Molecular Characterization of Three PRORP Proteins in the Moss *Physcomitrella patens*: Nuclear PRORP Protein Is Not Essential for Moss Viability

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Abstract

RNase P is a ubiquitous endonuclease that removes the 5' leader sequence from precursor tRNAs in all organisms. In *Arabidopsis thaliana*, RNA-free proteinaceous RNase P5s (PRORPs) seem to be enzyme(s) for pre-tRNA 5' -end processing in organelles and the nucleus and are thought to have replaced the ribonucleoprotein RNase P variant. However, the evolution and function of plant PRORPs are not fully understood. Here, we identified and characterized three PRORP-like proteins, PpPRR_63, 67, and 104, in the basal land plant, the moss *Physcomitrella patens*. PpPRR_63 localizes to the nucleus, while PpPRR_67 and PpPRR_104 are found in both the mitochondria and chloroplasts. The three proteins displayed pre-tRNA 5'-end processing activity in vitro. Mutants with knockout (KO) of the PpPRR_63 gene displayed growth retardation of protonemal colonies, indicating that, unlike Arabidopsis nuclear PRORPs, the moss nuclear PpPRR_63 is not essential for viability. In the KO mutant, nuclear-encoded tRNA^AAU^ (GUC) levels were slightly decreased, whereas most nuclear-encoded tRNA levels were not altered. This indicated that most of the cytosolic mature tRNAs were produced normally without proteinaceous RNase P-like PpPRR_63. Single PpPRR_67 or 104 gene KO mutants displayed different phenotypes of protonemal growth and chloroplast tRNA^AAU^ (ACG) accumulation. However, the levels of all other tRNAs were not altered in the KO mutants. In addition, in vitro RNase P assays showed that PpPRR_67 and PpPRR_104 efficiently cleaved chloroplast pre-tRNA^AAU^ (CCG) and pre-tRNA^AAU^ (UCU) but they cleaved pre-tRNA^AAU^ (ACG) with different efficiency. This suggests that the two proteins have overlapping function but their substrate specificity is not identical.

Introduction

RNase P is an endonuclease that removes the 5’ leader sequence from precursor tRNAs (pre-tRNAs). This endonucleolytic cleavage is an essential step in the production of mature tRNAs in all organisms, as well as in mitochondria and chloroplasts [1,2]. Bacterial, archaeal, and eukaryotic nuclear RNase P enzymes are ribonucleoprotein complexes composed of a catalytic RNA component and one or several proteins [3]. Therefore, it was believed that 5’-maturation of tRNAs in all organisms is catalyzed by ubiquitous ribonucleoprotein (RNP) RNase P enzymes. However, human mitochondrial RNase P was identified as an RNA-free enzyme composed of three proteins called mitochondrial RNase P protein 1 (MRP1), MRPP2, and MRPP3 [4]. MRPP3 contains two RNA-binding pentatricopeptide repeat (PPR) motifs and a conserved NYN metallo-nuclease domain [5], which are involved in the catalytic activity of mitochondrial RNase P. In a model plant, *Arabidopsis thaliana*, three MRPP3 homologs have been identified as RNase P enzymes; they are termed proteinaceous RNase P 1 (PRORP1), PRORP2, and PRORP3 [6]. *In vitro* cleavage assays using recombinant PRORP proteins have demonstrated that three PRORP proteins display RNase P activity [6,7]. PRORPs are single-protein RNase P enzymes, whereas human mitochondrial RNase P is a multi-subunit enzyme. PRORP1 localizes in both chloroplasts and mitochondria, whereas PRORP2 and 3 localize in the nucleus. PRORP1 is an essential gene, because *prorp1* mutants are embryonic lethal [6]. Single PRORP2 or PRORP3 knockout mutant lines show wild-type phenotypes, whereas the homozygous double mutation in *prop2* *prop3* results in embryonic lethality [7]. This indicates that PRORP2 and 3 have redundant functions and are essential for embryogenesis and plant growth.

