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Fertility restoration by *Ifr1* in rice with BT-type cytoplasmic male sterility is associated with a reduced level, but not processing, of *atp6-orf79* cotranscribed RNA

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Abstract BT-type cytoplasmic male sterility (CMS) in rice is associated with accumulation of unprocessed dicistronic RNA containing a duplicated *atp6* (B-*atp6*) and an unusual open reading frame, *orf79*, encoding a cytotoxic peptide in mitochondria. The male-sterile state of BT-type CMS is stably maintained by backcrossing the plants with line Taichung 65 (T65) that has no restorer gene and is completely suppressed by the presence of the *Rf1* gene through the processing of B-*atp6-orf79* RNA. A variant of the T65 line, T65(T), has a weak restoration function conferred by the *Ifr1* gene, which is genetically independent of the *Rf1* gene. However, little is known about the mechanism(s). In a study to examine whether the mechanism involved in fertility restoration by *Ifr1* is analogous to restoration mediated by *Rf1*, the transcript profile of B-*atp6-orf79* in male-sterile plants was compared to that in fertility restored plants obtained by crossing male-sterile plants with T65(T). The cellular level of unprocessed B-*atp6-orf79* RNA was reduced in the restored plants, but no change in processing efficiency or the quantity of B-*atp6-orf79* DNA was detected. These results suggest that *Ifr1* restores fertility through reducing either the transcription rate of B-*atp6-orf79* or the stability of its primary transcripts, a mechanism distinct from that involved in fertility restoration of BT-type CMS by *Rf1*.

Keywords: cytoplasmic male sterility; fertility restorer; mitochondrial DNA; *Oryza sativa*; RNA processing

Introduction

Cytoplasmic male sterility (CMS), caused by a mitochondrial-nuclear interaction that leads to a maternally inherited failure of affected plants to produce functional pollen (reviewed by Pring and Lonsdale 1985; Newton 1988; Levings and Brown 1989; Hanson 1991), is observed in more than 150 flowering plant species (Laser and Lersten, 1972). In many plant species, male fertility is restored by nuclear-encoded fertility restorer genes referred to as *Rf* (*Restorer of fertility*) or *Fr* (*Fertility restorer*) (Hanson and Bentolila 2004). The CMS/*Rf* system is commercially important for hybrid seed production because it eliminates the need for hand emasculation. Apart from its agronomic importance, CMS provides a clue to the interaction between the mitochondrial genome and the nuclear genome.

CMS is often associated with the presence of unusual open reading frames (ORFs) in the mitochondrial genome. Although these unusual ORFs that cause CMS have diverse structures, some of the mechanisms that restore fertility are common to different CMS/*Rf* systems. With the exception of maize *Rf2*, all restorers so far identified affect either the transcript profile and/or the accumulation of protein produced by the CMS-associated genes in mitochondria (Hanson and Bentolila 2004). In most cases, the protein product of the CMS-determining locus fails to accumulate in the presence of a restorer allele, which is associated with either a decrease in the level of CMS-associated transcripts or in the internal processing events that truncate these transcripts (reviewed by Chase 2007). Restoration of fertility through a reduction in the level of CMS-associated protein without a change in its transcript level (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003) or in the copy number of the mitochondrial subgenomic DNA molecule that encodes the CMS-associated protein (Janska et al. 1998) has also been reported. In addition, *Msh1*, a gene encoded by the nuclear genome, suppresses mitochondrial DNA (mtDNA) rearrangement (Shedge et al.

2007), the silencing of which induces male sterility associated with mtDNA rearrangement (Sandhu et al. 2007).

Genes that account for fertility restoration have been cloned in maize (Cui et al. 1996), petunia (Bentolila et al. 2002), rice (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004), radish (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003), and sorghum (Klein et al. 2005). The maize *Rf2* gene encodes an aldehyde dehydrogenase (Cui et al. 1996), while the rest of these restorer genes encode members of the pentatricopeptide repeat (PPR)-containing protein family (reviewed by Hanson and Bentolila 2004; Chase 2007). PPR proteins are proposed to function as site-specific, RNA-binding adaptor proteins that mediate interactions between RNA substrates and the enzymes that act on the RNA (Small and Peeters 2000; Lurin et al. 2004; Chase 2007; Schmitz-Linneweber and Small 2008). It has also been known that some PPR proteins have catalytic functions in chloroplast (Nakamura and Sugita 2008; Okuda et al. 2009).

The BT-type (also called *cms-bo*-type) CMS is the most fully characterized system of CMS in rice. In BT-type CMS, the cytoplasm ([*cms-bo*] cytoplasm) derived from rice line Chinsurah Boro II causes male sterility when combined with the nucleus from rice line Taichung 65 (abbreviated as T65), that carries no restorer gene (Shinjyo, 1969). In the mitochondrial genome of Chinsurah Boro II, there is an unusual chimeric sequence called *orf79*, which comprises a small portion of the *cox1* gene, and a sequence of unknown origin (Iwabuchi et al. 1993; Akagi et al. 1994), which encodes a cytotoxic peptide (Wang et al. 2006). The *orf79* is located downstream of a duplicated copy of the *atp6* gene called B-*atp6*, which is present in the mitochondrial genome of [*cms-bo*] cytoplasm in addition to the other copy of the *atp6* gene called N-*atp6*. The B-*atp6* gene is cotranscribed with *orf79* and produces a 2.0-kb RNA (B-*atp6-orf79* RNA), and accumulation of this RNA molecule is associated with CMS (Iwabuchi et al. 1993; Akagi et al. 1994).

Fertility restoration of BT-type CMS is controlled by a nuclear locus, *Rf1* (Shinjyo 1984). The *Rf1* gene also restores fertility against LD-type CMS, which involves a cytoplasm containing a B-*atp6-orf79* variant (Itabashi et al. 2009). The *Rf1* gene has been cloned and shown to encode a PPR-containing protein that promotes the processing of B-*atp6-orf79* RNA in mitochondria (Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004). A recent study indicated that the *Rf1* locus is a complex locus comprising two *Rf* genes, *Rf1a* and *Rf1b* (Wang et al. 2006). In the presence of the *Rf1a* gene, the cotranscribed 2.0-kb B-*atp6-orf79* RNA is processed into 1.5-kb RNA encoding ATP6 and 0.45-kb RNA encoding ORF79 by endonucleolytic cleavage at a site(s) located between the coding sequences of B-*atp6* and *orf79* (Iwabuchi et al. 1993; Akagi et al. 1994; Wang et al. 2006). Alternatively, the B-*atp6-orf79* RNA is degraded in the presence of the *Rf1b* gene (Wang et al. 2006). When the *Rf1a* and *Rf1b* genes are both present, the *Rf1a* gene has an epistatic effect over the *Rf1b* gene: the primary B-*atp6-orf79* transcript is processed by the function of RF1A, but the RNA fragments generated by the processing are not degraded by the function of RF1B. It is hypothesized that the inability of RF1B protein to destabilize the RNA fragments processed in the presence of RF1A protein is due to the loss of the recognition sequence necessary for RF1B-dependent RNA degradation (Wang et al. 2006). It has also been reported that the processed RNAs are partially degraded, and the processed *orf79* RNA, which remained after degradation, is not associated with the ribosome for translation, while the unprocessed B-*atp6-orf79* RNA is translatable (Kazama et al. 2008). Thus, the accumulation of unprocessed B-*atp6-orf79* RNA is crucial for BT-type CMS.

