferase gene; 35S pro., Cauliflower mosaic virus 35S promoter; HPT, hygromycin phosphotransferase gene. (b) Northern blot analysis of the transgenic rice. Mitochondrial RNA was isolated from calli of the CMS line (rf1rf1), the restorer line (Rf1RF1), and transgenic CMS line with Rf1, C1 and C2. The full length of the atp6-orf79 fragment was used as a probe.

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orf79 transcripts by primer extension (Figure 3a). In the restorer line (Rf1Rf1) and Rf1 transformant (C1), four specific bands positioned at TTGT were detected, indicating that the 5’ termini of the 0.5-kb orf79 transcripts were located at 52- to 55-bp upstream from the start codon of orf79 (Figure 3b). These signals were not detected in the CMS (rf1rf1) callus, because the processing did not occur in the CMS line. These multiple cleavage sites are the same as those previously reported (Wang et al., 2006). The processing sites were shown to be located at a single-stranded RNA region between stem loops in the predicted secondary structure (Figure 3b).

The Rf1 protein interacts directly with the intergenic region of atp6-orf79 transcripts

Accumulating evidence suggests that PPR protein is involved directly in various RNA processing steps, RNA stability and translation (Kotera et al., 2005; Meierhoff et al., 2003). Therefore, it is plausible to hypothesize that the Rf1 protein directly interacts with the atp6-orf79 transcripts to promote the processing event. To clarify this possibility, we performed an electrophoresis mobility shift assay (EMSA). A putative mature Rf1 protein, without an N-terminal mitochondrial signal peptide, was prepared as a fusion with Thioredoxin (Trx) using the protein expression system in Escherichia coli (Figure 4a). For EMSA, the Trx-fused recombinant Rf1 protein (Trx-rRf1) was incubated with the radiolabeled in vitro transcribed RNA BD4 (Figure 2). The BD4 RNA is an upstream region of the processing site that is expected to be a target site for the Rf1 protein. To estimate the relative binding affinity for the BD4 RNA, a constant concentration of labeled RNA probe was incubated with a range of the Trx-fused recombinant Rf1 protein (Trx-rRf1) concentrations. The specificity of the rRf1 protein–RNA complex was confirmed by the absence of complex in the control using Trx protein (Figure 4b). The apparent Kd value, determined as the concentration of protein at which 50% of the RNA probe was bound, was 1.68 × 10⁻⁷ M. The former characterized PPR protein, CRR4, displayed a similar Kd value of 3.0 × 10⁻⁸ M (Okuda et al., 2006), indicating that the Rf1 protein in this study has a sufficient ability to interact with the target RNA sequence. We next performed competition EMSA using non-labeled BD4 RNA and BD1 RNA that corresponds to the 5’ end of atp6 (Figure 2). As a result, the signal intensity of the rRf1 protein–RNA complex, at a five-fold excess of the self competitor (non-labeled BD4 RNA), is weaker than that of the BD1 RNA (Figure 4c). This indicates that the binding affinity of BD4 RNA with rRf1 is stronger than that of BD1 RNA. These data suggest that an atp6-orf79 transcript is a substrate of the Rf1 protein.

Processing of the atp6-orf79 transcripts reduces the translation of the ORF79 protein

In order to investigate the changes in the levels of the ORF79 protein, we prepared anti-rORF79 antibody raised against the recombinant peptide of ORF79 that lacks the first 29 amino acids showing homology to COXI. Western blot analysis using total protein detected a specific signal for ORF79 protein at the expected size of 8.9 kDa in the CMS line (rf1rf1), but not in the restorer line (Rf1Rf1) (Figure 5a). The ORF79 protein was also accumulated in the Rf1 transformant, although the level was lower than that in the CMS line (rf1rf1). The levels of ORF79 protein were further examined using isolated mitochondrial proteins (Figure 5b). The 8.9-kDa ORF79 protein was detected in both the CMS line (rf1rf1) and the C1 Rf1 transformant. The signal intensity of ORF79 protein in the Rf1 transformant was almost half of that of the CMS line (rf1rf1), which was detected in the same manner as when using the total protein. Because the C1 Rf1 transformant showed normal pollen fertility, our observations suggest that the complete disappearance of the ORF79 protein is not required for fertility restoration. There may be a threshold for the level of ORF79 protein that invokes pollen abortion.

