A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice

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Abstract A fertility restorer gene (*Rf-1*) of [*ms-bo*] cytoplasmic male sterility (CMS) in rice has been reported to be responsible for the processing of RNA of aberrant *atp6* of mitochondria. We have carried out map-based cloning of the *Rf-1* gene and found that a 4.7-kb genomic fragment of a restorer line promoted the processing of aberrant *atp6* RNA when introduced into a CMS line. The genomic fragment contained a single open reading frame encoding 18 repeats of the 35 amino acid pentatricopeptide repeat (PPR) motif. The cloned PPR gene is a possible candidate of *Rf-1*. A non-restoring genotype was identified to have deletions within the coding region.

Key words: Cytoplasmic male sterility; Restorer fertility gene; Pentatricopeptide repeat; *Oryza sativa* L.

1. Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in the inability to produce fertile pollen and is widely known in higher plants. It is usually attributed to mitochondrial defects. In most cases mitochondrial genome of CMS contains unusual chimeric genes that are cotranscribed with conventional mitochondrial genes [1]. In some cases, pollen fertility is recovered by a nuclear-encoded gene called a fertility restorer gene (*Rf*). CMS/*Rf* systems greatly facilitate hybrid seed production, because they eliminate the need for hand emasculation. Apart from its commercial importance for hybrid seed production, CMS/*Rf* systems offer an excellent opportunity to investigate the relationship between mitochondrial gene expression and a nuclear-encoded protein.

In many CMS/*Rf* systems, the transcript pattern of a CMS-associated gene is altered in the presence of the *Rf*-gene [1]. For example, in *Petunia*, CMS-associated abnormal protein termed PCF is greatly reduced by the action of the *Rf* gene [2]. In CMS of Kozena radish, the amount of CMS-associated protein, OFR125, was reduced considerably in the fertility restorer lines carrying the *Rf* gene [3]. Recently, *Rf* genes have been cloned in *Petunia* [4] and Kozena radish [5] by using a positional cloning strategy. Both of the *Rf* genes have been shown to encode a protein with mitochondrial transit peptide and pentatricopeptide repeat (PPR) motif. PPR protein contains a characteristic tandem array of a 35 amino acid motif, and therefore is termed a pentatricopeptide repeat. There are more than 450 members of the PPR family in *Arabidopsis thaliana* [6]. The majority of these proteins are predicted to be targeted to mitochondria or chloroplasts. The PPR proteins are considered to react with specific RNA in organelles and play a role in RNA processing or translation [7]. The identification of *Rf* genes in *Petunia* and Kozena radish as PPR-containing genes suggests that searching for PPR motif genes near known restorer loci should be a useful strategy to identify *Rf* genes in other species.

In rice (*Oryza sativa* L.), one particular CMS system has been obtained by combining the cytoplasm of Chinsurah Boro II (*indica* rice) with the nuclear genome of Taichung 65 (*japonica* rice). It is called the *ms-bo* type or BT type [8,9]. In this BT-type CMS line, pollen abortion is initiated after pollen mitosis. However, pollen fertility can be restored gametophytically by the gene product of a single dominant nuclear gene, *Rf-1* [8,9].

It has been reported that a unique sequence (*orf79*) located downstream from mitochondrial *atp6* causes male sterility. In CMS lines, the abnormal *atp6* (*B-atp6*) is transcribed as a 2.0-kb RNA consisting of normal *atp6* and a unique sequence containing *orf79*, whereas two discontinuous RNAs of 1.5 and 0.45 kb are generated from the 2.0 kb RNA by RNA processing in the presence of the *Rf-1* gene. RNA processing has also been shown to influence the sequential post-transcriptional editing of *atp6* RNA. Processing followed by complete editing of altered mitochondrial *atp6* RNA has been suggested to restore fertility of the CMS. The *Rf-1* gene is, therefore, considered to be involved in the efficiency of processing or editing of mitochondrial transcripts [10–12].

The locus of the *Rf-1* gene has been determined to lie on chromosome 10 [8]. Linkage analysis between *Rf-1* and several DNA markers, such as restriction fragment length polymorphism (RFLP) markers and polymerase chain reaction (PCR)-based markers, has been reported. Thus far, the *Rf-1* gene has been reported to be located within 13.6 cM between a PCR marker *fL601* and an RFLP marker *C16* [13]. However, cloning of the *Rf-1* gene has not yet been reported.

