A Pentatricopeptide Repeat Protein Is Required for RNA Processing of clpP Pre-mRNA in Moss Chloroplasts*

Mitsuru Hattori‡, Hiroshi Miyake‡, and Mamoru Sugita‡

*From the ‡Center for Gene Research, Nagoya University, Furo-cho 1, Chikusa-ku, Nagoya 464-8602 and the §Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

Pentatricopeptide repeat (PPR) proteins are encoded by the nuclear genome as a large gene family in land plants. PPR proteins play essential roles in organelle-related functions, mostly in RNA-processing steps in plastids and mitochondria. In the moss Physcomitrella patens, there is also a large gene family, but the moss PPR proteins are likely to be divergent from those of higher plants. To investigate the function of plastid PPR proteins, we have generated and characterized a PPR protein gene disrupted of P. patens. The PPR531-11-disrupted mosses displayed abnormal phenotypic characteristics, such as a significantly smaller protonemal colony, different chloroplast morphology, and incomplete thylakoid membrane formation. In addition, the quantum yield of photosystem II was reduced in the disrupted mosses. To further investigate whether disruption of the PPR531-11 gene affects chloroplast gene expression, we performed Northern blot and reverse transcription polymerase chain reaction analyses. These analyses revealed that PPR531-11 has a role in intergenic RNA cleavage between clpP and 5′-rps12 and in the splicing of clpP pre-mRNA. Western blot analysis showed that disruption of PPR531-11 resulted in a reduced level of ClpP, photosystem II reaction center protein D1, and the stromal enzyme, ribulose-bisphosphate carboxylase/oxygenase. These reductions might result in the severely retarded growth of the protonemal colony. Taken together, we propose a model where PPR531-11 function affects the steady-state level of ClpP, which regulates the formation and maintenance of thylakoid membranes in chloroplasts. This is the first evidence of a PPR protein controlling the protein expression level of ClpP.

The pentatricopeptide repeat (PPR)2 is a degenerate 35-amino acid repeating motif that is found in animals, fungi, and plants (1). The PPR motif is similar to but distinct from the tetratricopeptide repeat motif, a well characterized protein interaction motif that is composed of 34 amino acids (2). A particularly large gene family encoding PPR proteins exists in plants, from mosses (3) to flowering plants (4), but not in fungi and animals. For instance, the Arabidopsis thaliana and the rice (Oryza sativa) genomes encode more than 400 PPR proteins. Most plant PPR proteins are predicted to be targeted to the mitochondria or chloroplasts (4). Many PPR proteins play important roles in a wide range of physiological and developmental functions, i.e. cytoplasmic male sterility (5, 6), fertility restoration (7), photosynthesis (8, 9), chloroplast biogenesis (10), and early or late embryogenesis (11, 12).

Many chloroplast genes of land plants are cotranscribed as polycistronic pre-RNAs, which are then extensively processed into shorter mature RNA species (13). Recently, several lines of evidence that PPR proteins are involved in post-transcriptional regulation in chloroplast gene expression have accumulated. For instance, the maize PPR protein CRP1 is required for intergenic RNA processing of petB and petD dicistronic mRNA (8). The maize protein PPR2 was shown to exist in large macromolecular complexes in the chloroplast stroma and was suggested to function in the synthesis or assembly of the plastid translation machinery (10). The Arabidopsis HCF152 protein is a plastid RNA-binding protein that is involved in the 5′ processing and splicing of petB pre-mRNA (14, 15). The Arabidopsis CRR2 protein is essential for the processing of rps7-ndhB dicistronic mRNA (16). Thus, PPR proteins might be involved in certain steps of RNA maturation in a gene-specific manner. Interestingly, some PPR proteins have also been identified as DNA-binding proteins (12, 17, 18). Despite the large number of PPR proteins in plants, there is only fragmented information concerning the relationship between PPR proteins and their target RNA or DNA molecules.