The Arabidopsis and rice genomes do not encode RNase P RNA, whereas they do code for several homologs of RNP RNase P protein subunits found in mammals and yeasts [8]. The presence of ribonucleoprotein RNase P enzyme in plants remains obscure. Recently, Gutmann et al. (2012) suggested that Arabidopsis had entirely replaced the ribonucleoprotein RNase P enzyme by...
PRORP enzymes for tRNA maturation during plant evolution [7]. However, functional characterization of PRORP proteins is limited in Arabidopsis.

Unlike in land plant organelles, an RNA subunit of bacterial-type RNase P is encoded in the organellar genomes of the GlaucoPhyta (Cyanophora paradoxa) [9], the red alga Porphyra purpurea [10], and green algae (Nephroelmins olivacea, Ostreococcus tauri) [11,12]. These algae also possess a single nuclear gene for PRORP [6]. In C. paradoxa, RNase P activity in an extract of the photosynthesis organelle (cyanelle) is sensitive to micrococcal nuclease [13,14], indicating the existence of bacterial-type RNase P. The nuclear-encoded PRORP in the green alga O. tauri has been shown to cleave the 5′ leader of pre-tRNA in vitro [12,15]. Thus, algae seem to possess both a ribonuclceoprotein RNase P and a PRORP. However, the latter is not known to localize in either the organelles or the nucleus.

From these differences in the status of PRORP enzymes in algae and plants, the following questions have arisen: When were plant PRORP genes duplicated during plant evolution, which PRORP protein was targeted to the organelles and which to the nucleus, and was ribonuclease protein replaced by PRORP in plants? To answer these questions, we focused on PRORP Proteins of the basal land plant bryophyte Physcomitrella patens. Here, we report the molecular characterization of three P. patens PRORP-like proteins (PPPR_63, 67, and 104) that are members of the PPR protein family [16,17]. We also report the disruption mutants of each gene and show that nuclear-localized PPPR_63 is dispensable for moss viability and that organelle-localized PPPR_67 and PPPR_104 have functions that are redundant, but not completely so.

Results

The moss P. patens has three PRORP homologs

Among 105 Physcomitrella PPR proteins [17], PPPR_63 (protein ID, Phypa_174001), PPPR_67 (Phypa_177191) and PPPR_104 (Phypa_152956) appeared to be PRORP homologs (Fig. 1A) because these three PPR proteins contained at least two PPR motifs and an NYN metallonuclease domain and had 42%–55% amino acid identity with Arabidopsis PRORP proteins. Although algae and Chara have a single PRORP homolog, mosses and vascular plants have two or three PRORP homologs (Fig. 1B and Table S1). This suggests that PRORP genes were duplicated after the emergence of land plants.

PPPR_63 is localized in the nucleus, whereas PPPR_67 and 104 are found in the organelles

PPPR_67 and 104 possess an N-terminal transit peptide-like sequence targeting the organelles, whereas PPPR_63 does not. This suggests that PPPR_67 and 104 are likely organelle-localized PRORP1 homologs, whereas PPPR_63 seems to be a nuclear-localized PRORP2/PRORP3 homolog. To confirm this prediction we generated PPPR_63-green fluorescent protein (GFP) knockin (KI) mosses. The four independent KI lines obtained showed normal growth (Fig. S1), and GFP fluorescence was observed in the nuclei (Fig. 2A, b and c). GFP fluorescence was observed in the protoplasmic cells, buds, and young leaves (Fig. 2A, d–i) but not in mature leaves (Fig. 2A, j and k).

