Promoter Shuffling at a Nuclear Gene for Mitochondrial RPL27. Involvement of Interchromosome and Subsequent Intrachromosome Recombinations

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The Reclinomonas americana mitochondrial genome contains a mitochondrial ribosomal protein L27 (rpl27) gene, whereas the rpl27 gene is absent from all plant mitochondrial genomes examined to date. This suggests that plant mitochondrial rpl27 genes have been transferred previously from the mitochondrial genome to the nuclear genome. A nuclear cDNA encoding mitochondrial RPL27 was identified in rice (Oryza sativa). Three similar sequences were identified: rpl27-1 and rpl27-2 on chromosome 8 and rpl27-3 on chromosome 4. Harr plot analysis suggests that they were generated by inter- and intrachromosomal duplications. Interestingly, the transcribed rpl27 gene (rpl27-1) acquired a promoter sequence that was derived from the rice spt16 (Osspt16) gene, the homolog of a global transcription factor in yeast (Saccharomyces cerevisiae) located downstream from the rpl27-3 sequence on chromosome 4, after inter- and intrachromosomal recombination. Reverse transcription-PCR and promoter assay revealed that the rpl27 mRNAs were mainly transcribed from rpl27-1. A repeat of seven nucleotides (AATAGTT) was identified at the junction of rpl27-1 and rpl27-2 on chromosome 8, and the same repeat was also identified at the 5’ end of rpl27-2 and the 3’ end of rpl27-1. This repeat (AATAGTT) contains the hot-spot sequence AGTT, which is preferentially recognized by topoisomerase I in wheat (Triticum aestivum) germ, suggesting the involvement of topoisomerase I in this recombination. We here report the example of promoter shuffling and show that this promoter shuffling resulted from a recent segmental duplication through inter- and intrachromosomal recombination events.

The endosymbiont hypothesis of the origin of the mitochondria is generally accepted (Gray, 1992). It is thought that most of the genes of the ancestral endosymbiont have been either transferred to the nuclear genome or lost during evolution.

The complete mitochondrial genome sequences of various species have been determined. A limited number of genes are encoded, and their relative positions are largely conserved among vertebrate mitochondrial genomes (Gray, 1992). This is in marked contrast to the plant mitochondrial genome sequences of liverwort (Marchantia polymorpha), Arabidopsis (Arabidopsis thaliana), sugar beet (Beta vulgaris), rapeseed (Brassica napus), and rice (Oryza sativa), in which gene order and gene content are highly variable (Bullerwell and Gray, 2004).

The mitochondrial genome encodes 16 kinds of ribosomal protein genes that have already been lost from vertebrate mitochondrial genomes. In higher plant mitochondrial genomes, the situation is more complex because the numbers of ribosomal protein genes in mitochondrial genomes vary among higher plants (Adams and Palmer, 2003). A mitochondrial ribosomal protein gene missing from one plant species but encoded by another plant species is likely to be encoded by the nuclear genome in the former species because ribosomal proteins are essential for protein synthesis. These discrepancies in gene content strongly suggest that gene transfer from the mitochondrial genome to the nuclear genome is an ongoing process in higher plants. Several transfer events from the mitochondrial to the nuclear genome have been identified, and they further our understanding of the mechanism of gene transfer from the mitochondrial to the nuclear genome. These events include the RNA-mediated transfer of the cytochrome c oxidase subunit 2 gene in cowpea (Vigna unguiculata; Nugent and Palmer, 1991) and the alternative splicing involved in the expression of the ribosomal protein S14 gene in rice and maize (Zea mays; Figueroa et al., 1999; Kubo et al., 1999). Sequence duplication/recombination was involved in the genomic acquisition of the presequence of ribosomal protein S11 (Kadowaki et al., 1996). In addition to these findings, evidence was also presented on exchange for loci sharing RNA.
helicase-like target presequences, as well as loci sharing RNA binding protein target presequences, during the evolution of mitochondrial genes in Arabidopsis (Elo et al., 2003).