Processed orf79 transcripts are degraded and dissociated from the mitochondrial polysome

The western blot analysis presented here indicates that the accumulation of the ORF79 protein is blocked in the restorer
The orf79 transcripts would be co-transcribed from the promoter for atp6, which is identical to that of N-atp6. Therefore, the reduction of ORF79 protein should be given at the post-transcriptional and/or post-translational level. In order to determine the RNA stability of the orf79 transcripts, the levels of mitochondrial transcripts for orf79, as well as for atp6, were quantified by comparison with a series of known levels of in vitro synthesized RNAs for 2.0-kb atp6-orf79, 1.5-kb N-atp6 and 0.5-kb orf79RNA (Figure 6a). A comparison of signal intensities revealed that the CMS line (rf1rf1) contains 14 fmol of 2.0-kb atp6-orf79 transcripts, and 9.5 fmol of 1.5-kb normal atp6 transcripts, in the 2 lgo mitochondrial RNA. The 1.5-kb atp6 transcripts and 0.5-kb processed orf79 transcripts in the restorer line (Rf1Rf1) were estimated to be 16 and 6.6 fmol, respectively (Figure 6b). The 2.0-kb atp6-orf79 transcripts could not be detected in the restorer line with [cms-bo]Rf1Rf1, which means that the atp6-orf79 transcripts are processed nearly perfectly by the action of the Rf1 protein. Because the 0.5-kb orf79 transcripts were only generated from the processing of the atp6-orf79 transcripts, the processed orf79 transcripts should have accumulated equally in comparison with those of the unprocessed atp6-orf79 transcripts in the CMS line (rf1rf1). However, the actual level of the 0.5-kb processed

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Figure 3. The processing sites of atp6-orf79 transcripts are located at the single-stranded region of the intergenic region.
(a) The 5' termini of the processed orf79 transcripts determined by the primer extension using primers located in orf79, B-GSP12. The signals, TTGT, are detected in a restorer line (Rf1Rf1) and a transgenic cytoplasmic male sterile (CMS) line with Rf1 (C1).
(b) The schematic structure of the intergenic region between atp6 and orf79, and the predicted secondary structure. The 5' termini of the processed orf79 transcripts are indicated by arrowheads. The size difference of the arrowheads shows the difference in signal intensities.

Figure 4. A recombinant Rf1 protein binds to transcripts of the BD4 region, which is located in the intergenic region between atp6 and orf79, in vitro.
(a) Western blotting profile of the recombinant proteins expressed as a fusion protein with Thioredoxin (Trx) (Trx-Rf1) and that of Trx. In each lane, 240 ng of proteins was loaded and reacted with anti-polyhistidine antibody.
(b) An electrophoresis mobility shift assay (EMSA) was carried out using in vitro transcribed BD4 at a fixed concentration (0.5 nM) with a range of protein concentrations (0, 0.05, 0.15, 0.5, 1.5, 5 and 15 nM for lanes 1–7, respectively).
(c) An EMSA in the presence of a cold competitor, either BD4 or BD1, with purified Trx-Rf1 protein. A 10-fmol quantity of in vitro transcribed BD4 was used as a probe. The experiments were performed with a 5-, 10- and 15-fold molar excess of the non-radioactive RNAs.
antibody in the cytoplasmic male sterile (CMS) line (a) Western blot of the total protein from calli probed with the anti-rORF79 antibody in the cytoplasmic male sterile (CMS) line (rflrfl) and a transgenic CMS line with Rf1 (C1). The lower panel shows the Coomassie Brilliant Blue (CBB) staining.

(b) Western blot of mitochondrial protein from calli probed with anti-rORF79 antibody in the upper panel. The lower panel shows the CBB staining.

Figure 5. The presence of the Rf1 gene reduces ORF79 protein. (a) Western blot of the total protein from calli probed with the anti-rORF79 antibody in the cytoplasmic male sterile (CMS) line (rflrfl), the restorer line (RflRf1) and a transgenic CMS line with Rf1 (C1). The lower panel shows the Coomassie Brilliant Blue (CBB) staining. (b) Western blot of mitochondrial protein from calli probed with anti-rORF79 antibody in the upper panel. The lower panel shows the CBB staining.