To verify the organellar localization of PPPR_67 and 104, their N-terminal sequences fused to GFP (N67-GFP and N104-GFP) were transiently expressed in the protemata (Fig. 2B). GFP fluorescence of N67-GFP and N104-GFP was observed in both mitochondria and chloroplasts, coinciding with mitochon-

Figure 1. PRORP proteins are widely distributed among eukaryotes. (A) Schematic diagram of plant organelar PRORPs with a transit peptide (TP), RNA binding PPR motifs (P), and NYN metallonuclease domain (NYN). (B) Neighbor-joining phylogenetic tree of PRORPs and PRORP-like proteins. Representative PRORP protein sequences from evolutionarily distant plants were used for the phylogenetic analysis. Experimentally determined localization of PRORPs is presented as chloroplast (chl), mitochondria (mit), or nucleus (nuc). Bootstrap values >50 are indicated along branches. Protein ID or accession numbers of PRORPs and PRORP-like proteins are listed in Table S1. doi:10.1371/journal.pone.0108962.g001
These results indicated that the three moss PPR proteins had RNase P activity similar to that of Arabidopsis PRORPs [6,7,18].

Nuclear PpPPR_63 is dispensable for viability

To investigate the in vivo function of PpPPR_63, we generated two independent knockout (KO) mutants of PpPPR_63, Δ63-1 and Δ63-15. Both KO lines were verified to be null mutants. Gene structure of the Δ63-1 line is given as Figs. 4A and S2, and its absence of PpPPR_63 transcripts was verified by reverse transcription (RT)-PCR (Fig. 4B). Therefore, we used the Δ63-1 line for further analyses (hereafter named Δ63). In addition, to generate the complementation lines of the Δ63-1, we transformed the Δ63 moss with a transgene encoding the full length of PpPPR_63 or the mutated version and generated F63 and M63, respectively (Fig. S3). In M63, two aspartates (D460 and D461) in the NYN domain were mutated to alanines (A460 and A461). In the complementation lines F63 and M63, the introduced transgene was overexpressed (Fig. 4B). The KO mutant Δ63 and the complementation line M63 mosses displayed smaller protonemal colonies than the wild type (WT) (Fig. 4C), and they showed abnormal regeneration from leaves (especially defective growth of the sub-apical cells) (Fig. 4D). The complementation line F63 exhibited a phenotype the same as that of the WT. These results
indicated that PpPPR_63 was responsible for the mutant phenotype.

Knockout of PpPPR_63 slightly affects specific nuclear-encoded tRNAs

If nuclear PpPPR_63 could function as a bona fide RNase P, knockout of the PpPPR_63 gene could result in severely reduced accumulation of nuclear-encoded tRNAs. To investigate this possibility, we analyzed the accumulation levels of several nuclear-encoded tRNAs in the Δ63 mutant. For this analysis nuclear-encoded tRNA<sub>Asp</sub> (GUC) and tRNA<sub>Gln</sub> (CUG) were chosen, because expression of these tRNAs has been demonstrated to significantly decrease in the knockdown mutants of nuclear PRORP2/3 plants [7]. tRNA<sub>Asp</sub> (GUC) in the Δ63 mutant and M63 complementation line was slightly reduced to 70% of that in the WT and F63. Whereas, tRNA<sub>Gln</sub> (CUG) in the Δ63 mutant, M63 and F63 complementation lines accumulated to the same level as in the WT (Fig. 4E). Similarly, nuclear-encoded tRNA<sub>Ser</sub>
(AGA) and tRNA^{Lys} (CUU) levels were not affected in the mutants. This suggests that PpPPR_63 Pnase P activity may be limited to specific tRNA(s) including tRNA^{Asp} (GUC).

Knockout of PpPPR_104 results in a significant reduction in chloroplast tRNA^{Arg} (ACG)

To investigate the in vivo function of the PpPPR_67 and 104 genes, we generated and characterized their KO mutants (Figs. 5A, S4, S5, S6). The single gene KO mutants obtained, D_67 and D_104, were null mutants (Fig. 5B), and the protonemal colony of D_104 moss was smaller than that of the WT (Fig. 5C). A peunumbra and central part of the protonemal colony mostly consists of caulonemal and chloronemal filaments, respectively. In the D_104 moss, caulonemal filaments were poorly induced, resulting in a smaller colony. In contrast, PpPPR_67 KO moss (D_67) showed little difference from the WT. To generate double KO mutants, we carried out four times transformation experiments, such as introducing the PpPPR_104 KO plasmid into the PpPPR_67 KO line (D_67-1-17 line) and vice versa. However, no double KO mutants were obtained from the genotyping of 262 moss plants. This indicates that PpPPR_67 and 104 genes cannot be knocked out simultaneously. Since they may have redundant functions, single KO mutants are viable but double KO is probably lethal.