The moss Physcomitrella patens has recently emerged as a powerful model system in plant functional genomics (19). The genome sequences are known for the chloroplast (20), the mitochondrion (21), and the nucleus (22). Gene targeting is feasible in the nuclear and chloroplast genomes (23, 24). Furthermore, the P. patens gametophyte, the haploid phase of the life cycle, is dominant, making it possible to study the phenotypes of knockout directly without further crosses. We previously identified and characterized two chloroplast-localized PPR proteins, PPR513-10 and PPR566-6, from P. patens. Their genes were expressed differentially in protonemata grown under different light-dark conditions, suggesting that they have distinctive functions in chloroplasts (3). To investigate the function of chloroplast PPR proteins, we generated and characterized the...
A Novel PPR Protein in Moss

PPR531-11 (an isoform of PPR513-10) protein gene disruptant. In this study, a chloroplast PPR gene disrupted moss displayed abnormal phenotypic characteristics, such as significantly retarded growth of the protonemal colonies and smaller chloroplasts with an abnormal thylakoid membrane structure. Moreover, disruption of PPR531-11 resulted in aberrant RNA processing of clpP pre-mRNA. This is the first identification of an RNA species targeted by the moss chloroplast PPR protein.

EXPERIMENTAL PROCEDURES

Plant Material and Culture Conditions—The protonemata (juvenile gametophores) and the leafy shoots (adult gametophores) of P. patens subspecies patens were grown at 25 °C under continuous light (30 μmol/m²/s) on solidified BCD medium (25). For vegetative propagation, the protonemata were collected every 4 days and spread on fresh plates (26). Total cellular DNA and RNA and chloroplasts were isolated from the 4-day-old protonemata.

Isolation of DNA and RNA—Total cellular DNA was prepared by a cetyltrimethylammonium bromide method as described (27) and treated with RNase A (TaKara) to remove residual RNAs. Total cellular RNA was isolated with TRIzol (Invitrogen), chloroform, and isoamyl alcohol and finally treated with DNase I (TaKara). Chloroplast RNA was prepared from the isolated chloroplasts as described previously (28).

cDNA and Genomic DNA Sequencing—The genomic region of the PPR531-11 gene was amplified from total cellular DNA by PCR with the primers: 5'-GAGGAGCTGTGTTAGGGTCT-3' and 5'-GATCATTGGAACAAACTCGCCG-3' (3). The amplified DNA fragment (3879 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). cDNA derived from constitutively spliced mRNA coding for PPR531-11 was amplified and sequenced as described before (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

Moss Transformation—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5'-ATGCGCTGTTTGGCCGTGCTGCT-3' and P2, 5'-AGATATAATTTCTAGGGCTGATT-3'. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5'-ATGCGCTGTTTGGCCGTGCTGCT-3' and P2, 5'-AGATATAATTTCTAGGGCTGATT-3'. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

Moss Transformation—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5'-ATGCGCTGTTTGGCCGTGCTGCT-3' and P2, 5'-AGATATAATTTCTAGGGCTGATT-3'. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5’-ATGCGCTGTTTGGCCGTGCTGCT-3’ and P2, 5’-AGATATAATTTCTAGGGCTGATT-3’. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

Moss Transformation—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5’-ATGCGCTGTTTGGCCGTGCTGCT-3’ and P2, 5’-AGATATAATTTCTAGGGCTGATT-3’. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

Moss Transformation—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5’-ATGCGCTGTTTGGCCGTGCTGCT-3’ and P2, 5’-AGATATAATTTCTAGGGCTGATT-3’. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

Moss Transformation—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5’-ATGCGCTGTTTGGCCGTGCTGCT-3’ and P2, 5’-AGATATAATTTCTAGGGCTGATT-3’. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

Moss Transformation—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5’-ATGCGCTGTTTGGCCGTGCTGCT-3’ and P2, 5’-AGATATAATTTCTAGGGCTGATT-3’. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).