Although there have been several significant findings in this context, they are still limited, and the mechanism by which the promoter sequence of a newly translocated gene is acquired is unknown. Recently, whole-genome sequencing projects have been undertaken in many species, including humans (Homo sapiens), yeast (Saccharomyces cerevisiae), and Arabidopsis. These projects have revealed that inter- and intrachromosomal duplications occur in a complex manner (Arabidopsis Genome Initiative, 2000; Bailey et al., 2002; Dujon et al., 2004). During evolution, inter- and intrachromosomal duplications have occurred incidentally and have sometimes conferred a new function on a gene product or abolished its function, thus increasing genetic diversity. In higher plants, whole-genome sequencing projects have been conducted for Arabidopsis and rice. These genome sequences, together with full-length cDNA sequences, provide useful information about gene transfer events from organelles to the nucleus.

The mitochondrial genome of the heterotrophic flagellate Reclinomonas americana contains an rpl27 gene (Lang et al., 1997), whereas the rpl27 gene is absent from all plant mitochondrial genomes. We have identified a mitochondrial rpl27 gene in the rice nuclear genome. Detailed analysis shows that the rpl27 gene acquired a promoter sequence via inter- and intrachromosomal duplications from the rice spt16-related (Osspt16) gene, which is a homolog of the yeast spt16 gene. The flexibility of the rice nuclear genome structure and the process of promoter shuffling are also discussed.

RESULTS

Nuclear Genes Encoding Mitochondrial RPL27 in Rice and Arabidopsis

The numbers of genes in the mitochondrial genomes of higher plants vary. Comparative analysis of the genes lost in different species makes it possible to determine the mechanisms and processes of gene transfer from the mitochondrial genome to the nuclear genome (Adams and Palmer, 2003). The R. americana mitochondrial genome has the largest number of genes of any mitochondrial genome that has been completely sequenced to date (Lang et al., 1997). Investigation of the protein-coding genes in the database revealed that the rpl27 gene is encoded only in the R. americana mitochondrial genome; no other mitochondrial genome contains this gene. This suggests that the mitochondrial rpl27 gene has already been transferred from the mitochondrial genome to the nuclear genome in most species. To identify the mitochondrial rpl27 gene that was transferred to the nucleus in plants, a TBLASTN search of the National Center for Biotechnology Information (NCBI) database was conducted using R. americana mitochondrial RPL27 as the query. Two full-length cDNAs were found in both the rice and Arabidopsis databases. The rice clones show 49% (GenBank accession no. AK061690) and 50% (GenBank accession no. AK063072) amino acid identity relative to R. americana RPL27, and the Arabidopsis clones show 51% (GenBank accession no. AY046039) and 54% identity (GenBank accession no. AK118574) to R. americana RPL27. The deduced amino acid sequences of RPL27 from rice, Arabidopsis, R. americana, Escherichia coli, and Cyanobacterium were compared (Fig. 1). The alignment strongly suggests that the four clones found in rice and Arabidopsis are RPL27 and that these four clones have several conserved domains and extended amino acid sequences at their N and C termini.

The localization of these four clones from rice and Arabidopsis was predicted using three computer programs: TargetP (Emanuelsson et al., 2000), Predotar version 0.5 (http://www.inra.fr/predotar/), and Mitopred (Guda et al., 2004). Almost all programs concurred in predicting that AK063072 and AK118574 are mitochondrial RPL27 proteins and AK061690 and AY046039 are plastid RPL27 proteins; the only exception was the target organelle predicted for AY046039 by Predotar version 0.5.

Evaluation of the Targeting Signal of RPL27

In addition to the amino acid sequences conserved during evolution (AK063072, amino acids 40–120; AK061690, amino acids 61–138), the two rice clones have additional peptide sequences at both their N termini (AK063072, 1–39; AK061690, 1–60) and C termini (AK063072, 121–145; AK061690, 139–195). Mitochondrial proteins encoded by the nuclear genome carry a targeting signal that allows the protein to be sorted from the cytoplasm to the mitochondria. Most mitochondrial matrix proteins, such as ribosomal proteins, contain a targeting signal at the N terminus called the presequence (Pfanner and Geissler, 2001). It was assumed that the N-terminal sequence of rice RPL27 (AK063072) might function as a presequence. To monitor the subcellular localization of predicted mitochondrial RPL27 (AK063072), a reporter experiment was performed using the targeting signal described above and green fluorescent protein (GFP).