To investigate whether organellar tRNAs were affected in the PpPPR_67 and 104 KO mutants, we performed northern blot analyses by using all organelle-encoded tRNA probes. The chloroplast tRNA^{Arg} (ACG) level significantly decreased in D_104 but not in D_67 (Fig. 5D). All the other tRNAs, including chloroplast tRNA^{Pro} (GAA) and mitochondrial tRNA^{Lys} (GCA) that were used for the in vitro RNase P cleavage assay, were not altered in the WT and the three KO mutants (Figs. 5D, S7, S8). This result suggests that PpPPR_67 and PpPPR_104 have redundant functions as RNase P in moss organelles.

Discussion

The first two questions to be addressed here were: When were plant PRORP genes duplicated during plant evolution and where were the respective PRORP proteins localized or targeted to? Our present study provides evidence that PRORP genes were duplicated after the emergence of the early land plants, i.e. mosses. The moss P. patens has three PRORP-like proteins displaying RNase P activity in vitro. Arabidopsis PRORP1 is localized in both the mitochondria and chloroplasts, and PRORP2 and 3 are localized in the nucleus [6]. On the other hand, the moss P. patens has two PRORP1-like proteins, PpPPR_67 and 104, both of which are dual-targeted to the mitochondria and chloroplasts, and one nuclear-localized PRORP2/3-like PpPPR_63. Thus, duplicated PRORP gene products are likely targeted to either the organelles or the nucleus.

PpPPR_67 and 104 cleave chloroplast pre-tRNA^{Arg} (ACG) with different efficiency

Because chloroplast tRNA^{Arg} (ACG) levels decreased in the D_104 but not in the D_67, we assumed that PpPPR_104 and PpPPR_67 would have distinct RNase P activity against chloroplast pre-tRNA^{Arg} (ACG). To test this possibility, three chloroplast pre-tRNA^{Arg} isoacceptors were used as substrates for in vitro RNase P cleavage assay. This assay revealed that PpPPR_67 and 104 efficiently cleaved pre-tRNA^{Arg} (CCG) and pre-tRNA^{Arg} (UCU) but they cleaved pre-tRNA^{Arg} (ACG) with different efficiency (Fig. 6). These results suggest that the function of their RNase P is not completely redundant.
Nuclear PpPPR_63 is involved in normal plant growth and branch formation of protonemata

In Arabidopsis, the double KO mutation of PRORP2 and 3 resulted in embryonic lethality [6]. In contrast, a null mutant of PpPPR_63 displayed smaller protonemal colonies than the WT and also showed abnormal branch formation of filamentous protonemata (Fig. 4). This indicates that unlike Arabidopsis nuclear RPORPs, the moss nuclear PpPPR_63 is dispensable for plant viability. The nuclear-encoded tRNAAsp (GUC) level in the PpPPR_63 KO mutant was slightly decreased (to 70% of that in WT mosses), but most nuclear-encoded tRNA levels were not altered. This result indicates that most of the cytosolic mature tRNAs were produced normally, without proteinaceous RNase P-like PpPPR_63. This does not concur with the results observed from functional analysis of Arabidopsis PRORP2 and 3 [6,7]. In Arabidopsis mutant plants where PRORP3 was absent and the PRORP2 level was decreased, the steady-state levels of five nuclear-encoded tRNAs investigated were significantly reduced, to 30% of the tRNA levels in control plants [7]. These observations indicate that Arabidopsis nuclear PRORP2 and 3 function as master RNase P enzymes, while the moss PpPPR_63 is not the sole RNase P enzyme in the nucleus.