Figure 6. Degradation of processed orf79 transcripts revealed by quantitative northern blot analysis. (a) Northern blot analysis of 2 μg of mitochondrial RNA in the cytoplasmic male sterile (CMS) line (rflrfl) and the restorer line (rflrfl). To compare the signal intensity of each band, in vitro transcribed RNA mix (100, 30 and 10 fmol) was loaded as a quantitative standard in the upper panel, and an orf79 RNA mix (10 and 3 fmol) was loaded in the lower panel. (b) The actual measured signal intensities. B.G. means background level. The relative value of each signal compared with the 2.0-kb orf79 transcripts in the CMS line (rflrfl) is also shown in parenthesis.

Table 1. Relative value of processed orf79 transcripts in each line

<table>
<thead>
<tr>
<th>RNA mix (fmol)</th>
<th>orf79 transcripts (relative value)</th>
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<tbody>
<tr>
<td>100</td>
<td>1.00 (0.55)</td>
</tr>
<tr>
<td>30</td>
<td>9.5 (0.64)</td>
</tr>
<tr>
<td>10</td>
<td>9.5 (0.68)</td>
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orf79 transcripts in restorer line was about 50% of the 2.0-kb unprocessed transcripts in the CMS line (rflrfl) (Figure 6a). The same extent of degradation was also observed in the

Figure 7. The association of atp6, processed orf79 and atp6-orf79 transcripts with the polysome. Mitochondrial extracts were fractionated on sucrose gradients and divided into 10 fractions of equal volumes. An equal proportion of the RNA purified from each fraction was analyzed by northern blot hybridization. (a) Polysomal association of atp6 and processed orf79 transcripts in the restorer line (RflRf1). The upper panel shows the profile of 1.5-kb atp6 transcripts. (b) Polysomal association of orf79 and atp6-orf79 transcripts in the cytoplasmic male sterile (CMS) line (rflrfl). The polysomal profile was the same as that of the restorer line (RflRf1).

1.5-kb atp6 band in the restorer line (RflRf1) (Figure 6a). This suggests that the processed orf79 transcripts, as well as the processed atp6, are easily degraded in mitochondria.

The absence of ORF79 protein in the restorer line (RflRf1) cannot only be explained by the degradation of the processed orf79 transcripts. Therefore, we next addressed the translational efficiency of the orf79 transcripts, as judged by association with the polysome. Mitochondrial lysates were fractionated in sucrose density gradient, and the translational state of transcripts was revealed by hybridization (Figure 7). The fraction containing 26S rRNA indicates the fractions of the polysome (lanes 1–6, Figure 7a). The distribution of the 26S rRNA was similar between the CMS (rflrfl) and the restorer (RflRf1) lines (data not shown for the CMS line, rflrfl). In the restorer line with [cms-bo]RflRf1, processed atp6 transcripts were associated with the polysome, but the processed orf79 transcripts were not (Figure 7a). Ninety percent of the orf79 transcripts were found in the upper fractions, which did not contain the polysome, indicating that they are in non-translational states (lanes 6–10, Figure 7a).

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In contrast to the restorer line (Rf1Rf1), both unprocessed atp6-orf79 transcripts and N-atp6 transcripts were found in polysome fractions in the CMS line (rfr1f1) (Figure 7b). The ORF79 protein is likely to be co-translated from unprocessed atp6-orf79 transcripts in the CMS line (rfr1f1).

Discussion

ORF79 in BT-type CMS rice

It is well known that the expression of aberrant mitochondrial protein directly leads to cytoplasmic male sterility. The presence of a restorer gene drastically reduces the level of CMS-associated aberrant protein. For example, PCF and ORF125 are known as CMS-associated proteins in Petunia and Kosena radish CMS plants, respectively (Iwabuchi et al., 1999; Nivison and Hanson, 1989). Similar to that of other CMS plants, the expression of ORF79 protein impairs functional pollen production in BT-type CMS rice (Wang et al., 2006). Recently, Wang et al. have shown that orf79 encodes a cytotoxic peptide responsible for CMS, based on the observation that transgenic rice plants expressing orf79 caused gametophytic male sterility (Wang et al., 2006). It has been reported that ORF79 protein accumulates specifically in the microspores of a BT-type CMS line, but is absent in young seedling leaves. In our current study, we found that ORF79 protein accumulated in the calli of the BT-type CMS line, but is absent in the microspores of the BT-type CMS line (Wang et al., 2006). Additionally, we showed that the ORF79 protein accumulates in callus mitochondria, in contrast to the previous report that suggested the ORF79 protein only accumulates in the microspore of the CMS line (Wang et al., 2006). It is interesting to explore why the aberrant ORF only impairs pollen maturation, even though the protein is not harmful in somatic cells.