—To disrupt the PPR531-11 gene, the genomic region of the gene was amplified from pMH1-1 with the primers: P1, 5’-ATGCGCTGTTTGGCCGTGCTGCT-3’ and P2, 5’-AGATATAATTTCTAGGGCTGATT-3’. The amplified DNA fragment (3288 bp) was cloned into pGEM-T Easy vector (Promega, Madison, WI) to generate plasmid pMH1-1 and was sequenced using an ABI3100 Genetic Analyzer (PerkinElmer Life Sciences, Applied Biosystems) and appropriate primers as described previously (3). The amplified DNA fragment was sequenced and amplified as described above (3). Nucleotide sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB267806 (PPR531-11 gene) and AB267854 (PPR531-11 cDNA)).
A Novel PPR Protein in Moss

3'; RL1, 5'-GACTAGATTAACGTGGGTATG-3' and RL2, 5'-TGTAGAAAAACATTAGTGC-3'; D1, 5'-CGATTGGTTTGAAGACGTCTTTG-3' and D2, 5'-AAGCTGCTCCAT-3'.

ATACCTAACAA-3' and D3, 5'-TAACGCTTTAATAATTATG-3' and D2.

Primer Extension Analysis—To determine the RNA processing site of clpP pre-mRNA, primer extension was carried out using isolated chloroplast RNA (10 μg) and 5'-32P-labeled primer, 5'-TGGAGTCCCTCAAACGAGAGAGTTTGTCTAGAG-3' (nucleotide positions 13571–13537) (20). Reverse transcription was performed using ReverTra Ace (Toyobo). Sequence ladders were amplified with the same primers and a Thermo Sequenase Primer Cycle sequencing kit (Amersham Biosciences). The products and sequence ladder were loaded onto a 6% denaturing polyacrylamide gel.

Chloroplast Isolation—4-day-old protonemata (30 g of fresh weight) were incubated for 1 h at 25 °C in 8% mannitol and 2% Driserase 20 (Kyowa Hakko Kogyo). The protoplasts were suspended in buffer (330 mM sorbitol, 30 mM HEPES-KOH (pH 7.5), 2 mM EDTA, and 0.1% bovine serum albumin), and broken by passing the suspension through two layers of nylon mesh (20 μm pore size). The chloroplast pellet was collected by centrifugation at 4000 × g for 10 min at 2 °C (28).

Immunoblot Analysis of Chloroplast Proteins—Chloroplast protein was extracted from the isolated chloroplasts using buffer (2% Triton X-100, 10 mM Tris–HCl, 20% glycerol and 3 mM dithiothreitol). 9 μg of extract, and each protein dilution were loaded on SDS-12% or 15% (for detection of ClpP) PAGE gels. For immunoblotting, the gel was transferred to Immobilon™ Transfer Membranes (Millipore). The incubation and immunodetection were performed using ECL plus (Amersham Biosciences) protocols. Antibodies were provided by M. Ikekchi (α-spinach D1), T. Hisabori (α-TF1-B), F. Sato (α-spinach PsbO), and A. Watanabe (α-rice ClpP). α-Rice ClpP, α-spinach D1, and α-tobacco cp28 were used at a dilution of 1:5000, and α-TF1-B and α-spinach PsbO at a dilution of 1:10000.