DNA encoding the amino acid sequence of the N-terminal extension (residues 1–40 of AK063072, corresponding to Fig. 1) of the predicted mitochondrial RPL27 was ligated to the gfp gene, and the fused protein was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The chimeric gene was introduced into tobacco (Nicotiana tabacum) Bright-Yellow 2 (BY-2) suspension-cultured cells, and its expression was monitored 6 h after its introduction using confocal laser-scanning microscopy. The locations of the GFP protein and the mitochondria were visualized as the green color of GFP...
(Fig. 2A) and the red color of MitoTracker Red (Fig. 2C), respectively. If the GFP protein was targeted to the mitochondria, a yellow color should be observed when the green and red colors merge. After the fluorescent signals from GFP and MitoTracker Red were merged, almost all the GFP spots became yellow (Fig. 2B). These results strongly suggest that the GFP fusion protein containing the N-terminal portion of AK063072 were localized to the mitochondria, and that the mitochondrial-targeting signal occurs within the 40 amino acids of the N-terminal extension. These results strongly suggest that clone AK063072 encodes a gene for mitochondrial RPL27 protein.

Inter- and Intrachromosomal Duplications around the Mitochondrial rpl27 Gene

The rice nuclear genome has been completely sequenced, and the genome sequence is available (http://rgp.dna.affrc.go.jp/). To determine the locus of the mitochondrial rpl27 gene in the nuclear genome (AK063072), a BLASTN search of the rice genome database (http://riceblast.dna.affrc.go.jp/) was conducted. The mitochondrial rpl27 gene was identified on chromosome 8. Interestingly, a sequence homologous but not identical to that of the rpl27 gene was also found on chromosome 4. This suggests that the rpl27 gene and the homologous region on a different chromosome have an evolutionary relationship.

To understand the relationship between these sequences, Harr plot analysis of the rpl27 gene on chromosome 8 and the rpl27-related sequence on chromosome 4 was performed (Fig. 3). This indicated that 29 kb of chromosome 4 and 32 kb of chromosome 8 share a common DNA sequence around the rpl27 gene. This strongly suggests that an interchromosomal duplication occurred between chromosome 4 and chromosome 8 in the past. The sequence similarity of the overall region duplicated between chromosome 4 and chromosome 8 is 97%, suggesting a relatively recent event.

Detailed analysis identified a tandem duplication of 5.2 kb within chromosome 8 (Fig. 3). The intrachromosomal duplication created a new 5.2-kb sequence containing an additional rpl27 sequence. There is no base substitution between the two 5.2-kb regions, which suggests that the intrachromosomal duplication...
were carefully analyzed. Mitochondrial rpl27 Promoter Shuffling in the Mitochondrial intrachromosomal duplications around the rpl27 some effect on a gene, the effects of the inter- and intrachromosomal duplication. Because duplication may have chromosomal duplications have been found in rice (Arabidopsis Genome Initiative, 2000; Bailey et al., 2002; Dujon et al., 2004). Furthermore, large-scale interchromosomal duplications have been found in rice (Paterson et al., 2004; Salse et al., 2004). Duplications can allow new functions to be conferred on the duplicate gene; they can also generate gene families or disrupt functional genes. Because duplication may have some effect on a gene, the effects of the inter- and intrachromosomal duplications around the rpl27 gene were carefully analyzed.

Promoter Shuffling in the Mitochondrial rpl27 Gene

The rpl27 gene translocation event from the mitochondrial genome to the nuclear genome and the subsequent inter- and intrachromosomal duplication events ultimately resulted in the presence of three copies of the rpl27 sequence on chromosome 4 and chromosome 8 (Fig. 4A). We designated the AK063072 sequence on chromosome 8 that was identified as a full-length cDNA, rpl27-1. The homologous sequence upstream from AK063072 on chromosome 8 was designated rpl27-2. The sequence on chromosome 4 homologous to that of AK063072 was designated rpl27-3. It is interesting that a sequence similar to the first exon (5’ untranslated region [UTR], 5’ UTR) of rpl27-1 on chromosome 8 was identified approximately 1.6 kb downstream from rpl27-3 on chromosome 4. Detailed analysis showed that this sequence was submitted previously as a full-length cDNA (GenBank accession no. AK121570) and that the cDNA was deduced to encode a homolog of the yeast spt16 gene (Belotserkovskaya et al., 2003). We designated the AK121570 sequence Osspt16.