The male-sterile state of BT-type CMS was originally reported to be maintained stably by backcrossing the male-sterile plants with the T65 line (Shinjyo 1969). T65 was established from a cross between two Japanese strains in 1923, but has diverged into two subtypes T65(R) and T65(T) (also referred to as T65A and T65B, respectively [Sano et al. 1992]), which have slightly different responses to photoperiod

(Tsai 1986, 1993). Interestingly, while T65(R) confers no ability to restore fertility against BT-type CMS, T65(T) confers a weak restoration function (Sano et al. 1992). Self-fertilization of plants obtained by a cross between the CMS line with the genotype [*cms-bo*]*rf1*/*rf1*, a homozygote of the *rf1* allele carrying the [*cms-bo*] cytoplasm, and T65(T) resulted in an increase in the extent of fertility, and the degree of fertility gradually increased with repeated self-fertilization (Sano et al. 1992). As a consequence, almost fully fertile plants were obtained in the F_6 generation. The partial fertility (approximately 8% seed set) observed in the F_1 plants was maintained by backcrossing T65(T) with the F_1 plants.

A series of genetic experiments were done to characterize the gene(s) responsible for the partial fertility restoration. In brief, [*cms-bo*]*rf1*/*rf1* × [T65(R) × T65(T)] F_1 resulted in 1:1 segregation of completely sterile and partially fertile plants; [*cms-bo*]*rf1*/*rf1* × (T65(T) × [*cms-bo*]*Rf1*/*Rf1*) F_1 resulted in 1:1:2 segregation of completely sterile, partially sterile, and fully fertile plants, respectively. Based on these results, the gene responsible for fertility restoration was considered to be a single dominant gene conferred by T65(T) and to be genetically independent of the *Rf1* gene (Sano et al. 1992). This restorer gene was designated *Instability of fertility restoration 1* (*Ifr1*; Sano et al. 1992). However, no attempt to address the mechanism of fertility restoration by *Ifr1* has been reported. Whether mechanisms analogous to those mediated by *Rf1* are involved in the fertility restoration by *Ifr1* is not even known.

In this study to understand the mechanisms of partial fertility restoration by the *Ifr1* gene, we carried out a comparative analysis that focused on the transcript profile of B-*atp6-orf79* using [*cms-bo*]*rf1*/*rf1* plants and fertility restored plants obtained by crossing [*cms-bo*]*rf1*/*rf1* plants with T65(T) carrying the *Ifr1* gene. The results indicated that fertility restoration by *Ifr1* was correlated with a decrease in the unprocessed B-*atp6-orf79* transcript, but via a mechanism that differed from that involved in endonucleolytic cleavage of the transcript, which is known to be caused by the *Rf1*

locus.

Materials and methods

Plant materials

Four rice (*Oryza sativa* L.) lines, [*cms-bo*]*Rf1*/*Rf1*, [*cms-bo*]*rf1*/*rf1*, T65(R), and T65(T), were used. The [*cms-bo*]*Rf1*/*Rf1* line has the [*cms-bo*] cytoplasm derived from Chinsurah Boro II and its nuclear restoring gene *Rf1*, which were introduced into the T65(R) line from Chinsurah Boro II by successive backcrosses. A completely male-sterile line [*cms-bo*]*rf1*/*rf1* was made by backcrossing the [*cms-bo*]*Rf1*/*Rf1* × T65(R) F_1 plants with T65(R). The [*cms-bo*]*rf1*/*rf1* line was maintained by crossing with T65(R). Plants having partial fertility as a consequence of crossing between the male-sterile [*cms-bo*]*rf1*/*rf1* line and the T65(T) line were also used. These include the BC_7F_1 plants derived from backcrossing [*cms-bo*]*rf1*/*rf1* × T65(T) F_1 plants with T65(T), and F_3 and F_9 selfed progenies of the BC_7F_1 plants. For the F_3 generation, two plant populations (F_3-1 and F_3-2), which were independently derived from the BC_7F_1 plants, were used.

Analysis of pollen fertility

Pollen grains were harvested from spikelets a day before anthesis and stained with iodine-potassium iodide (I_2-KI) solution (Nelson 1962). For each plant line, pollen grains from 12-18 anthers of 2-3 spikelets were analyzed. Pollen fertility was estimated based on the frequency of stainable pollen grains.

DNA extraction and Southern blot analysis

Total DNA was extracted from leaf tissues using Nucleon Phytopure (Amersham Bioscience) according to the manufacturer's instructions. Total DNA (3 μg) digested with *Eco*RI was fractionated by electrophoresis on a 0.9% agarose gel. DNAs were transferred to nylon membranes and allowed to hybridize with a labeled probe. Hybridization, labeling of probes, washing of membranes after hybridization and detection of signals were carried out using AlkPhos Direct Labeling and Detection System (Amersham). The coding region of the *atp6* gene was amplified by PCR using specific primers atp6F1 (5′-CTGAGGGGTAGAATTTGAATTCC-3′) and atp6R1 (5′-TAAGGGACCAAGATCTCCTATGA-3′) and used as a probe.

DNA sequence analysis

The nucleotide sequences covering the entire coding regions of the *Rf1a* and *Rf1b* genes were amplified from total DNA by PCR using specific primers designed to anneal to the 5′- and 3′-untranslated regions. The analyzed sequences contained the 782-bp 5′-upstream region and 1029-bp 3′-downstream region of the *Rf1a* gene, and the 45-bp 5′ upstream region and 449-bp 3′ downstream region of the *Rf1b* gene, in addition to the coding regions of these genes. The following primer pairs were used for PCR: for *Rf1a*, rf1F1 (5′-CTTGGGAGATAGGATATGGAGAGAGAA-3′) and rf1R1 (5′-AAACATCTTTGGAACGCTTCTTGAG-3′); and for *Rf1b*, rf1bF1 (5′-AAAAACATGCCACTCGTAGCTAGAAAAA-3′) and rf1bR1 (5′-GGGGAATCTTTGTCACTGCAAGTTTAGC-3′). After confirmation of the amplification of a single DNA species by gel electrophoresis, the PCR products were purified using Get *pure* DNA Kit-Agarose (Dojindo), then the purified fragments were sequenced using BigDye Terminator Cycle Sequencing v1.1 Ready Reaction Kit

(Applied Biosystems) and a DNA sequencing system (ABI 3130, Applied Biosystems). DNA sequences were analyzed using the program ATGC ver. 4 (Genetyx Co.).

RNA extraction and cDNA synthesis

Total RNA from leaves of three-month-old plants and mature anthers was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions, with the modification that we removed the genomic DNA from the RNA fraction using DNase I (Takara Bio). Total RNA (0.5 μg) was used as the template for cDNA synthesis. The cDNA synthesis reaction mixture was prepared by mixing 2 μl of 5× reaction buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl, 15 mM $MgCl_2$), 1 μl of 0.1 M dithiothreitol (DTT), 0.25 μl RNaseOUT inhibitor (Invitrogen), 1 μl of 50 μM random 9-mer primer (TaKaRa Bio), 2 μl of 2.5 mM dNTPs, the RNA solution, and water, to a final volume of 9.5 μl. After the addition of 0.5 μl of M-MLV reverse transcriptase (Invitrogen), cDNA synthesis was done at 42°C for 1 h. The reverse transcriptase was inactivated by heating the sample at 99°C for 1 min.