Post-transcriptional control of mitochondrial gene expression

The expression of a mitochondrial gene largely depends on nuclear-encoded protein factors. It is well known that the mitochondrial gene expression systems differ among species, probably resulting from the adaptation of the host genetic system (Gagliardi et al., 2004). The plant mitochondrial gene is extensively controlled at the post-transcriptional level, involving cis- and trans-splicing, RNA editing, maturation of the transcript termini and RNA stability. The regulatory apparatus is largely unknown in plant mitochondria, in contrast with that of chloroplasts (Barkan and Goldschmidt-Clermont, 2000; Giege and Brennicke, 2001).

Here, we clearly showed that the expression of the orf79 gene is regulated post-transcriptionally in terms of both RNA stability and translation. The quantitative analysis for the atp6-orf79 transcripts revealed that the processing induces destabilization of orf79 transcripts in the fertile line (Figure 6). The processing also seems to reduce the atp6 transcripts in the restorer line (Rf1Rf1). The RNA stability is reported as a major determinant of the steady-state RNA level in plant mitochondria (Giege et al., 2000; Holec et al., 2006). The transcript levels of orf79, as well as atp6, could be controlled under the same mechanism, and the destabilization contributes, to some extent, to the avoidance of the expression of the aberrant gene products. Destabilization of CMS-associated gene transcripts in the fertility restorer line has also been reported in the fertility restorer line of the PET1-type Sunflower CMS. In Sunflower PET1-CMS plants, the atp4-orf522 has been reported as a CMS-associated gene in the mitochondria. The dicistronic atp4-orf522 transcripts are destabilized in the fertile line by polyadenylation (Gagliardi and Leaver, 1999). The addition of the 3’ poly(A) tail increases the stability of cytoplasmic mRNA in eukaryotic cells. In contrast, this process mediates rapid RNA decay in plant mitochondria and chloroplasts (Gagliardi et al., 2001; Kudla et al., 1996). The polyadenylation could be
involved in the destabilization of processed orf79 transcripts. The atp6-orf79 transcripts have the same 3' end as the orf79 transcripts. Selective degradation of the orf79 transcripts might depend on the translational state of the transcripts (see below).

The 50% reduction of processed orf79 transcripts does not solely explain the absence of ORF79 protein in the restorer line (Rf1RF1). Sedimentation analysis revealed that 90% of processed orf79 transcripts, which remained after degradation, were not associated with ribosome for translation (Figure 7). The plant mitochondrial translational system is so far largely unknown, but the three conserved sequence blocks can be found at the 5' untranslated region of several mitochondrial genes (Pring et al., 1992). The functionality of these blocks has not yet been determined, but they are thought to be translational initiation and/or regulation signals. All three blocks are found in the 5' untranslated region (5'-UTR) of atp6, whereas only one of the blocks is found in the 5'-UTR of the processed orf79 transcripts at the position of between −35 and −26 bp from the start codon. The 5'-UTR often contains a translational regulatory element in various organisms, including yeast mitochondria. It is possible to hypothesize that some of these blocks are functional for translation, but the block in the processed orf79 transcripts is not sufficient for translation activation. The unprocessed atp6-orf79 transcripts may be co-translated in the CMS line (rf1rf1) as mammalian mitochondrial RNA. Mammalian mitochondrial RNA does not have 5' leader sequences to facilitate ribosomal binding. All mitochondrial genes are transcribed as a single transcript, and are translated subsequently (Taaman, 1999). In contrast, chloroplast transcripts become active in translation after cleavage of the 5' end. Our results demonstrate a unique feature of translational control of CMS-associated dicistronic genes in plant mitochondria. Further research is necessary to reveal the detailed action for translation of plant mitochondrial transcripts.