It is unlikely that the slight reduction of the tRNAAsp (GUC) level resulted in growth retardation and abnormal branch formation in the PpPPR_63 KO mutant. PpPPR_63 may have a versatile function rather than pre-tRNA 5'9-end processing. Arabidopsis PRORP proteins are involved in the maturation of not only tRNAs but also mRNA or snoRNA [7]. Similarly, PpPPR_63 may have a versatile function rather than pre-tRNA 5'-end processing.

Organelle-localized PpPPR_67 and 104 have functions not completely redundant for tRNA accumulation

In this study, we generated single KO mutants of PpPPR_67 and PpPPR_104 but failed to obtain double KO mutants. Similarly, KO mutation of Arabidopsis PRORP1 was shown to

Figure 5. PpPPR_67 and PpPPR_104 KO mosses. (A) Schematic structure of PpPPR_67 and 104 KO loci. The nptII cassette was inserted into the 5th exon of PpPPR_67 and the 4th exon of PpPPR_104. (B) Detection of PpPPR_67 and 104 transcripts by RT-PCR. (C) Two-week-old protonemata colonies of the PpPPR_67 KO line (Δ67) and the PpPPR_104 KO line (Δ104). Scale bars = 5 mm. (D) Steady-state levels of chloroplast (chl) tRNAAsp isoacceptors with different anticodons, chl tRNAPhe (GAA) and mitochondrial (mit) tRNAAsp (GCA).

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Figure 6. Pre-tRNA cleavage assay of the recombinant proteins r67 and r104. Reactions containing 500 nM of chloroplast 5'-32P-pre-tRNAAsp (ACG) (upper), tRNAAsp (CCG) (middle), or tRNAAsp (UCU) (lower) and 500 nM of recombinant protein were quenched at 1, 5, 15, and 30 min points, resolved on 8% denaturing PAGE, and analyzed with a phosphoimager.

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result in embryonic lethality [6]. This suggests that PpPPR_67 and 104 have redundant functions. However, it is interesting to observe that a single mutant of PpPPR_67 and 104 displayed different phenotypes of protonemal growth and chloroplast tRNA^ARG\(^{\text{CCG}}\) were cleaved with efficient cleaved by PpPPR_104 than by PpPPR_67, whereas pre-tRNA^ARG\(^{\text{ACG}}\) and pre-tRNA^ARG\(^{\text{UCU}}\) were cleaved with similar efficiency by both proteins. This is the first evidence that PRORP enzymes have substrate specificity of tRNA processing. The anticodon of P. patens tRNA^ARG\(^{\text{CCG}}\) (AGC) is modified that including inosine in chloroplasts, and tRNA^ARG\(^{\text{ICG}}\) can accumulate of mature tRNA^ARG\(^{\text{ACG}}\). Therefore, the chloroplast pre-tRNA^ARG\(^{\text{ACG}}\) by PpPPR_104 is essential for the mitochondrial tRNACys, and nuclear tRNAAsp were amplified by PCR for the future.

Materials and Methods

Accession numbers

The sequences reported in this paper have been deposited in the DDBJ/GenBank/EMBL databases with accession numbers: PpPPR_63 mRNA, AB983707; PpPPR_67 mRNA, AB983708; PpPPR_104 mRNA, AB808584.

Plant material and preparation of DNA and RNA

The moss P. patens subsp. patens was grown at 25°C under continuous light (30 μmol photon m\(^{-2}\) s\(^{-1}\)), and genomic DNA and total cellular RNA were prepared from the moss protonemata as described previously [22].

Preparation of recombinant proteins

DNAs corresponding to the proteins (1–595 residues for PpPPR_63, 119–791 for PpPPR_67, and 125–993 for PpPPR104) were amplified by using the primers listed in Table S2 and cloned into pBAD/Thio-TOPO vector (Invitrogen). Proteins were expressed for 6 h at 18°C in E. coli BL21 and purified as described previously [23].