Quantitative analyses of DNA and RNA by real-time PCR

For analyzing changes in the copy number of mitochondrial genes, we ran a quantitative PCR using 5 ng of total DNA and SYBR Premix ExTaq Perfect Real Time (TaKaRa Bio) with the DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). The amount of the *actin* gene, a single copy gene in the rice nuclear genome, was used as a control for the analysis. The PCR conditions were: 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 20 s, and 78°C for 2 s. Fluorescence was quantified before and after the incubation at 78°C to monitor the formation of primer-dimers. The following primer pairs were used for the PCR: for the total N-*atp6* and B-*atp6* genes, atp6F3

(5′-CGAGGCCAGTTGAGATCAGT-3′) and atp6R3 (5′-GCCCTCTCAAATGGTTCAAA-3′); for the B-*atp6-orf79* genes, 2.0F1 (5′-AGGAGCCGAAGATTTTAGGG-3′) and 2.0R2 (5′-TTAGTCCCTCGGGTAGTGGA-3′); for the *cox2* gene, cox2F2 (5′-TAGGATCTCAAGACGCTGCAAC-3′) and cox2R2 (5′-CGATAGTAGTTCCATGAACAATCC-3′); for the *cob* gene, cobF2 (5′-CTACCGATCCATGCCATTCT-3′) and cobR2 (5′-GTTGACATCCGATCCAACCT-3′); and for the *actin* gene, OsRAc1-F4 (5′-TGGTGGTACCACTATGTTCCCT-3′) and OsRAc1-R4 (5′-ACAATGGATGGGCCAGACTC-3′).

For analyzing changes in the transcript levels of mitochondrial and nuclear genes, we ran a quantitative reverse transcription-mediated PCR (RT-PCR) using a 1-μl sample of the reaction mixture of cDNA synthesis. The mRNA level of the *actin* gene was used as a control for the analysis. The PCR was done as described. A reaction mixture without reverse transcriptase was used as a control to confirm that no amplification occurred from genomic DNA contamination of the RNA sample. The lack of amplification from such samples was examined by both gel electrophoresis of PCR products and real-time PCR. The following primer pairs were used for the PCR: for the *Rf1a* gene, Rf1a-RTF7 (5′-ATTAGATCCAAAACAATTTATAGG-3′) and Rf1a-RTR7 (5′-ATAAGATAATCAGATCAGAAACC-3′); for the *Rf1b* gene, Rf1b-RTF1 (5′-GCTGGGGTTTACATGTCCAA-3′) and Rf1b-RTR1 (5′-ATTCAGTGAGAAGGCCAACA-3′); for the *orf79* gene (both processed and unprocessed transcripts of the B-*atp6-orf79* genes), 0.45F3 (5′-TCTGGTCCGATGGCTCTTCTCC-3′) and 0.45R3 (5′-CCCACCACGAATAGTCAACC-3′); and for the unprocessed transcript of the B-*atp6-orf79* genes, primers 2.0F1 and 2.0R1. Primers specific for the *Rf1a* and *Rf1b* genes were designed by aligning nucleotide sequences of the 10 PPR-containing genes

at the chromosomal region of *Rf1* with the CLUSTAL W Multiple Sequence Alignment Program version 1.8 (http://clustalw.genome.jp) (Thompson et al. 1994), and differences were detected. For the total N-*atp6* and B-*atp6*, *cox2*, *cob*, and *actin* genes, the same primers used for quantitative analysis of DNA were used. In all PCR experiments, amplification of a single DNA species was confirmed by both melting curve analysis of real-time PCR and gel electrophoresis of PCR products. The data were obtained from three biological replicates except for the RT-PCR analysis of F_9 plants for which we ran two replicates. Differences between plants were statistically analyzed using the Fisher's protected least significant difference (PLSD) test.

Results

Effects of the *Ifr1* gene on pollen fertility

To examine the effects of the *Ifr1* gene on pollen fertility, we harvested pollen grains before anthesis and treated them with I_2-KI. The ratio of stainable pollen grains produced in [*cms-bo*]*Rf1*/*Rf1* and [*cms-bo*]*rf1*/*rf1* plants was almost 100% and 0%, respectively (Fig. 1g, h; Table 1) as reported previously (Itabashi et al. 2009). In contrast, both stainable and non-stainable pollen grains were produced in plants carrying the *Ifr1* gene, namely, BC_7F_1, F_3-1, F_3-2 and F_9 plants (Fig. 1i-l; Table 1). In addition, a considerable number of stainable pollen grains were only partially stained and only a small number of pollen grains were completely stained in these *Ifr1* carriers (Fig. 1m-o). These results are consistent with a previous observation that *Ifr1* carriers have partial seed fertility (Sano et al. 1992). No differences were detected in the morphology of flower organs before anthesis between these plants (Fig. 1a-f).

The *Rf1a* and *Rf1b* genes of the T65(T) line and the fertility restored plants are identical to those of the T65(R) line

The BT-type CMS can be restored gametophytically by either of two closely linked genes at the *Rf1* locus, *Rf1a* or *Rf1b* (Wang et al. 2006). Previous genetic analysis indicated that the T65(T) line carries the *rf1* allele (namely, *rf1a* and *rf1b* alleles) and that the effect of partial fertility restoration conferred by the *Ifr1* gene in the T65(T) line is independent of the *Rf1* locus (Sano et al. 1992). To examine whether the functions of the *Rf1a* and *Rf1b* genes are involved in the process of partial fertility restoration conferred by the genetically-independent *Ifr1* gene, we analyzed the nucleotide sequences of the *Rf1a* and *Rf1b* genes from the T65(T) line and fertility restored plants. For a comparison, the nucleotide sequences of the same genes in the T65(R) line, which carries the *rf1* allele and does not induces fertility restoration, were also analyzed. The nucleotide sequences of both the *Rf1a* (from 782-bp upstream of the initiation codon to 1029-bp downstream of the stop codon) and *Rf1b* (from 45-bp upstream of the initiation codon to 449-bp downstream of the stop codon) genes of T65(T) and F_9 generation of the fertility restored plants were completely identical to those of the T65(R) line (data not shown; the nucleotide sequence data have been deposited in the DDBJ/EMBL/GanBank database under accession numbers AB470406-AB470409). Therefore, the presence and absence of the fertility restoration ability in T65(T) and T65(R), respectively, were not ascribed to a difference in the nucleotide sequences of the *Rf1a* and *Rf1b* genes.

The nucleotide sequence of the *Rf1a* gene of these plants had a premature stop codon as reported previously for the *rf1* allele from other rice lines (Kazama et al. 2003; Akagi et al. 2004; Komori et al. 2004; Wang et al. 2006), indicating that the gene encodes a nonfunctional protein. For the *Rf1b* gene, a previous analysis indicated that a base substitution resulting in an amino acid change was correlated with the loss of

fertility-restoration function: A and G residues at nucleotide position 1235 of the coding region of the *Rf1b* gene were commonly detected in various rice lines carrying the *Rf1b* and *rf1b* alleles, respectively (Wang et al. 2006). Our analysis indicated that T65(R), T65(T), and fertility restored plants had a G residue at position 1235.