The combination of the RNA degradation and translational block diminishes 95% of the production of ORF79 protein from processed orf79 transcripts in the restorer line (Rf1RF1). As mentioned earlier, the successful maturation of pollen seems to depend on a subtle reduction of the accumulation of the ORF79 protein. Therefore, we favor the mechanism in which the suppression at both steps, RNA degradation and translational block, is guaranteed for pollen maturation in the restorer line (Rf1RF1). Several fertility restorer genes have been isolated in various plants, including Petunia and Ogura/Kosena radish (Iwabuchi et al., 1999; Nivison and Hanson, 1989). The mechanism of how the fertility restorer genes reduce the accumulation of CMS-associated protein in mitochondria is still unknown in the aforementioned plants. Further analysis of CMS/Rf systems would give us an opportunity to learn about the relationship between mitochondrial gene expression and nuclear-encoded genes.

The role of PPR protein in the fertility restoration of CMS plants

In this report, we showed that the Rf1 protein directly binds the intergenic region of the atp6-orf79 transcripts to promote RNA cleavage. Plant mitochondrial DNA exhibit extraordinarily large and complex features in comparison with their animal counterparts, because of their larger size (200–2400 kb), multipartite structure and large non-coding regions. The non-coding region comprises sequences derived from chloroplasts and nuclear genomes, as well as considerable sequences of unknown origin containing many freestanding open reading frames (Kubo et al., 2000; Notsu et al., 2002; Unsel et al., 1997). It is widely known that the CMS-associated region often appears to arise from the aberrant recombination of mitochondrial genomes. The PPR proteins have been identified as fertility restorer genes: Rf-PPR592 in Petunia, Rfo/RK in Ogura/Kosena radish and Rf1 in BT-type rice CMS (Akagi et al., 2004; Bentolila et al., 2002; Brown et al., 2003; Koizuka et al., 2003; Komori et al., 2004). Interestingly, it has been reported that there are highly homologous PPR protein genes near each Rf gene (Akagi et al., 2004; Bentolila et al., 2002; Desloire et al., 2003; Koizuka et al., 2003; Komori et al., 2004). The Rf1 gene in rice appears to have arisen from the fusion between two adjacent PPR genes called PPR8-2 and PPR8-3 (Kazama and Toriyama, 2005). In a recent study, the PPR protein gene family derived by duplication was proposed (Rivals et al., 2006). The PPR protein genes might have been duplicated to adapt to the rapid change of mitochondrial genetic organization. Further functional analysis of CMS/Rf systems, as well as other mitochondrial PPR proteins, will provide insight as to how the nuclear–cytoplasmic interaction is maintained and has evolved.

Experimental procedures

Vector construction for transformation

The full-length Rf1 was amplified by PCR using primers 5'-GGATCCACCTCTCCGTATAAGACAACTG-3' and 5'-GGATCCGC-AGCTAAAGATTC-3' (where the underlined letters indicate BamHI sites) and the plasmid Xba 4.0 (Kazama and Toriyama, 2003), which contains the complete open reading frame of Rf1. To fuse the double HA tag and six-repeated His tags to the C-terminal of the Rf1 protein, the PCR fragment was subcloned into the BamHI site of p35S-DHA-His-NosT (Kagaya et al., 2002). The DNA fragments containing tagged Rf1 were subcloned into the BamHI-Sacl site of modified pBl101 (Ariizumi et al., 2002).

Plant materials and introduction of Rf1

Two near-isogenic lines, a CMS line with [cms-bo]rf1rf1 and a restorer line with [cms-bo]Rf1RF1, were used throughout this work (Kazama and Toriyama, 2003). A CMS line with [cms-bo]rf1rf1 was used for transformation following the method of Yoko et al. (1997).
Hygromycin-resistant calli and plants were selected, transplanted to soil in pots and grown in a greenhouse. The integration of the introduced Rf1 was confirmed by northern blot analysis using an atp6-orf79 probe, according to the method used in a previous report (Kazama and Toriyama, 2003). The T1 calli were used in all experiments.