Preparation of pre-tRNA

DNAs representing precursors of chloroplast tRNA\(^{\text{Phe}}\), mitochondrial tRNA\(^{\text{Cys}}\), and nuclear tRNA\(^{\text{Asp}}\) were amplified by PCR from P. patens genomic DNA with oligonucleotide pairs T7-cpF5 and cpF3, T7-mtC5 and mtC3, and T7-mdGTGC5 and mdGTGC, respectively (Table S2). The 5′ oligonucleotides contained the T7 promoter sequence. The pre-tRNA\(^{\text{Phe}}\), pre-tRNA\(^{\text{Cys}}\), and nuclear pre-tRNA\(^{\text{Asp}}\) respectively 142, 153, and 140 nt long and were designed with respective 5′ leaders of 42, 51, and 40 nt and 5′ trailers of 27, 32, and 25 nt. Amplified DNAs (200 ng) were transcribed with T7 RNA polymerase (TaKaRa) for 1 h at 37°C, then digested with 5 U of RNase-free DNase I (TaKaRa) and 20 U of RNase Inhibitor (TaKaRa) for 15 min at 37°C. Synthesized pre-tRNAs were purified by phenol chloroform extraction.

For preparation of chloroplast pre-tRNA\(^{\text{Asp}}\) isocceptrors, the corresponding genes were cloned and in vitro transcribed. The obtained pre-tRNAs were dephosphorylated with Thermosensitive Alkaline Phosphatase (Promega) and were 5′-end radiolabeled with \(\text{[\text{32P]}\text{ATP}}\) and polynucleotidylase (TaKaRa). Labeled RNAs were purified from the gel after 8% polyacrylamide gel electrophoresis (PAGE) containing 7M urea. For preparation of chloroplast pre-tRNA\(^{\text{Asp}}\) isoacceptors, the corresponding genes were cloned and in vitro transcribed. The obtained pre-tRNAs were dephosphorylated with Thermosensitive Alkaline Phosphatase (Promega) and were 5′-end radiolabeled with \(\text{[\text{32P]}\text{ATP}}\) and polynucleotidylase (TaKaRa). Labeled RNAs were purified from the gel after 8% polyacrylamide gel electrophoresis (PAGE) containing 7M urea.

Pre-tRNA cleavage assay

Cleavage assays were performed as described previously [7]. Recombinant protein (100 ng) and in vitro-synthesized pre-tRNA (2 μg) were incubated for 1 h at 25°C in a 20 μl mixture of 20 mM Tris-HCl (pH 8.0), 30 mM KCl, 4.5 mM MgCl\(_2\), 20 μg/ml bovine serum albumin, and 2 mM DTT. A half of the reaction mixture was separated on 8% polyacrylamide-7 M urea gels and stained with ethidium bromide.

For cleavage assays of three chloroplast pre-tRNA\(^{\text{Asp}}\) isoacceptors, the 5′-end radiolabeled pre-tRNAs (500 nM each) and the recombinant proteins (500 nM each) were incubated and aliquots were withdrawn at the indicated times. Dried PAGE gels were analyzed by Storm 820 (GE Healthcare).

Determination of the 5′-end of tRNA

The \(\text{[32P]}\) 5′-end-labeled oligonucleotides cp-F3 or mt-C3 (Table S2) and 200 ng of in vitro-cleaved RNAs were mixed and reverse transcribed with ReverTraAce (ToYoBo) for 1 h at 42°C, then...
digested with RNase A and precipitated with ethanol. The sequence ladders were obtained by using template DNA, the radiolabeled primer cp-F3 or mt-C3, as described previously [22].

Subcellular localization

The DNA region from the 6th intron to the last codon of the *PpPPR_63* gene was inserted in-frame into the GFP coding region of pGFPmutNTPII (http://moss.nibb.ac.jp). The 3’-flanking region of *PpPPR_63* was inserted downstream of the nptII cassette of pGFPmutNTPII. The resultant plasmid p63-GFPKI (Fig. S1) was linearized with NotI and introduced into *P. patens* particle bombardment as described previously [23]. For N67-GFP and N104-GFP, cDNAs corresponding to an N-terminal part of the proteins (residues 1-112 and 1-105, respectively) were amplified by using the appropriate primers (Table S2) and cloned into pGEM-T Easy (Promega). The KO plasmid from moss genomic DNA by PCR using the appropriate primers (Figs. S4 and S5) were generated by the GPS-M mutagenesis System (NEB). The KO plasmids previously [24].