The mRNA levels of *Rf1a* and *Rf1b* genes are not changed in the fertility restored plants

Although the nucleotide sequences of the *Rf1a* and *Rf1b* genes of T65(T) and the F_9 plant were completely identical to those of T65(R) and these genes encode proteins that are correlated with recessive genotypes, whether an increase in the level of the expression of these genes might occur and result in partial fertility restoration had not been known. To examine such a possibility, we analyzed the mRNA levels of the *Rf1a* and *Rf1b* genes in anther tissues by quantitative RT-PCR. No significant difference in the mRNA level of the *Rf1a* gene was detected between the fertility restored plants, namely, BC_7F_1, F_3-1, F_3-2 and F_9 plants, and the CMS line carrying the *rf1* allele derived from the T65(R) line (Fig. 2a). This analysis also revealed that the mRNA levels of the *Rf1a* gene in these plants were approximately 50 times lower than that in plants carrying the dominant *Rf1* allele (Fig. 2a). The lower mRNA level can be due to the destabilization of mRNA caused by a base substitution that led to premature termination of translation, which is known as nonsense-mediated mRNA decay (reviewed by Maquat, 2004). The mRNA level for the *Rf1b* gene did not differ significantly among any of the plants analyzed (Fig. 2b). These results indicate that the partial restoration of fertility conferred by the *Ifr1* gene of T65(T) can be attributed to neither a change in nucleotide sequences of the *Rf1a* or *Rf1b* genes nor a change in the mRNA level of these genes.

The DNA amount of B-*atp6-orf79* in the fertility restored plants is unchanged

In common bean, a decrease in the copy number of a mitochondrial subgenomic DNA molecule, on which the *pvs-orf239* genes responsible for CMS is located, is associated with restoration from the CMS phenotype by the fertility restorer gene in the nuclear genome (Janska et al. 1998). To examine whether the observed reduction in the level of unprocessed B-*atp6-orf79* transcript in the fertility restored plants is accompanied by a change in the amount of mtDNA molecule on which the B-*atp6-orf79* genes are located, we ran a Southern blot analysis and real-time PCR analysis. The mtDNA fragments that contain the N-*atp6* and B-*atp6-orf79* genes could be distinguished by Southern blot analysis of the *Eco*RI-digested leaf DNA with a probe specific to the *atp6* gene: the probe hybridized 2.1-kb and 1.5-kb fragments containing the N-*atp6* and B-*atp6-orf79* genes, respectively, as reported previously (Kadowaki and Harada 1989; Kadowaki et al. 1990; Iwabuchi et al. 1993). The intensity of the hybridization signals of N-*atp6* and B-*atp6* appeared very similar to each other, and no changes in relative intensities between these signals were detected between CMS line and any of the restored plants (data not shown).

The amount of genes located on the mitochondrial genome was also analyzed by quantitative PCR using the *actin* gene, a single copy gene encoded by the nuclear genome, as an internal control. No significant differences in the amount of B-*atp6-orf79*, total *atp6*, *cox2*, and *cob* genes were detected between the CMS line and the restored plants in leaf tissues (Fig. 3). In anther tissues, differences were detected only between limited plants: between F_3-1 and F_3-2 plants in the amount of total *atp6*; between [*cms-bo*]*Rf1*/*Rf1* and F_3-1 plants and between F_3-1 and F_3-2 plants in the amount of *cox2*. There was no difference in the amount of B-*atp6-orf79* and *cob* between any of these plants in anther tissues. These results indicate that no quantitative change associated with the fertility restoration has occurred in the mtDNA.

The processing efficiency of the B-*atp6-orf79* transcript in fertility restored plants was as low as that in the CMS line

To examine whether the B-*atp6-orf79* primary transcript is cleaved at a site(s) between B-*atp6* and *orf79* in fertility restored plants obtained by crossing [*cms-bo*]*rf1*/*rf1* plants with T65(T) carrying the *Ifr1* gene, we performed quantitative RT-PCR. Two primer sets were designed, one amplifying the *orf79* region from both processed and unprocessed RNA, the other amplifying the region that encompasses the processing site from the RNA that remained unprocessed (Fig. 4a). The processing efficiency of the transcript of B-*atp6-orf79* was calculated by the relative amount of RNA containing the processing site (corresponding to unprocessed B-*atp6-orf79* RNA) vs. the *orf79* RNA.

The relative amounts of unprocessed RNA vs. *orf79* RNA in both the leaf and anther tissues were lower in the [*cms-bo*]*Rf1*/*Rf1* line than in the [*cms-bo*]*rf1*/*rf1* line (Fig. 4b). These results indicate that processing at the site occurred more efficiently in the [*cms-bo*]*Rf1*/*Rf1* line, as reported previously based on a Northern blot analysis (Iwabuchi et al. 1993). The relative amounts of unprocessed RNA vs. *orf79* transcripts in the fertility restored plants, BC_7F_1, F_3-1, F_3-2 and F_9, were similar to those in the [*cms-bo*]*rf1*/*rf1* line, and a statistically significant difference was detected only between the [*cms-bo*]*rf1*/*rf1* line and F_3-1 plants in anther tissues, for which the ratio was rather higher in the F_3-1 (Fig. 4b). These results indicate that an increase in the efficiency of the processing of the B-*atp6-orf79* primary transcripts was not involved in the restoration of fertility in the restored plants.

The cellular level of unprocessed RNA is reduced in the fertility restored plants

Although no increase in the efficiency of RNA processing was detected, there still remained a possibility that the absolute level of unprocessed B-*atp6-orf79* RNA per cell

might have changed in the restored plants. Therefore, the mean level of unprocessed RNA per cell was evaluated using the ratio of the level of unprocessed B-*atp6-orf79* RNA to the mRNA level of the *actin* gene encoded in the nuclear genome.

The relative amount of unprocessed B-*atp6-orf79* RNA vs. *actin* mRNA in [*cms-bo*]*Rf1*/*Rf1* plants was lower than that in [*cms-bo*]*rf1*/*rf1* plants as expected (Fig. 5a). In fertility restored plants, BC_7F_1, F_3-1, F_3-2 and F_9, the relative amounts of unprocessed B-*atp6-orf79* RNA vs. *actin* mRNA were also significantly lower than those in [*cms-bo*]*rf1*/*rf1* in both leaf and anther tissues (Fig. 5a). Similarly, the relative amounts of *orf79* RNA vs. *actin* mRNA in fertility restored plants were lower than those in [*cms-bo*]*rf1*/*rf1* plants (data not shown). These results indicate that the fertility restoration by *Rf1* and that by *Ifr1* were both correlated with a decrease in the level of unprocessed B-*atp6-orf79* transcript, although the efficient processing of the B-*atp6-orf79* primary transcript was involved in the fertility restoration by *Rf1* but not in that by *Ifr1* (Fig. 4b). The total levels of N-*atp6* and B-*atp6* RNA in the [*cms-bo*]*rf1*/*rf1* and the fertility restored plants did not differ significantly (Fig. 5b), indicating that the decrease in the level of unprocessed B-*atp6-orf79* RNA in the presence of *Ifr1* was not accompanied by a change in the total level of *atp6* RNA.

The transcript levels of two unlinked genes, *cox2* and *cob*, were also analyzed. No significant difference in the level of *cox2* was detected in anther tissues between [*cms-bo*]*rf1*/*rf1* and any of the fertility restored plants, although differences in the leaf tissues were detected between [*cms-bo*]*rf1*/*rf1* and BC_7F_1, F_3-2, and F_9 plants (Fig. 5c). Similarly, no significant difference in the level of *cob* was detected between [*cms-bo*]*rf1*/*rf1* and fertility restored plants except for between anther tissues of [*cms-bo*]*rf1*/*rf1* and F_3-2 plants (Fig. 5d). The cause of the difference between fertility restored plants is not known at present. It might reflect slight fluctuation of gene expression level as a consequence of hybridization between different plant lines and/or subsequent self-fertilization. In any case, these results suggest that changes in the

transcript levels of *atp6*, *cox2*, and *cob* are not necessary to restore fertility in the presence of *Ifr1*, while changes in the level of unprocessed B-*atp6-orf79* transcript are unequivocally correlated with the process.