In this study, we followed a positional cloning strategy to identify the *Rf-1* gene. After mapping of the *Rf-1* locus within 1 cM, we searched for the open reading frame (ORF) predicted to carry mitochondrial transit sequence and PPR motif using a database of rice genome sequences, and introduced each PPR gene into callus of an *rf-1*/*rf-1* CMS line. One of

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the PPR genes, which encodes a 791-aa protein with 18 PPR repeats, has been demonstrated to promote the processing of aberrant *atp6* RNA in the same manner as the *Rf-1* gene does. A recessive allele was shown to have deletions. The cloned PPR gene is a possible candidate of the *Rf-1* gene.

2. Materials and methods

2.1. Plant materials

The nuclear and cytoplasmic genotypes and phenotypes of the plant materials used in this study are listed in Table 1. We used near-isogenic lines which differ at the *Rf-1* gene locus. These lines have been derived from a backcross of Chinsurah Boro II×Taichung 65 [8]. They are [*normal*]*rf-1*/*rf-1* Taichung 65 as a maintainer line (BTB), $B_{21}F_1$ [*ms-bo*]*rf-1*/*rf-1* as a CMS line (BTA), and $B_{12}F_8$ [*ms-bo*]*Rf-1*/*Rf-1* as a restorer line (BTR). The genotype of Nipponbare is *rf-1*/*rf-1*, and that of Milyang 23 is *Rf-1*/*Rf-1* [13].

2.2. Mapping of Rf-1 locus

PCR primers flanking simple sequence repeats were designed based on the rice genome sequence of the DNA databases (the TIGR Rice Genome Project, http://www.tigr.org/tdb/rice/). Primer pairs that detected polymorphism between BTA and BTR were used as DNA markers for mapping (Table 2). Recombinants were investigated in 1121 BC_1F_1 plants of BTA×BTR, all of which had *Rf-1*/*rf-1* genotype. Prediction of the PPR gene was carried out using RiceGAAS (http://ricegaas.dna.affrc.gp.jp/). Mitochondrial targeting peptide was predicted using PREDOTAR version 0.5 (http://inra.fr/Internet/Produits/Predotar/), TARGETP (http://www.cbs.dtu.dk/services/TargetP/) and MITOPRT (http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter).

2.3. Screening of BAC clones

Primers for amplifying several kinds of DNA fragments in *Rf-1* locus were designed based on the DNA sequences of Nipponbare (accession no. AC068923 in GenBank databases; Table 2). These primers were used for synthesizing DIG-labeled probes and screening of BAC clones. Hybridization filters and BAC clones of rice cultivar Milyang 23 were obtained from Clemson University Genomics Institute (Clemson, SC, USA). Nucleotide sequences were determined using ABI Prism BigDye terminator cycle sequencing kits (Applied Biosystem, Foster City, CA, USA).

2.4. Transformation vector and plants

The BAC clone was digested with *Xba*I or *Sac*I, and each fragment containing the PPR gene was identified and inserted into the same site in pBI101HYG where a gene for hygromycin resistance had been inserted into pBI101 [14]. The resultant transformation vector was transferred into *Agrobacterium tumefaciens* strain EHA105 [15], and then introduced into the BTA line by the method of [16]. Hygromycin-resistant calli were selected and used for DNA and Northern blot analysis.

2.5. Northern blot analysis

Total RNA was isolated from 100 mg of calli of BTA, BTR and F_1(BTA×BTR), and transformants using the RNeasy Plant mini kit (Qiagen, Hilden, Germany), and 10 μg of each RNA was subjected to Northern blot analysis. A part of the *B-atp6* gene of BTA was amplified using primer j [11] and primer GSP (5′-AGGGGTGGGATATTTGCCTGGTCCACC-3′) and labeled with digoxigenin using the PCR DIG probe synthesis kit. Hybridization, washing and detection were performed according to the manufacturer's instructions of the DIG DNA labeling and detection kit (Roche Diagnostics, Quebec, Canada).