RNA and polysome extraction and analysis

Rice callus was induced in solid N6CI medium containing 2 mg l$^{-1}$ 2,4-dichlorophenoxyacetic acid, 300 mg ml$^{-1}$ casein acid hydrolysate and 10 ml proline from mature rice seeds, and were cultured in a growth chamber under light (55 mol m$^{-2}$sec$^{-1}$) at 30°C (Yokoi et al., 1997). The secondary callus was transferred to a fresh medium every 2 weeks, and 4-day cultured callus was subjected to the mitochondrial isolation as described by Zeltz et al. (1996). The mitochondrial lysate (100 μl) was layered onto 10-ml sucrose gradients that were prepared, centrifuged and fractionated as described by Barkan (1988). RNA was purified from each fraction by Isogen-LS (Nippon Gene, http://www.nippongene.com). For northern blot analysis, RNA samples were fractionated in denaturing formaldehyde gels, transferred to nylon membranes and blotted with a gene-specific probe, as described previously (Hattori et al., 2004). Gene-specific DNA probes were generated by amplification of each gene using the following primers: for N-atp6 and atp6-orf79, 5′-TCTCCCTTCTTAGACACGACG-3′ and 5′-GGATTCCGTGTCATATTGG-3′; for atp6, 5′-CTTGGTCCACC-3′; for 1.5-kb atp6 and 0.5-kb orf79 used in Figure 6 were generated by in vitro transcription from PCR fragments, in which a T7 promoter was incorporated into the 5′ PCR primer. Each PCR fragment was amplified using the following primers: for BD1, 5′-(5′-TAATACGACTCATATAGGGGCGTTGGTTTTTCC-3′); for ori2, 5′-CTTGGTCCACC-3′; for BD4, 5′-(5′-CTTGAATGCGTCTATACGG-3′). In vitro transcription was performed with T7 RNA polymerase (TaKaRa Bio, http://www.takara-bio.com) with 5 mM each of ATP, GTP, CTP and 0.25 mM UTP in the presence of 80 mCi [α-32P]UTP (3000 Ci mmol$^{-1}$). Transcripts were gel purified after electrophoresis in 5% polyacrylamide gel containing 7 M urea, eluted from the gel slice by incubation in 0.5 M CH3COONH4, 1 mM EDTA and 8% glycerol (w/v) at 25°C.

RNA probes used for the binding assay, BD1 and BD4, were generated by in vitro transcription from PCR fragments in which a T7 promoter was incorporated into the 5′ PCR primer. Each PCR fragment was amplified using following primers: for BD1, pBD1F (5′-TAATACGACTCATATAGGGGCGTTGGTTTTTCC-3′)/pBD1R (5′-GACATTCCGCTATACGACGACG-3′); and for BD4, pBD4F (5′-TAATACGACTCATATAGGGGCGTTGGTTTTTCC-3′)/pBD4R (5′-CTTGAATGCGTCTATACGG-3′). In vitro transcription was performed with T7 RNA polymerase (TaKaRa Bio, http://www.takara-bio.com) with 5 μM each of ATP, GTP, CTP and 0.25 mM UTP in the presence of 80 mCi [α-32P]UTP (3000 Ci mmol$^{-1}$). Transcripts were gel purified after electrophoresis in 5% polyacrylamide gel containing 7 M urea, eluted from the gel slice by incubation in 0.5 M CH3COONH4, 1 mM EDTA and 0.2% SDS overnight at 45°C and by ethanol precipitation. RNA were resuspended in deionized water and stored at −30°C.

Electrophoresis mobility shift assays

For EMSA, various fmol quantities of purified Rf1 recombinant protein was incubated with a [α-32P]-labeled RNA probe in 20 μl of 10 mM Tris–HCl, pH 8.0, 30 mM KCl, 6 mM MgCl2, 0.05 mM EDTA, 2 mM DTT and 8% glycerol (w/v) at 25°C for 15 min. For competition studies, the competitor RNAs were pre-incubated with proteins for 5 min before the labeled RNA was added. Samples were then subjected to electrophoresis in 6% (w/v) native polyacrylamide gel (29:1) using Tris-borate-EDTA as the electrophoresis buffer. Gels were dried and imaged with a BAS2000 (Fuji Photo Film, http://www.fujifilm.com). The Kd value was determined from the concentration of protein at which 50% of the RNA probe was bound.