Discussion

CMS has been attributed to chimeric ORFs present in the mitochondrial genomes in many plants (Hanson and Bentolila 2004). The unique proteins encoded by these ORFs were associated with the dysfunction of the mitochondria as well as the consequent male-sterility. In the BT-type CMS of rice, the chimeric gene *orf79* is present downstream of B-*atp6* and is thought to cause male-sterility due to the toxicity of the encoded protein. A high level of unprocessed B-*atp6-orf79* RNA is associated with the CMS phenotype (Iwabuchi et al. 1993; Akagi et al. 1994), and the restoration of fertility was associated with a decrease in the level of unprocessed B-*atp6-orf79* RNA through a mechanism involving the processing or degradation of the transcript (Iwabuchi et al. 1993; Akagi et al. 1994; Wang et al. 2006). A previous report also indicated that overexpression of *orf79,* which is fused to the nucleotide sequence encoding the mitochondrial transit peptide, caused a decrease in the extent of male fertility (Wang et al. 2006). Accordingly, the decrease in the level of unprocessed RNA in the fertility restored plants observed in this study could account for their partial restoration of fertility. The fact that unprocessed B-*atp6-orf79* transcript levels in all the *Ifr1* carriers were higher and lower than those in *Rf1* and *rf1* homozygotes, respectively, in anther tissues (Fig. 5a) is consistent with the partial restoration by *Ifr1*.

Because there was no difference in the level of unprocessed B-*atp6-orf79* RNA relative to *orf79* RNA between the CMS plants and the fertility restored plants, the

possibility that *Rf1a*, whose effect on the B-*atp6-orf79* locus is to cleave the transcript at an internal position between B-*atp6* and *orf79*, is involved in the fertility restoration by *Ifr1* should be excluded. The nucleotide sequence of the *Rf1a* gene in T65(T) and in a fertility restored plant (F_9) was identical to that of the maintainer line T65(R) and contained a premature stop codon, which confers no fertility restoration to CMS plants. In addition, the mRNA level of *Rf1a* in the fertility restored plants was as low as that in the [*cms-bo*]*rf1*/*rf1* plants. These facts are consistent with the notion that *Rf1a* is not the factor involved in the fertility restoration by *Ifr1*. Similarly, the present results indicated that neither a change in nucleotide sequence nor a change in the mRNA level of *Rf1b* accounts for the fertility restoration by *Ifr1*. The remaining possibility for the involvement of *Rf1b* in this phenomenon may be that the level of RF1B protein and/or its activity is modulated by the presence of *Ifr1*, which might result in the reduction of unprocessed B-*atp6-orf79* transcript. It is also possible that other PPR protein gene(s) in the region adjacent to the *Rf1a* or *Rf1b* genes or in the other regions of the genome is activated by *Ifr1*, although the functions of these genes are uncharacterized. It is not likely that the *Ifr1* gene is involved in the regulation of mtDNA copy number and/or rearrangement that results in quantitative changes in mtDNA because no difference in the copy number of mitochondrial genes was detected between the CMS line and fertility restored plants. Overall, the decrease in the level of unprocessed RNA per cell without any change in the efficiency of RNA processing or the quantity of mtDNA suggests that either transcription rate or stability of the transcripts of B-*atp6-orf79* was reduced in the restored plants. The *Ifr1* gene is likely to encode a novel protein that brings about such changes in mitochondria directly or indirectly.

A gradual increase in the extent of fertility with an increase in the number of self-fertilizations of the hybrids between [*cms-bo*]*rf1*/*rf1* and T65(T) was detected previously (Sano et al. 1992). In addition, the high level of fertility observed in the F_{10} generation reverted to a low level through recurrent backcrossing with T65(T) plants

(Sano et al. 1992). Although the present results indicated that the fertility restoration by *Ifr1* was correlated with a decrease in the unprocessed B-*atp6-orf79* transcript, no prominent difference in the level of the unprocessed RNA was detected between different generations of the fertility restored plants. In this context, it is tempting to speculate that certain epigenetic changes in the nuclear-encoded gene(s) that slightly affect fertility might accumulate with recurrent self-fertilization. In addition, although the frequency of stainable pollen grains in the *Ifr1* carriers was higher than that in [*cms-bo*]*rf1*/*rf1* plants, no increase in stainable pollen grains was observed with an increase in the number of self-fertilizations. Accordingly, we tentatively assume that *Ifr1* may act not only on gametogenesis but also on a process after gametogenesis (e.g., anthesis). Such possibilities, as well as the lack of strong correlation between the increase in the level of fertility and the level of unprocessed B-*atp6-orf79* RNA, still need to be examined.

CMS can function as a mechanism of reproductive isolation in plants (Koide et al. 2008). A previous analysis of the distribution of genes that restore BT-type CMS among various rice strains indicated that 13% of examined strains functioned in partial fertility restoration (Shinjyo 1975) like that conferred by *Ifr1*. A recent report also indicated the presence of rice strains carrying *Rf* genes that confer fertility restoration of various degrees (Tan et al. 2008). It is possible that some of these strains have genes similar to *Ifr1*, although their genetic relationships are unknown. The presence of *Ifr1*, in addition to *Rf1*, indicates that multiple factors are involved in fertility restoration of BT-type CMS and have been harbored redundantly in the rice genome, allowing the maintenance of interfertile relationships during rice evolution.

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Legends of figures

Fig. 1 Flower morphology and pollen grains of fertile, male sterile, and fertility restored rice plants. **a-f** Morphology of flower organs before anthesis. Flowers of [*cms-bo*]*Rf1/Rf1*, [*cms-bo*]*rf1/rf1*, BC_7F_1, F_3-1, F_3-2, and F_9 plants are shown. A scale bar denotes 1 mm. **g-l** Population of pollen grains produced in plants listed in panel **a-f**. Pollen grains were harvested from spikelets a day before anthesis and treated with I_2-KI. A scale bar denotes 100 μm. **m-o** Pollen grains with a different degree of staining in *Ifr1* carrier (F_3-2): completely stained pollen (**m**); non-stainable pollen (**n**); partially stained pollens (**o**). A scale bar denotes 40 μm.

Fig. 2 Comparison of the mRNA levels of *Rf1a* and *Rf1b* genes in the CMS line and fertility restored plants. The *Rf1a* (**a**) and *Rf1b* (**b**) mRNA levels relative to the *actin* mRNA level in anther tissues were assessed by quantitative RT-PCR for the following plants: [*cms-bo*]*Rf1/Rf1*, [*cms-bo*]*rf1/rf1*, BC_7F_1, F_3-1, F_3-2, and F_9 plants. The *Rf1a* and *Rf1b* mRNA levels relative to the *actin* mRNA level in [*cms-bo*]*rf1/rf1* plants were assigned a value of 1. Data are means ± SE. Statistic analysis was done using Fisher's PLSD test. Means that are not indicated by the same letter are significantly different ($P < 0.01$).