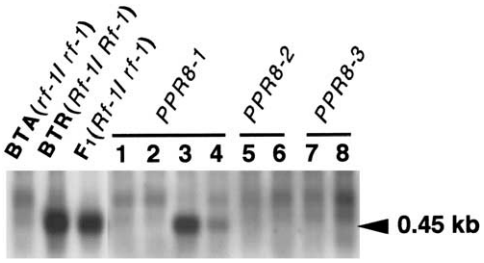

Fig. 1. Detection of 0.45-kb RNA specific for *Rf-1* genotype in transgenic calli. Total RNA was isolated from calli of BTA (*rf-1*/*rf-1*), BTR (*Rf-1*/*Rf-1*), F_1 (*Rf-1*/*rf-1*) and transgenic BTA lines with *PPR8-1* (Nos. 1–4), *PPR8-2* (Nos. 5 and 6) and *PPR8-3* (Nos. 7 and 8). The RNA gel-blot was probed with a portion of the 3′ end of *B-atp6*.

2.6. Reverse transcription (RT)-PCR analysis

Poly(A)+RNA was isolated from anthers at the microspore stage of BTR using Isogen (Nippongene, Tokyo, Japan) and Dynabeads mRNA direct kit (Vertas, Tokyo, Japan). RT-PCR was carried out as described previously [17]. PCR primers F58 5′-TCCGTATAAGACAAACTGCG-3′, R1498 5′-TTGTTACAGGTGCACAAACC-3′, F1026 5′-CCAGATGTTGTTACTTATAGCTT-3′ and R2577 5′-AAGATCCCTCCTCTAATAGG-3′ were used for amplification of cDNA. Rapid amplification of cDNA ends, 3′-RACE, was carried out using the 3′-RLM RACE kit (Ambion, Woodward Austin, USA). The gene-specific primers were F1026 (5′-CCAGATGTTGTTACTTATAGCTT-3′) for the first PCR and F2276 (5′-TGGCTGTACTGTTGACTCTG-3′) for the second nested PCR.

3. Results and discussion

3.1. Identification of candidate genes

The nuclear and cytoplasmic genotypes and phenotypes of the plant materials used in this study are listed in Table 1. In order to determine the genetic distance between DNA markers and the *Rf-1* locus, we produced BC_1F_1 plants of BTA×(BTA×BTR). Since F_1 plants (BTA×BTR) have the genotype *Rf-1*/*rf-1* and segregate fertile pollen with *Rf-1* and sterile pollen with *rf-1*, only *Rf-1* pollen participates in the fertilization when pollinated to BTA carrying *rf-1*/*rf-1*. All the BC_1F_1 plants, therefore, have the *Rf-1*/*rf-1* genotype. As for the locus of a DNA marker, it is segregated in the BC_1F_1 plants in accordance with the linkage to the *Rf-1* gene. Thus, it is expected that plants showing the genotype defined by a DNA marker identical to BTA indicate the recombinants between the locus for the DNA marker and the *Rf-1* locus.

Based on the draft sequences of BAC clones of Nipponbare (the TIGR Rice Genome Project), we designed PCR primers flanking simple sequence repeats (SSR) and RFLP markers. Eventually six recombinants were identified using the RFLP marker GEND713, and four recombinants using CT502 (Table 2). The recombination values were calculated to be 0.54 and 0.36 cM, respectively. The SSR markers, AT801 and AT501, and the RFLP marker, GEND811, did not yield

Table 1
The plant materials used in this study

	Genotype	Nuclear background	Phenotype
BTA	[*cms-bo*]*rf-1*/*rf-1*	Taichung 65	sterile
BTB	[+]*rf-1*/*rf-1*	Taichung 65	fertile
BTR	[*cms-bo*]*Rf-1*/*Rf-1*	Taichung 65	fertile
Nipponbare	[+]*rf-1*/*rf-1*	Nipponbare	fertile
Milyang 23	[+]*Rf-1*/*Rf-1*	Milyang 23	fertile