Isolation of mitochondria and mitochondrial RNA

Mitochondria and mitochondrial RNA were isolated from callus as described by Tanaka et al. (2004) and Zeltz et al. (1996), respectively.

Preparation of protein extracts

Total protein of each callus was homogenized in liquid nitrogen and extracted with extraction buffer (100 mM Tris–HCl, pH 8.0, 20 mM NaCl, 5 mM MgCl2, 3 mM DTT, 2 mM EDTA, 1% (w/v) Tween 20, 1% (w/v) Nonidet P-40, 1% (w/v) Triton X-100, protease inhibitor

Expression and purification of recombinant Rf1 protein

To produce the mature protein without mitochondrial signal peptide, the mitOP program was used to define the signal peptide and the cleavage site. The corresponding DNA sequence of the mature protein was amplified by PCR using primers 5′-GGATCCGTCACCTATAGGGGCGTTGGTTTTTCC-3′ and 5′-GGATTCCGTGTCATATTGG-3′ and the plasmid XbaA 4.0 (Kazama and Toriyama, 2003), which contains the complete open reading frame of Rf1. For expression in E. coli, the PCR product was inserted in frame into the pBAD/Thio-TOPO vector (Invitrogen, http://www.invitrogen.com). Thus, the mature protein was fused with thioredoxin (18 kDa) and six His residues at the N and C termini, respectively. Expression and purification of the bacterially produced protein were performed according to the manufacturer’s protocol. Briefly, the LMG194 strain of E. coli containing the vector described above was grown in RM medium (40 mM Na$_2$HPO$_4$, 20 mM KH$_2$PO$_4$, 10 mM NaCl, 20 mM NH$_4$Cl, 2% casamino acid (Difco, BD, http://www.bd.com), 0.2% glucose and 10 mM MgCl$_2$) at 37°C to an OD$_{600}$ of 0.5. Expression was induced by 0.2% arabinose, and the incubation was continued for 2 h at 37°C. Cells were harvested by centrifugation and were lysed in 50 mM Tris–HCl, pH 8.0, 500 mM KCl, 2 mM imidazole, 0.5% Triton X-100, 10 mM MgCl$_2$, 10% glycerol (w/v) and 1 mg ml$^{-1}$ lysozyme. After incubation for 40 min at 4°C and sonication, the soluble proteins were collected by centrifugation at 20 000 g for 15 min. Trx-Rf1 was purified further by binding to nickel-nitrilotriacetic acid agarose resin (Qiagen, http://www.qiagen.com), eluted with imidazole and dialyzed against buffer (20 mM Tris–HCl, pH 8.0, 60 mM KCl, 12.5 mM MgCl$_2$, 0.1 mM EDTA, 15% glycerol).
Production of antibodies

For the production of antibodies, the orf79 open reading frame lacking the 5’ terminal 87-bp region homologous to the coxl (orf79.N) was amplified by PCR using primers 5’-GGAT-CCGCCGCTGGTTGCTTGGCT-3’ (where the underlined letters show the BamHI site) and 3’-GAGCTCGGATATTTGCCTGGTCCACC-3’ (where the underlined letters show the SalI site). This PCR fragment was digested with BamHI and SalI, and was subcloned into the BamHI and SalI sites of pQE30 (Qiagen), such that sequences encoding a 6xHis tag were fused to the 5’ end of the orf79.N. The bacterial expressed protein was purified by affinity chromatography and was then injected into mice (OPERON, http://www.operon.com).

Western blot analysis

Proteins were separated by Tris-Tricine SDS-PAGE using an acrylamide concentration at 10% (w/v), and were electroblotted to polyvinylidene fluoride (PVDF) membranes (Immobilan-P, 0.45 mm; MILLIPORE, http://www.millipore.com). Primary antisera were mouse anti-ORF79 (1:10 000), or anti-6xHis (1:10 000; Sigma-Aldrich, http://www.sigmaaldrich.com). Signals were detected using the ECL Western Blotting Analysis System (GE Healthcare, http://www.gehealthcare.com).

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References


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(Complete Mini; Roche, http://www.roche.com). After centrifugation at 3000 g for 10 min, supernatants were used as total protein. Mitochondrial protein was isolated from the purified mitochondria described above. Purified mitochondria were resuspended with extraction buffer, described above, and the supernatant was used as mitochondrial protein.