Fig. 3 Comparison of the amount of B-*atp6-orf79*, total B-*atp6* and N-*atp6*, *cox2*, and *cob* DNA in the CMS line and restored plants. The B-*atp6-orf79* (**a**), total B-*atp6* and N-*atp6* (**b**), *cox2* (**c**), and *cob* (**d**). DNA levels relative to the *actin* DNA level in leaf (black bars) and anther (gray bars) tissues were assessed by quantitative PCR for the following plants: [*cms-bo*]*Rf1/Rf1*, [*cms-bo*]*rf1/rf1*, BC_7F_1, F_3-1, F_3-2, and F_9 plants. The DNA levels of the analyzed genes relative to the *actin* DNA level in [*cms-bo*]*rf1/rf1* plants were assigned a value of 1. Data are means ± SE. Statistic analyses of leaf DNA

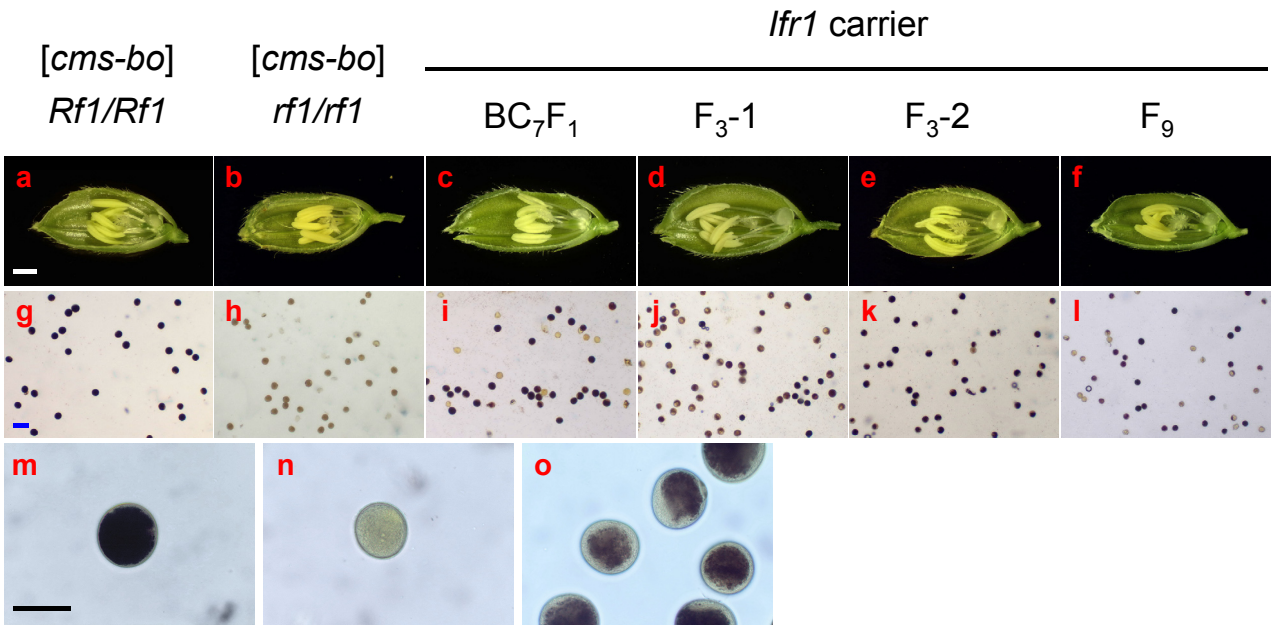
and anther DNA were done independently using Fisher's PLSD test. Means that are not indicated by the same letter are significantly different ($P < 0.05$). Note that there was no significant difference between plants in the levels of any of the genes analyzed in leaf tissues and those of B-*atp6-orf79* and *cob* in anther tissues.

Fig. 4 Analysis of the processing efficiency of B-*atp6-orf79* transcript. **a** Structure of mtDNA region containing B-*atp6* and *orf79*. The coding regions of B-*atp6* and *orf79* are indicated by boxes. The DNA regions common to B-*atp6* and N-*atp6* and present in the B-*atp6-orf79* locus but not in the N-*atp6* locus are indicated by a black line and a broken line, respectively. The site of processing for cleaving the primary transcript is indicated by a vertical triangle. Positions of primers for RT-PCR are indicated by horizontal arrows. PCR using primers 2.0F1 and 2.0R1 amplifies the region encompassing the processing site from unprocessed B-*atp6-orf79* RNA but does not amplify the region from processed RNA. PCR using primers 0.45F3 and 0.45R3 amplifies the *orf79* region from both unprocessed and processed RNA. **b** Comparison of the processing efficiency of B-*atp6-orf79* RNA in the CMS line and restored plants. The levels of unprocessed B-*atp6-orf79* RNA relative to the *orf79* RNA level in leaf (black bars) and anther (gray bars) tissues were assessed by quantitative RT-PCR for the following plants: [*cms-bo*]*Rf1/Rf1*, [*cms-bo*]*rf1/rf1*, BC_7F_1, F_3-1, F_3-2, and F_9 plants. The unprocessed B-*atp6-orf79* RNA level relative to the *orf79* RNA level in [*cms-bo*]*rf1/rf1* plants was assigned a value of 1. Data are means ± SE. Statistic analyses of leaf RNA and anther RNA were done independently using Fisher's PLSD test. Means that are not indicated by the same letter are significantly different ($P < 0.05$).

Fig. 5 Comparison of the mean cellular levels of unprocessed B-*atp6-orf79*, total B-*atp6* and N-*atp6*, *cox2*, and *cob* RNA in the CMS line and restored plants. The

unprocessed B-*atp6-orf79* (**a**), total B-*atp6* and N-*atp6* (**b**), *cox2* (**c**), and *cob* (**d**) RNA levels relative to the *actin* mRNA level in leaf (black bars) and anther (gray bars) tissues were assessed by quantitative RT-PCR for the following plants: [*cms-bo*]*Rf1/Rf1*, [*cms-bo*]*rf1/rf1*, BC_7F_1, F_3-1, F_3-2, and F_9 plants. The RNA levels of the analyzed genes relative to the *actin* mRNA level in [*cms-bo*]*rf1/rf1* plants were assigned a value of 1. Data are means ± SE. Statistic analyses of leaf RNA and anther RNA were done independently using Fisher's PLSD test. Means that are not indicated by the same letter are significantly different ($P < 0.05$) in each panel. Note that the level of unprocessed B-*atp6-orf79* RNA is significantly lower in all restored plants than in [*cms-bo*]*rf1/rf1* in both leaf and anther tissues. There was no significant difference between plants in the levels of *cob* in leaf tissues.