An interchromosomal duplication event that occurred between the sequences on chromosome 4 and chromosome 8 resulted in the translocation and duplication of the whole rpl27 gene and part of the Osspt16 gene (Fig. 4A). There are two Osspt16-related sequences on chromosome 4 and chromosome 8 (Fig. 4A). The deduced open reading frame (ORF) of Osspt16 on chromosome 8 is 161 amino acids shorter than that on chromosome 4. There are two possibilities regarding the origin of the Osspt16 sequence. One is that the sequence on chromosome 8 is a truncated form that originated on chromosome 4. The other possibility is that the sequence on chromosome 4 is an extended form that originated on chromosome 8. A BLASTX search of the NCBI database was conducted using the Osspt16 sequence as the query, and two expressed sequence tags were identified in maize (GenBank accession no. AF545812) and Arabidopsis (GenBank accession no. NM_117139). When the expressed sequence tags from maize and Arabidopsis were aligned with Osspt16, they were seen to be highly conserved in terms of the size of the ORF (data not shown). This strongly suggests that the Osspt16 gene on chromosome 4 is the original form. The first exon of rpl27-1 shares 94.7% sequence similarity with the first exon of Osspt16 on chromosome 4 (Fig. 5, hatched boxes). The first exon of rpl27-1 is located in the UTR and the translational ATG codon is present in the second exon. Because spliced full-length cDNAs of rpl27-1 and Osspt16 were identified in the rice full-length cDNA database (knowledge-based Oryza molecular biological encyclopedia), both sequences should have promoter sequences in their 5’ regions. The sequences around the 5’ ends of the cDNAs (possible transcriptional initiation sites) of rpl27-1 and Osspt16 were compared (Fig. 5B) and showed a high degree (97.3%) of similarity. In short, the entire first exon, UTR, and promoter sequences of the rpl27-1 locus shared sequence similarity with the Osspt16 locus. These results strongly suggest that the 5’ UTR of rpl27-1, including the promoter sequence, is derived from the Osspt16 gene.

Assay for Transcription Activity of the Duplicated rpl27 Gene with the Osspt16 Promoter

Interc chromosomal duplication followed by intrachromosomal duplication created two additional
copies of rpl27-related sequences on chromosome 8 (rpl27-1 and rpl27-2) from the original copy on chromosome 4 (rpl27-3). However, questions still remain regarding when and how the rpl27-related sequences acquired transcriptional apparatus. First, to measure the transcriptional activity of the three rpl27 sequences, the promoter regions of the three rpl27-related sequences and that of Ossp16 (used as a control) were fused to a β-glucuronidase (GUS) reporter gene and these constructs were transfected into rice protoplasts. The transcriptional activities of rpl27-2 and rpl27-3 were less than one-seventh that of rpl27-1 (Fig. 4B). In short, this result indicates that the transcriptional activity of the rpl27-1 gene increased more than 7-fold after acquiring the Ospp16 promoter by promoter shuffling.

Nucleotide substitutions at three positions were identified in the ORFs of rpl27-1 and rpl27-3. This nucleotide dissimilarity allowed us to distinguish the RNAs transcribed from rpl27-1 and rpl27-3. Reverse transcription (RT)-PCR was performed using a primer set designed to amplify an internal part of the rpl27 ORF, and the RT-PCR products obtained were sequenced. The sequences of 30 independent clones revealed that all of them were transcribed from rpl27-1 and no clone was transcribed from rpl27-3. In short, the rpl27 gene is transcribed from chromosome 8 but negligibly from chromosome 4.

There are no nucleotide differences between the two copies of the rpl27-related sequences on chromosome 8, rpl27-1 and rpl27-2, except in the first exon of rpl27-1. This sequence is unique to rpl27-1 and is absent from rpl27-2 and rpl27-3 (Fig. 4A). Therefore, it is difficult to distinguish the transcription of rpl27-2 from that of rpl27-1 using their internal sequences, as described for