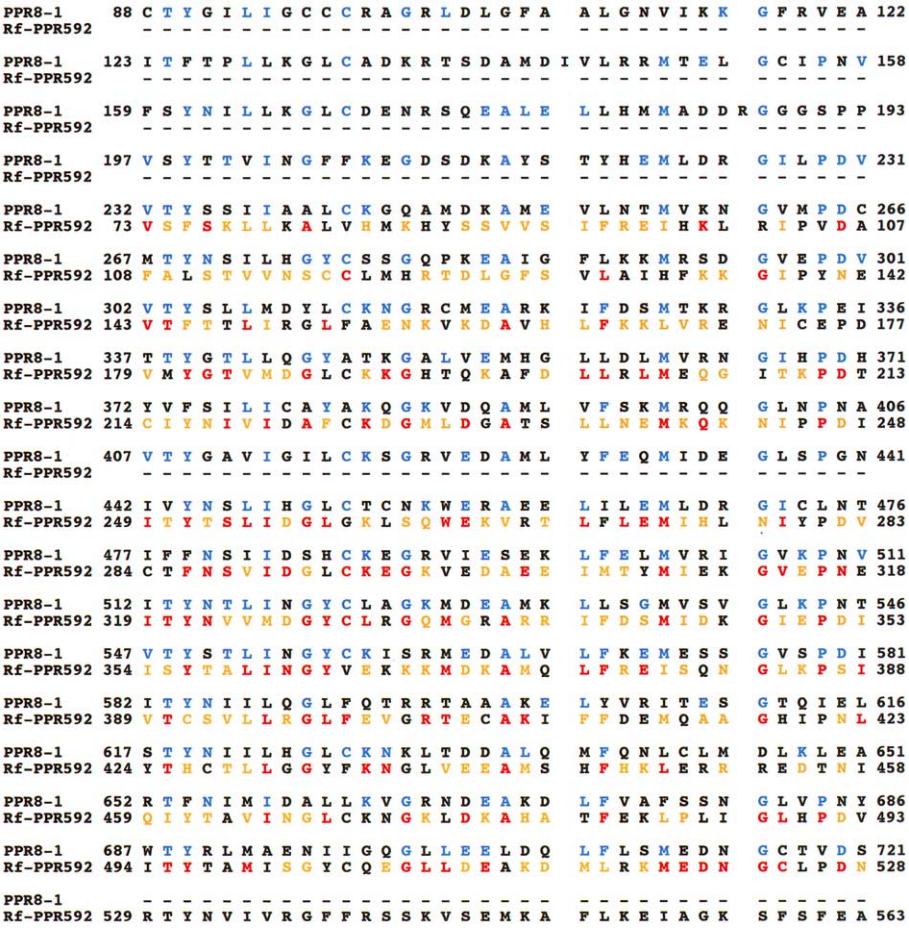

Fig. 2. PPR8-1 contains 18 PPR repeats and shows noticeable similarity to the PPR motifs of *Petunia* restorer gene, *Rf-PPR592*. PPR motifs were identified in the predicted amino acid sequence of PPR8-1 of Milyang 23 by the Pfam software (http://www.sanger.ac.uk/Software/Pfam/). Amino acid residues identical to the Pfam-derived consensus are shown in blue. The nucleotide sequence data reported appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession numbers AB106867 and AB110016. The PPR motifs of rice PPR8-1 and *Petunia* Rf-PPR592 are aligned using the CLUSTAL W software (http://www.ddbj.nig.ac.jp/E-mail/clustalw-j.html). Identical and similar amino acid residues in *Petunia* Rf-PPR592 are shown in red and orange, respectively.

any recombinants (Table 2). This region was found to be covered by four BAC clones (accession numbers AC068950, AC092489, AC068923 and AC079888 in the GenBank databases). The physical distance between GEND713 and CT502 was estimated to be 370 kb. We searched for ORFs containing a mitochondrial targeting presequence and PPR motif in the genome of Nipponbare, even though the genotype of Nipponbare is *rf-1*/*rf-1*, using a rice genome automated annotation system, RiceGAAS, and found four such genes (Predgene14, Predgene15 and Predgene18 of the BAC clone AC068923, and Predgene12 of the BAC clone AC079888). Three genes (Predgene14, Predgene15 and Predgene18) existed in a tandem array. The genes were named *PPR8-1*, *PPR8-2* and *PPR8-3*, respectively. Alignment of these genes revealed that they were quite similar to each other.

3.2. Complementation test

A BAC clone containing the *PPR8-1*, *PPR8-2* and *PPR8-3*

Table 2
Number of recombinants identified using the designated DNA markers in BAC clones of Nipponbare

Number of recombinants	Name of marker	Primer sequence (5′–3′)	Accession No. of BAC clone
6	GEND713	AGATGTCAAGACCTTCGCGT TGAAACTGAGAAGGCTTGCC	AC068950
0	GEND811	GTCAGTGATGTCATCAGTTG CTTGGCTAGCTAACCCGAAC	AC068923
0	AT801	TGCCGGTATTTTGGTACGTC TGGATTCCTAATTGCAGCTC	AC068923
0	AT501	TCTCCGTGAGACGGTTATGT CGATGATCTGTCATCCTGTA	AC079888
4	CT502	CACACGATGGATGGATGGAT TCCAACTCATGACACATGGC	AC079888