Figure 1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Rf1/Rf1
[cms-bo]
rf1/rf1
lfr1 carrier
BC7F1
F3-1
F3-2
F9
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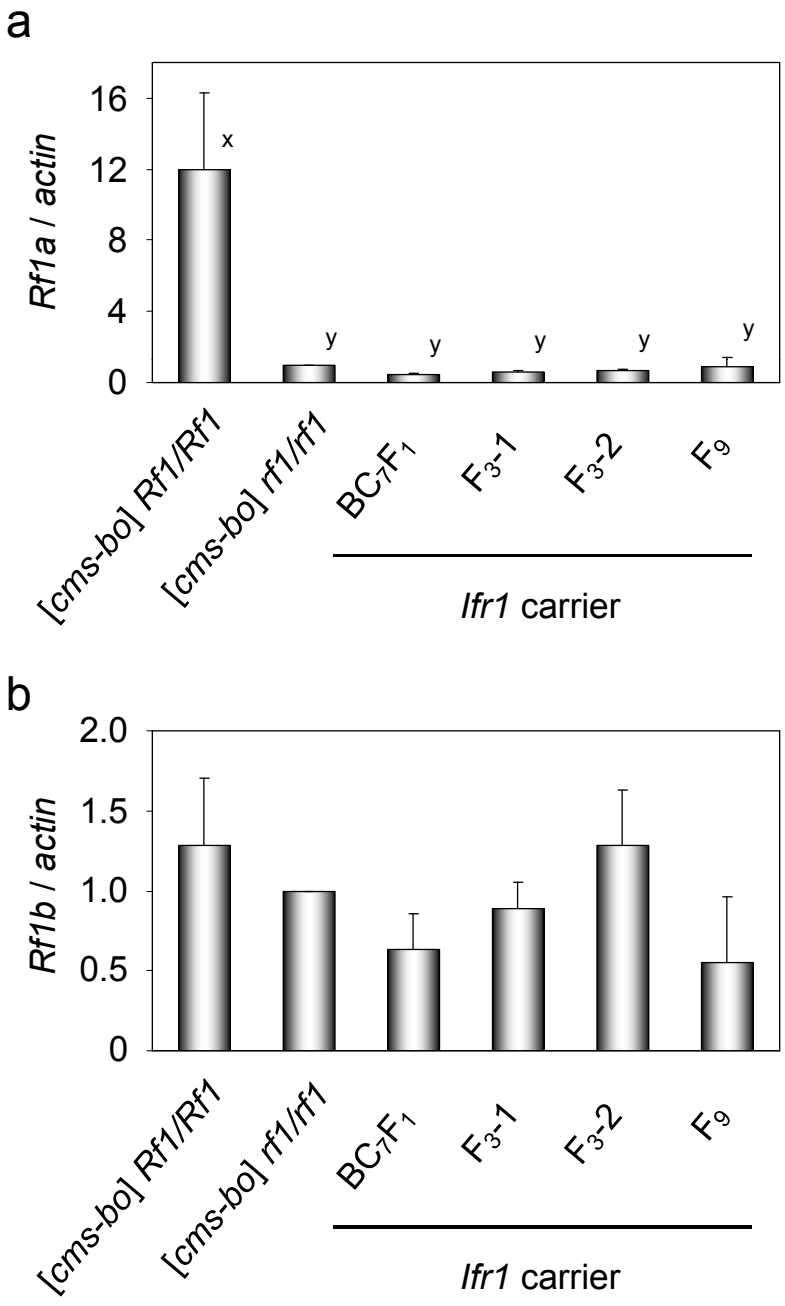
h
i
j
k
l
m
n
o

Figure 2


a
16
12
8
4
0
Rf1a / actin
x
y
y
y
y
y
[cms-bo] Rf1/Rf1
[cms-bo] rf1/rf1
BC7F1
F3-1
F3-2
F9
Ifr1 carrier
b
2.0
1.5
1.0
0.5
0
Rf1b / actin
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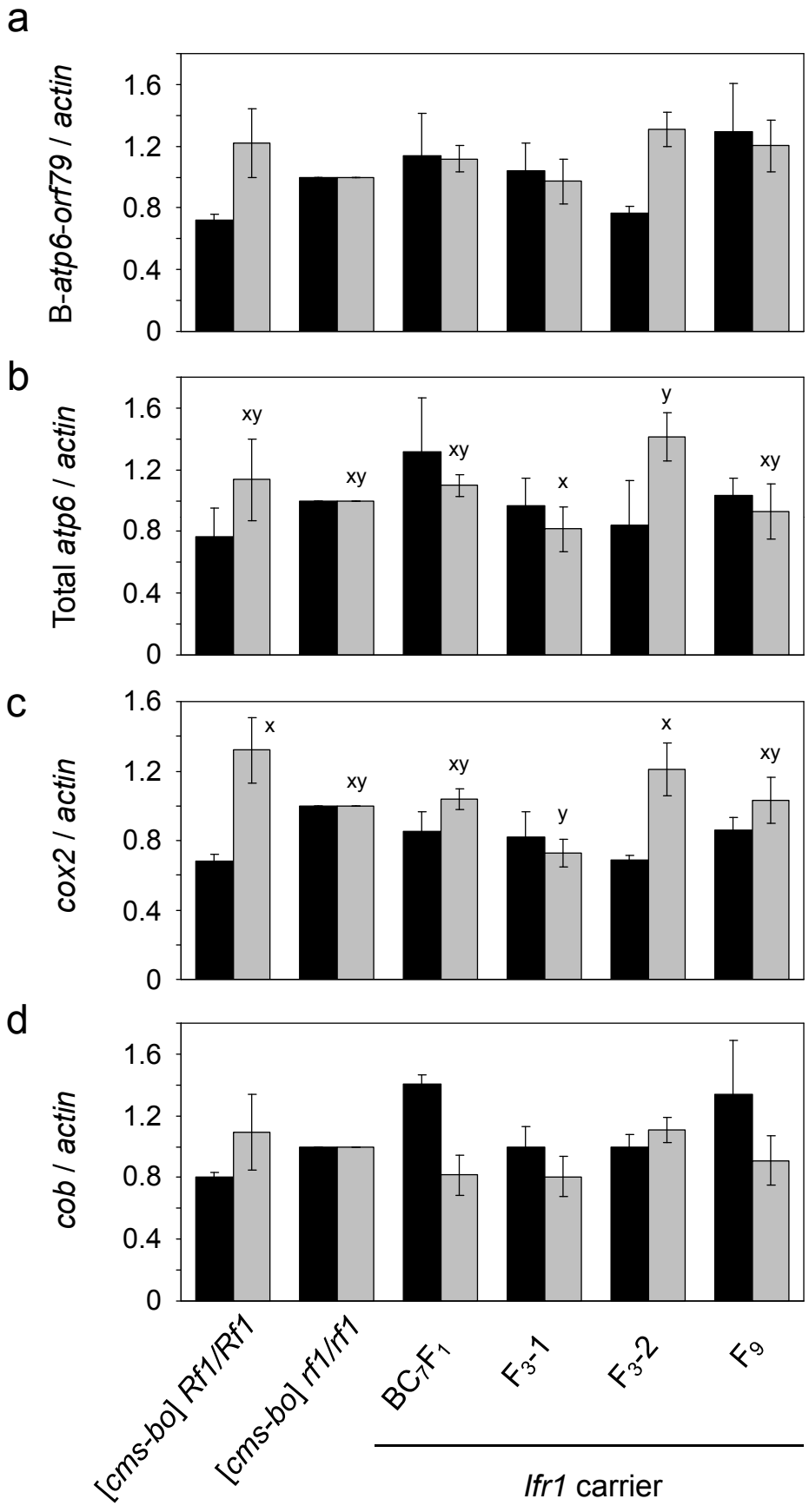
F9
Ifr1 carrier