Figure 4. Schematic representation of promoter acquisition by the rpl27 gene mediated by inter- and subsequent intrachromosomal duplications and the relative promoter activities of each rpl27 sequence. A, Schematic representation of promoter acquisition by the rpl27 gene mediated by inter- and subsequent intrachromosomal duplications. The white arrow shows the past rpl27 gene transfer event from the mitochondrial genome to the nuclear genome. Horizontal arrows indicate transcribed regions identified from the full-length cDNA sequence. Exons and introns are indicated by boxes and thick horizontal lines, respectively. Black dotted lines indicate the regions duplicated. Black triangles show tandem repeats (AATAGTT) that are likely to have been involved in the intrachromosomal duplication. Black lines below rpl27-3 (a), rpl27-2 (c), rpl27-1 (d), and Ospp16 (b) indicate the cloned regions for transient promoter assays. B, Relative GUS/LUC activities. Effects of the inter- and intrachromosomal duplications on the promoter of the rpl27 gene. Values show the activity of each construct relative to that of the rpl27-3 construct (a). The characters a, b, c, and d to the left of the graph correspond to those in A. The experiment was repeated three times per plasmid, and the means are shown by black bars. Standard errors are indicated by lines extending from the bars. The graph represents an experiment performed with a single preparation of protoplasts. Numbers in the graph show the extent of activation (-fold) for each construct relative to that of the rpl27-3 construct (a).
Alternatively, RT-PCR was performed using a 5' primer derived from the unique sequence upstream from rpl27-2 that is not found in rpl27-1 and a 3' primer derived from the evolutionarily conserved rpl27 sequence. However, no PCR product was detected (data not shown). This strongly suggests that there is no transcription from rpl27-2. The results described above demonstrate that the majority of rpl27 transcripts are derived from rpl27-1 on chromosome 8.

These results suggest that the rpl27 gene was translocated from chromosome 4 to chromosome 8 by interchromosomal duplication, and that a subsequent intrachromosomal duplication on chromosome 8 created a new gene with a foreign promoter that originated from the Osspt16 gene.

**DISCUSSION**

The mitochondrial rpl27 genes are absent from mitochondrial genomes of plants, e.g. liverwort, Arabidopsis, and rice (Bullerwell and Gray, 2004). This suggests that translocation of mitochondrial rpl27 genes occurred before the divergence of monocots and dicots. Genes for mitochondrial RPL27 have been identified in the nuclear genome of Arabidopsis and rice (Fig. 1). Thanks to mass genome-sequencing projects, a large number of segmental duplications have been found in the genomes of many species (Eichler and Sankoff, 2003). Segmental duplications have contributed to an increase in gene-copy number and genome size. In addition, in this study we have shown that segmental duplication also bestowed an already existing promoter sequence to a new gene, which was transferred from the mitochondrial genome to the nuclear genome during evolution. The rpl27-1 gene acquired a promoter sequence derived from the rice Osspt16 gene, the homolog of a global transcription factor in yeast, located downstream from the rpl27-3 sequence on chromosome 4, after inter- and intrachromosomal recombination. RT-PCR and promoter assays revealed that rpl27 mRNAs were mainly transcribed...

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**Figure 5.** Schematic representations and alignment of genomic DNA sequences of the rpl27-1 and the Osspt16 genes. A, Schematic representations of exons and introns of the rpl27-1 and the Osspt16 genes determined from full-length cDNAs. Exons and introns are indicated by boxes and oblique lines, respectively. Horizontal lines indicate 5' and 3' flanking regions. Hatched boxes corresponding to hatched lines in B show exons of the rpl27-1 and Osspt16 genes that share sequence similarity. Black and white arrows indicate the transcription initiation sites of rpl27-1 and Osspt16, respectively. Scale bar is 1 kb. ATG and TAA show translation initiation and stop codons, respectively. The nucleotide sequences between the two dotted lines, from rpl27-1 and Osspt16, are aligned in B. Alignment of genomic DNA sequences around the transcription initiation sites of the rpl27-1 and the Osspt16 genes. About 500 bp upstream from the transcription initiation site, the first exon, and a part of the first intron of each gene are aligned. Black background indicates nucleotides identical between the rpl27-1 and the Osspt16 genes. A splicing donor site is shown by a box.
from rpl27-1. No such novel findings have been observed in two Arabidopsis rpl27 genes and one rice-chloroplast rpl27 gene. Hence, we have focused our experimental analysis on rice mitochondrial rpl27 only.

We have demonstrated that rice mitochondrial rpl27-1 contains targeting information within 40 amino acids of its N terminus. To search for sequences homologous with these 40 amino acids, BLASTN and TBLASTN searches against the rice genome database were conducted. However, we did not find any homologous sequences with those in the rice genome. Regarding Arabidopsis mitochondrial rpl27, we did not find any sequences in the Arabidopsis genome that were homologous with its N-terminal portion.