**(Table S2)** and cloned into pKSPGFP9 [23]. The resultant plasmid was cotransfected with pMr-RFP into *P. patens* protoplasts and GFP and RFP fluorescence were observed as described previously [24].

Generation of KO lines

The 5’ region (2960 bp) of *PpPPR_63*, a 2958-bp region of *PpPPR_67*, and a 3032-bp region of *PpPPR_104* were amplified from moss genomic DNA by PCR using the appropriate primers (Table S2) and cloned into pGEM-T Easy (Promega). The KO plasmid p63KO4-13A (Fig. S2) was generated by insertion of the nptII cassette into the 4th intron of the *PpPPR_63* gene by using the GPS-M mutagenesis System (NEB). The KO plasmid p67KO-1 and p67KO-2 (Figs. S4 and S5) were generated by insertion of the nptII cassette into HindIII in the 4th exon, or into StuI in the 5th exon, respectively, of the *PpPPR_67* gene. The KO plasmid p104KO (Fig. S6) was generated by insertion of the nptII cassette into the BglII site in the 4th exon of the *PpPPR_104* gene. These KO plasmids were digested with NotI and introduced into *P. patens* protoplasts and GFP and RFP fluorescence were observed as described previously [24].

Generation of complementation lines

For F63, the *PpPPR_63* cDNA coding region (1785 bp) amplified by PCR using specific primers (Table S2) was cloned into the SacI site of the overexpression vector pOX9WZ1 [25]. The mutated *PpPPR_63* (M63: D460A/D461A) was amplified from the F63 construct by using the primers 63DA-F and 63DA-R. Both constructs were linearized with NotI and introduced into the KO line Δ63.

Northern blot analysis

Total cellular RNA (5 μg) was separated on 10% polyacrylamide-7 M urea gels and transferred to nylon membranes. Hybridization and washing were performed at 42°C. The gene-specific oligonucleotide probes (Table S2) were labeled with DIG-dUTP and terminal deoxynucleotidyl transferase (Roche). Signals were acquired with EZ-capture (ATTO).

Supporting Information

**Figure S1 Generation of *PpPPR_63*-GFP knockin (KI) mosses.** (A) Schematic of insertion of p63-GFPKI into the 3’ terminal end of the *PpPPR_63* coding region. Translated and untranslated regions are represented by black and white boxes, respectively. Primers P1 to P3 used for PCR, and the expected amplified DNA sizes, are shown. (B) PCR analysis using the indicated primer pairs. The predicted 4.5- and 1.8-kb fragments were amplified from the KI lines. (C) Protonemal colonies of wild-type (WT) and KI mosses grown for 2 weeks on BCDATG medium plates without antibiotics. KI-10 was used for further analyses (Fig. 2). (ZIP)

**Figure S2 Generation of *PpPPR_63* knockout (KO) moss.** (A) Schematic diagrams of the *PpPPR_63* gene, the KO construct (p63KO-13), and the generated knockout locus. A Genetica G418-resistance gene (nptII) cassette was inserted into the 4th intron of *PpPPR_63*. Translated and untranslated regions are represented by black and white boxes, respectively. Primers P4 to P7 used for PCR and the expected amplified DNA sizes are shown. The DNA probe used in (C) is represented by grey bars. (B) PCR analysis using the indicated primer pairs. The predicted 1.9-, 2.2- and 5.5-kb fragments were amplified from the KO lines; 3.0 kb was amplified from the WT. (C) Total cellular DNAs (20 μg) from wild type (WT) and KO mosses were digested with *SphI* and hybridized with the *PpPPR_63* probe in (A). The probe was labeled with DIG-dUTP. Predicted hybridized fragments were detected in WT and KO mosses. (EPS)