Figure 3


a
B-atp6-orf79 / actin
b
Total atp6 / actin
c
cox2 / actin
d
cob / actin
1.6
1.2
0.8
0.4
0
xy
x
y
[cms-bo] Rf1/Rf1
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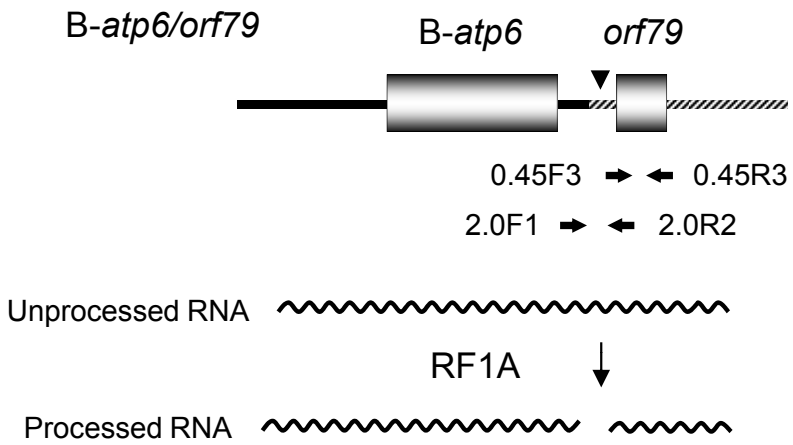
BC7F1
F3-1
F3-2
F9
Ifr1 carrier

Figure 4

a


B-atp6/orf79
B-atp6
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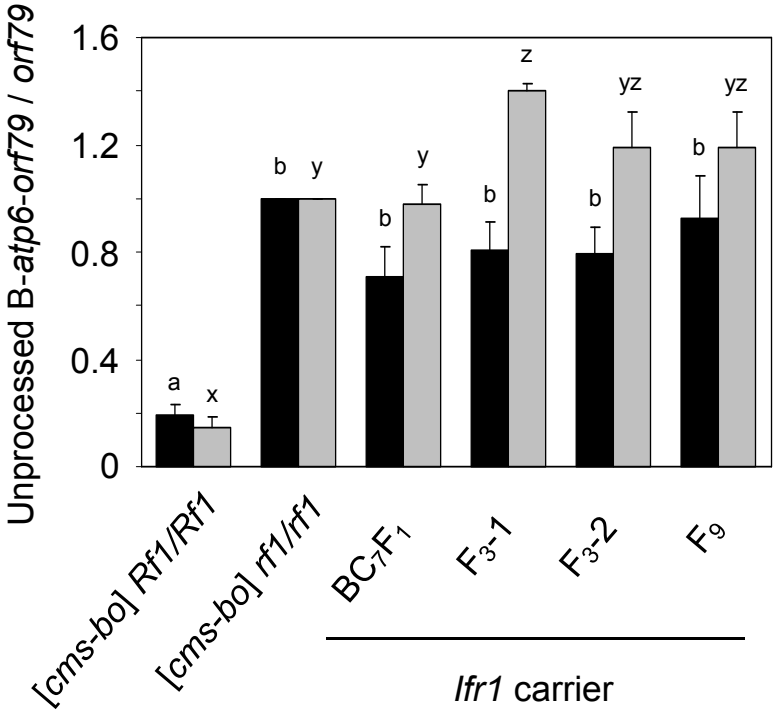
0.45R3
2.0F1
2.0R2
Unprocessed RNA
RF1A
Processed RNA

b


Unprocessed B-atp6-orf79 / orf79
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1.2
0.8
0.4
0
a
x
b
y
b
y
b
z
b
yz
b
yz
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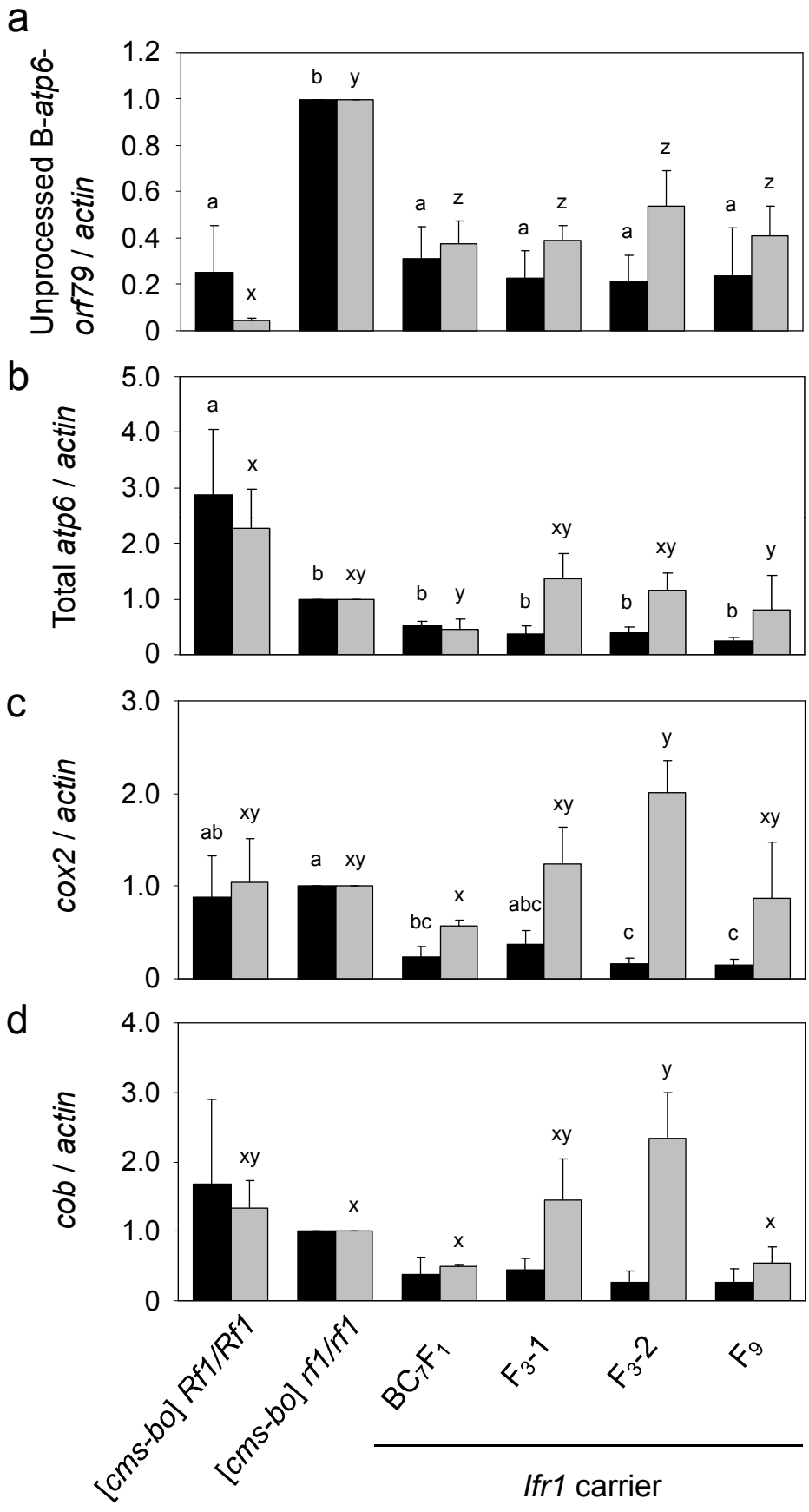
BC7F1
F3-1
F3-2
F9
Ifr1 carrier

Figure 5


a
Unprocessed B-atp6-orf79 / actin
1.2
1.0
0.8
0.6
0.4
0.2
0
a x b y a z a z a z a z
b
Total atp6 / actin
5.0
4.0
3.0
2.0
1.0
0
a x b xy b y b xy b xy b y
c
cox2 / actin
3.0
2.0
1.0
0
ab xy a xy bc x abc xy c y c xy
d
cob / actin
4.0
3.0
2.0
1.0
0
xy x x xy x y x
[cms-bo] Rf1/Rf1
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BC7F1
F3-1
F3-2
F9
Ifr1 carrier