Nucleotide sequence similarity around the 5’ portion of mitochondrial rpl27 was not found when Arabidopsis and rice were compared. However, a comparison of the N-terminal amino acid sequences revealed that 27 amino acids were conserved between the two (Fig. 1). It is well known that mitochondrial targeting signals are loosely conserved and enriched in specific amino acids (Arg, Leu, and Ser), although a characteristic property of presequences is the high tendency to form an amphipathic α-helix that presents one positively charged polar group at the end (Arg, Lys, Leu, or Ile; Muto et al., 2001). A common profile of the Tom20 binding segment was observed at the conserved amphipathic α-helix structure in rice (FKRWA) and Arabidopsis (LKRWA; Fig. 1). This strongly suggests that presequences of the mitochondrial rpl27 genes in higher plants are of the same origin. However, they are quite divergent while retaining the same biological function during evolution.

There are two OsSpt16 sequences. One is located on chromosome 4 and the other on chromosome 8. It is possible that the OsSpt16 sequence on chromosome 4 is the original form and that the OsSpt16 sequence on chromosome 8 is a duplicated form. It is also possible that these sequences on chromosome 4 and chromosome 8 arose independently from the ancient mitochondrial genome. However, the latter possibility is unlikely for the following reasons. No spt16 gene has been identified in any plant mitochondrial genome, including lower plants, such as liverwort, suggesting that gene transfer of spt16 was completed a long time ago during evolution or the spt16 gene has never been encoded by the mitochondrial genome. Two DNA segments from chromosome 4 and chromosome 8 containing OsSpt16 showed 97% DNA sequence identity, although one of the two sequences seems to be nonfunctional. This evidence strongly suggests that the two DNA segments were duplicated relatively recently, rather than the translocation of the DNA segments into the nuclear genome from the plastidic genome occurring before the divergence of monocots and dicots. In addition, the spt16 gene is one of two components of the facilitates chromatin transcription (FACT) complex. The FACT complex is widely conserved in eukaryotic cells (Belotserkovskaya et al.,
The regulation of gene transcription in prokaryotes and eukaryotes is fundamentally different. The FACT complex is indispensable for the elongation of polymerase II in yeast; however, we identified no spt16 gene in the database for bacteria such as *E. coli*. This suggests the spt16 gene is a eukaryotic gene and not a prokaryotic gene. Mitochondria are widely accepted as a descendant of prokaryotes, such as *α* proteobacterium. Hence, it is less likely that the two sequences including spt16 were transferred from mitochondria to the nuclear genome independently.

Inter- and intrachromosomal recombinations are believed to have been involved in the acquisition of the promoter sequence by the *rpl27* gene, which was translocated from the mitochondrial genome to the nuclear genome. Therefore, we undertook to determine the process of this intrachromosomal recombination event. A repeat of seven nucleotides (AATAGTT) was identified at the junction of the duplicated sequences on chromosome 8 and the same repeat was also identified at the 5′ and 3′ ends of the duplicated sequences on chromosome 8 (Fig. 4A). It is possible that this 7-bp repeat was involved in the intrachromosomal recombination event or is a footprint of the sequence duplication. Illegitimate recombination is mediated by topoisomerase I, which recognizes small sequence duplication. Illegitimate recombination is an intragenic tandem duplication of 3.3 kb. Each of the tandem repeats is flanked by the 3-bp sequence AAT, indicating that a 3-bp microhomology is involved in generating the tandem duplication (Park et al., 2004). It is extremely interesting that the repeat identified in the *rpl27* region (AATAGT)TT contains the 3-bp microhomology (AAT) involved in generating the tandem duplication in the *Tdv-τ* mutant of morning glory (Fig. 6B). We speculate that topoisomerase I and the AATAGTT repeat were implicated in the recombination described above, resulting in the promoter shuffling involving the *rpl27* gene, as well as in the *rpl27* sequence duplication (Fig. 6C).

**MATERIALS AND METHODS**

**Plant Material**

Etiolated seedlings of rice (*Oryza sativa* L. var Nipponbare) were used as the plant material.

**Sequencing Procedures**

Purified DNA was used for cycle sequencing with the Dye Terminator cycle sequencing quick start kit (Beckman Coulter). PCR was performed with an initial denaturation step at 96°C for 1 min followed by 30 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 4 min. PCR products were precipitated and subjected immediately to sequence analysis using the CEQ 2000 XL DNA analysis system (Beckman Coulter).

**Database Analysis**

The available rice genome database (http://rgp.dna.affrc.go.jp/) and the BLAST programs in the RiceBLAST search service (http://riceblast.dna.affrc.go.jp/) were used. Default values were used for all parameters in the TBLASTN and BLASTN programs. Subcellular localization of the protein was predicted with TargetP ( Emanuelsson et al., 2000), Predotar version 0.5 (http://www.inra.fr/predotar/), and Mitopred (Guda et al., 2004).