RESULTS

Structure of the PPR531-11 Gene—In a previous study (3), we showed that two PPR proteins, PPR513-10 and PPR566-6, are plastid-localized in P. patens. Expression of the PPR513-10 gene is regulated in a light-dependent manner, suggesting PPR513-10 plays an important role in plastid function. Hence, we selected the PPR513-10 protein to investigate its function in plastids. Before this analysis, we amplified the genomic region using primers designed from the PPR513-10 cDNA (3) and determined that the genomic sequence consisted of...
seven exons and six introns. In this analysis, we noticed that the cDNA encoding PPR513-10 was derived from an alternatively spliced mRNA. We then isolated and sequenced the cDNA, which encodes a polypeptide of 531 amino acids with 11 PPR motifs (hereafter designated as PPR531-11, Fig. 1). There were two splicing donor sites at the border of exon 5 and intron 6 of the gene. If 312 bp or 258 bp of intron 6 were spliced out, mRNA encoding PPR513-10 or PPR531-11, respectively, would be produced (Fig. 2A). RT-PCR analysis showed that the mRNA encoding PPR531-11 was predominant in the moss protonemal cells (data not shown).

Knockout of the PPR531-11 Gene Results in Significantly Reduced Growth of Protonemata—To investigate the function of PPR531-11, we constructed and characterized PPR531-11 knock-out mosses (Fig. 2A). Among several G418-resistant mosses, we isolated two lines, 4-4 and 2-30, and performed Southern blot analysis to verify the targeted disruption (Fig. 2B). Probing with PPR531-11 gene detected the predicted 2.6-kb signal in the wild-type moss. By contrast, a 4.6-kb signal appeared in the G418-resistant moss lines, corresponding to the 2.0-kb nptII cassette integrated into the PPR531-11 locus (Fig. 2A). A uniform population of the transformed moss genome in the transgenic moss was further verified by PCR analysis (Fig. 2C). These results indicated the correct insertion of the nptII cassette into the targeted PPR531-11 gene locus. Furthermore, the absence of PPR531-11 transcript in the 4-4 and 2-30 transgenic lines was verified by RT-PCR (Fig. 2D). A band of 500 bp was detected in the wild-type moss but not in the transgenic PPR531-11 disruptants. By contrast, actin gene transcript was detected in the wild-type and PPR531-11 disruptant mosses. This result clearly indicates that PPR531-11 transcripts are absence from the disruptants, which are probably PPR531-11-deficient. Because the two disruptants displayed the same external phenotypes, the 4-4 transgenic moss was selected as the representative PPR531-11 disruptant and was further characterized.

The PPR531-11 disruptant, transgenic line 4-4, did display abnormal protonemal colonies. Compared with the wild-type moss colony, the protonemal colonies were significantly smaller in the disrupted moss. This phenotype was not caused by G418, because G418 was not added to the growth medium.
for phenotypic characterization. Even though glucose (0.5%, 1%, or 3%) and/or ammonium tartrate (5 mM) was supplemented in the BCD medium, the size of the moss colony was not restored in the disruptant. A concentration of 0.15 M (2.8%) glucose is known to induce caulonemal filaments (34). Such a small colony might result in poor induction of caulonemal filaments (Fig. 3, A and B). Generally, chloronemal cells contain more chlorophyll than caulonemal cells (35). The chlorophyll content was 632.7 ± 28.2 (n = 3) µg/g fresh weight in the wild-type and 982.7 ± 10.3 in the disruptant colonies. This also supports the fact that the induction of caulonemal filaments is retarded in the disruptants. The gametophores were somewhat smaller than those of the wild-type moss (Fig. 3C). The number of gametophores formed in the colony was ~50 in the disruptant, less than half of that of the wild-type (Fig. 3D). To investigate the functional status of the photosynthetic apparatus of the disruptant, 4-4, we measured quantum yield of PSI photochemistry (φPSII). The score of the disruptant was 30% lower than that of the wild-type. In addition, \( F_{m}'/F_{m} \) and \( F_{o}'/F_{o} \) were reduced by 40 and 28%, respectively, but the photochemical quenching (\( q_{p} \)) was not changed (Table 1). This indicated that the electron transfer from PSII to PSI was not interrupted, but the efficiency of PSII or accumulation of active PSII complexes was reduced in the disruptant.