Primer sequences used for making DNA markers are listed.

genes was screened from BAC libraries of cultivar Milyang 23, whose genotype is *Rf-1*/*Rf-1*. We determined that BAC clone OSIMBb0046F08 (Clemson University Genomics Institute, Clemson, SC, USA) contained *PPR8-1*, *PPR8-2* and *PPR8-3* genes. A 4.7-kb *Xba*I fragment containing the *PPR8-1* gene, a 9.5-kb *Sac*I fragment containing the *PPR8-2* gene, and a 8.7-kb *Sac*I fragment containing the *PPR8-3* gene were then subcloned. Each of the genomic fragments was introduced into a CMS line, BTA (*rf-1*/*rf-1*), by *Agrobacterium*-mediated gene transfer. At least four independent transgenic calli for each clone were selected based on hygromycin resistance.

Examination of *B-atp6* RNA in transgenic callus was expected to be used as an indicator of the presence of *Rf-1* instead of observing the pollen fertility in the complementation test. We focused on the transcripts of 0.45 kb that had been reported to be specific for *Rf-1* genotype and derived from the processing of the *B-atp6* RNA by the action of *Rf-1* [11,12]. As shown in Fig. 1, transcripts of *B-atp6* were investigated in transgenic calli by Northern blot analysis. A portion of the 3′ end of *B-atp6* containing *orf79* was used as a probe. A 0.45-kb band was detected in BTR (*Rf-1*/*Rf-1*) and F_1 (BTA×BTR), but not in BTA (*rf-1*/*rf-1*), as previously reported [11,12]. In transgenic callus lines with the 4.7-kb *Xba*I fragment containing the *PPR8-1* gene, the 0.45-kb band specific for *Rf-1* genotype was detected. This band was detected in two transgenic lines out of four lines investigated (Fig. 1, Nos. 1–4). In contrast, the band was not detected in the transgenic lines with the 9.5-kb *Sac*I fragment containing the *PPR8-2* gene or in those with the 8.7-kb *Sac*I fragment containing the *PPR8-3* gene (Fig. 1, Nos. 5–8). This result indicates that protein encoded by the *PPR8-1* gene promotes the production of the 0.45-kb RNA of *B-atp6* in the same manner as the product of the *Rf-1* gene does.

3.3. Sequencing of the PPR8-1 gene

Sequencing of the 4.7-kb *Xba*I fragment of Milyang 23 revealed that it contained a single ORF consisting of 2376 nucleotides. The ORF predicted a protein of 791 amino acids, a mitochondrial targeting presequence of 26 amino acids and 18 repeats of pentatricopeptide (Fig. 2). A cDNA corresponding to this ORF was isolated from BTR using RT-PCR, and its sequence was identified to be the same as the genomic sequence of Milyang 23. Comparison of the nucleotide sequences with those of Nipponbare (*rf-1*/*rf-1*) revealed that there is a one-nucleotide deletion and a 574-nucleotide deletion in the middle of the *PPR8-1* gene, which cause the creation of a stop codon in the fifth PPR. There are 22 nucleotide substitutions other than the deletions.

Comparison of amino acid sequences of the rice *PPR8-1* and the fertility restorer gene of *Petunia*, *Rf-PPR592* [4], indicates that each PPR is noticeably similar; the 5th–18th PPR of the PPR8-1 shows 29% identity and 68% similarity to the 1st–13th PPR of the *Petunia* Rf-PPR592 (Fig. 2). The significant similarity between the rice PPR8-1 and the *Petunia* Rf-PPR592 suggests that this PPR region has a certain common role in the modification of mitochondrial RNAs.

3.4. Conclusion

In the current study, we focused on the transcripts of 0.45 kb that were derived from the processing of the *B-atp6* RNA by the action of *Rf-1*. A small piece of transgenic callus was demonstrated to be sufficient for quick examination of the transcripts. One of the PPR genes, the *PPR8-1* gene, was shown to be responsible for the production of the 0.45-kb RNA, in the same manner as the *Rf-1* gene in the restorer line (Fig. 1). Further investigation of the fertility of the transgenic plants is needed in order to conclude that the *PPR8-1* gene is the *Rf-1* gene. Nevertheless, it is clear that the *PPR8-1* gene promotes the processing of aberrant *B-atp6* RNA of the CMS rice. Characterization of the *PPR8-1* gene will provide new insights into the interaction between nuclear-encoded protein and mitochondrial RNA.

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