**RT-PCR Analysis of Transcripts**

Total RNA was isolated and further purified by incubation with RNase-free DNAse I (TaKaRa), according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using 1 μg of purified RNA, 0.5 units of Moloney murine leukemia virus reverse transcriptase, and 20 pmol of random hexamer primer (BD Biosciences). The resultant cDNAs were used as templates to amplify the mitochondrial *rpl27* cDNA. Amplification of mitochondrial *rpl27* cDNA was performed with forward (5′- TCTACGATTTCACGAG-AGG-3′) and reverse (5′- CAGCGTTCAATTCTAGACTG-3′) primers and LA Taq DNA polymerase (TaKaRa). cDNA was denatured at 94°C for 5 min and amplified with 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. PCR products were subsequently cloned into the pCR2.1-TOPO plasmid vector (Invitrogen). Independent clones were sequenced using universal primers.

**GFP Expression Analysis**

A nucleotide sequence thought to encode the targeting signal of rice *RPL27*, AK063072 (RPL27N), was amplified by PCR. The PCR product and the S6STGFP vector encoding the CaMV 35S promoter and 3′ nopaline synthase (NOS) transcription terminator (Chiu et al., 1996) were doubly digested with *Sal* and *Nco* I and ligated in frame as follows: CaMV35S::sGFP(S6ST):NOSTer. The nucleotide sequence of the resultant plasmid was confirmed by DNA sequencing. Plasmid DNA (10 μg) was precipitated onto 1.0 μm spherical gold beads (Bio-Rad), and the beads were bombarded into suspension-cultured tobacco (*Nicotiana tabacum*) BY2 cells using a PDS-1000 particle delivery system (Bio-Rad). After bombardment, the cells were incubated in the dark at 24°C for 6 h. GFP fluorescence was visualized with a confocal laser-scanning microscope, as described previously (Arimura and Tsutsumi, 2002).
Staining of Mitochondria with MitoTracker Dyes

BY-2 suspension cells were incubated with 500 nM MitoTracker Red CMXRos (Invitrogen) in modified Murashige and Skogow medium enriched with 0.2 mg L⁻¹ 2,4-D for 5 min at 24°C and washed three times in the above medium.

Oligonucleotide Primers for GFP and GUS Fusion Constructs

The putative promoter region of each gene and deduced targeting signal of predicted mitochondrial RPL27 (A0K83072) were amplified by PCR for GFP-fused protein, forward (5'-GAGAGCTCCAGACGTTTTTTC-3') and reverse (5'-GTCGCCATGGCTTTTTC-3') primers, containing SbfI (GTCGAC) and Ncol (CCATTG) sites, respectively. For GFP-fused protein, F1 (5'-GAGAGCTCCAGACGTTTTTTC-3'), R1 (5'-TTCGCCTCAATCACCTGAA-3'), F2 (5'-TTCGACAATCACCTGAA-3'), R2 (5'-TTTTCGACTGACCTGACAA-3') primers were used, into which a HindIII (AAGCTT), BamHI (GGATCC), or SbfI (GTCGAC) site was introduced (underlined). F1 and R2 primers were used for rpl27-2 and rpl27-3. Sequences from −966 to −6 and from −1,000 to −6 relative to the translation initiation codons of rpl27-2 and rpl27-3, respectively, were cloned. F2 and R2 primers were used for Osopt16. Sequences from −3,213 to −13 relative to the translation initiation codon of Osopt16 were cloned. F2 and R1 primers were used for rpl27-1. Sequences from −3,472 to −3 relative to the translation initiation codon of rpl27-1 were cloned.

Transient GUS Expression Assay Using Rice Protoplast

The putative promoter region of each gene, amplified by PCR, was ligated upstream from the GUS reporter gene:‘−3’ NOS transcription terminator (Wu et al., 1998). The prepared GUS plasmid (10 μg) was transfected into rice protoplasts using the electroporation method described previously (Wu et al., 1998). Each transfection included 10 μg of the pAHC18 plasmid, which contains a ubiquitin promoter-luciferase (LUC)-encoding construct (Bruce et al., 1989), as an internal standard. GUS activities were normalized to the LUC activity.

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LITERATURE CITED