**Figure S3 Generation of *PpPPR_63* complementation lines into Δ63-1.** (A) Schematic diagrams of *PpPPR_63* loci of wild type and Δ63-1. Primers P8 and P9 and the expected fragment sizes for PCR analysis are shown. (B) The *PpPPR_63* coding region (1.8 kb) amplified from the wild type cDNA was cloned into the *SacI* site of the overexpression vector, pOX9WZ1, which harbors a rice actin promoter, a c-myc tag and the Zeocin resistance cassette. The mutated *PpPPR_63* (M63: D460A/D461A) gene was modified from the F63 construct. (C) The predicted 1.8- and 3.0-kb fragments derived from the transgenic lines and wild type (WT), respectively, were amplified. The predicted 5.0-kb fragments were not amplified from Δ63-1 under the PCR conditions. F63-3 and M63-8 plants were used for further analysis as F63 and M63, respectively, in Fig. 4. Transgenic lines V-7 and V-8 were generated by transferring the vector only into the Δ63-1 moss. (EPS)

**Figure S4 Generation of *PpPPR_67* knockout (KO) lines.** (A) Schematic diagrams of the *PpPPR_67* gene (WT), the KO construct (p67KO-1), and the generated KO locus. A Genetica G418-resistance gene (nptII) cassette was inserted into the HindIII site of the 4th exon of *PpPPR_67*. Primers used for PCR and the expected amplified DNA sizes are shown. (B) The predicted 5.0-, 2.1- and 1.6-kb fragments were amplified from the transgenic lines, and 3.0 kb was amplified from the WT. The KO line 67-1-17 was used in Figs. S7 and S8. (EPS)

**Figure S5 Generation of *PpPPR_67* knockout (KO) lines.** (A) Schematic diagrams of the *PpPPR_67* locus, the KO construct (p67KO-2), and the targeted KO locus. A Genetica G418-resistance gene (nptII) cassette was inserted into the *SacI* site of the 5th exon of *PpPPR_67*. Primers and the amplified DNA sizes for PCR analysis are shown. (B) The predicted 5.0-, 2.3- and 1.5-kb fragments were amplified from the transgenic lines, and 3.0 kb was amplified from the WT. The KO line 67-2-2 was used as Δ67 in Figs. 5, S7, and S8. (EPS)

**Figure S6 Generation of *PpPPR_104* knockout (KO) line.** (A) Schematic diagrams of the *PpPPR_104* locus, the KO construct (p104KO), and the targeted KO locus. A Genetica G418-resistance gene (nptII) cassette was inserted into the BglII site of the 4th exon of *PpPPR_104*. Primers used for PCR and the expected fragment sizes are shown. (B) The predicted 5.0-, 1.8-
and 1.3-kb fragments were amplified from the transgenic line Δ104-7, and 3.0 kb was amplified from the wild type (WT). The KO line Δ104-7 was used as Δ104 in Figs. 5, S7, and S8.

**Figure S7** Northern blot analysis of chloroplast tRNAs. Total cellular RNAs (5 μg) from wild type (WT), Δ67-1-17, Δ67-2-2, and Δ104-7 protonemata were separated on 8% polyacrylamide containing 7 M urea and transferred to nylon membranes. Chloroplast tRNA gene-specific oligonucleotide probes (Table S2) were labeled with DIG-ddUTP and terminal deoxynucleotidyl transferase (Roche).

(TIF)

**Figure S8** Northern blot analysis of mitochondrial tRNAs. Total cellular RNAs (5 μg) were subjected to northern blot analysis as described in Fig. S7. Mitochondrial tRNA gene-specific oligonucleotide probes (Table S2) were labeled as described in Fig. S7.

(TIF)

**Tables**

| Table S1 | List of PRORP and PRORP-like proteins from various organisms. (XLSX) |
| Table S2 | Oligonucleotides used in this study. (XLSX) |

**References**

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