PPR531-11 Disruptant Has Abnormal Chloroplasts—We also observed the morphology of the chloroplasts. The disruptant contained more chloroplasts per cell than the wild type (Fig. 3E). No apparent morphological differences in the chloroplasts were found in chloronemal and caulonemal cells, whereas the difference in chloroplast size was remarkable in gametophore cells of the disruptant (Fig. 3F). We then analyzed morphological changes in chloroplasts in the protonemata and gametophores by electron microscopy (Fig. 4). Although large starch granules accumulated in the protonemal chloroplasts from both wild-type and disruptant, thylakoid membranes were stacked differently. In the wild-type, several layers of straight inner membranes were stacked to form two distinct membrane regions, grana and stroma lamellae. In the disruptant, however, most membranes were arranged in a pairwise manner with a wide distance between the layers and they did not form two distinct membrane regions. These observations indicated that the PPR531-11 disruption resulted in abnormal stacking of thylakoid membranes.

Disruption of PPR531-11 Impaired Processing of clpP Pre-mRNA—A deficiency of plastid-localized PPR proteins results in abnormal RNA processing or accumulation of specific chloroplast gene transcripts in Arabidopsis (14, 15) and maize (8). We therefore expected that targeted disruption of the PPR531-11 gene would give rise to abnormal accumulation of plastid transcripts in the moss. To investigate this possibility, we initially performed DNA microarray analysis using the Physcomitrella chloroplast DNA chip (32). 100 signals were detected from 108 DNA fragments, and the signal intensity and RNA accumulation ratios of the PPR531-11 disruptant relative to the wild-type were analyzed. However, we could not identify the candidate genes quantitatively affected by PPR531-11 disruption (data not shown). We then performed Northern blot analysis to find aberrant transcript profiles. For this analysis, we selected 12 intron-containing plastid genes, 3′-rps12, ndhB, ycf66, petB-petD, ycf3, rps16, rpl2, ndhA, atpF, rpc1, and clpP. Because, some of PPR proteins are known to involve in RNA splicing. Most of the transcript patterns were not largely different in the wild-type and disruptant, except for clpP (Fig. 5A). Probing with a two-intron containing clpP gene, the predominant spliced and matured clpP mRNA (0.6-kb) and less abundant 1.7-kb transcript were detected in the wild-type. By contrast, the 3.2- and 1.7-kb transcripts accumulated to substantial levels, but the 0.6-kb transcript appeared at a much lower level (~35% of the wild-type level) in the PPR531-11 disruptant. The clpP gene is known to be cotranscribed with the downstream 5′-rps12 and rpl20 genes in tobacco (36). The gene-specific probes for 5′-rps12 and rpl20 also gave the longest 3.2-kb transcript, indicating the occurrence of a primary transcript of clpP to rpl20 in the moss. In the wild type, the 3.2-kb primary transcript was processed to produce the 1.7-kb unspliced clpP pre-mRNA, which was then immediately spliced to form the mature 0.6-kb mRNA (Fig. 5B). In the PPR531-11 disruptant, such RNA-processing events were obviously impaired.

To determine a cleavage site between clpP and 5′-rps12 in the primary transcript, primer extension analysis was carried out. The major cleavage sites were mapped to be ~53 or ~52 relative to the translation initiation codon of 5′-rps12 in both the wild-type and the disruptant (Fig. 5C). In the disruptant, numerous but small amounts of longer primer extension products were also detected. These could be primer-extended products from the aberrantly accumulated primary transcripts of 3.2 kb. The results indicated that the PPR531-11 disruption severely affected RNA processing of clpP pre-mRNA.

![A Novel PPR Protein in Moss](image)

**FIGURE 4. Representative images of chloroplast ultrastructure.** Electron microscopic pictures of the 4-day-old protonema (a and b) and 30-day-old gametophore (c and d) cells of the wild-type (a and c) and disrupted (b and d) mosses. Scale bars, 500 nm.

**TABLE 1**

Fluorescence parameters for WT and disruptant (4-4)

| Parameter | WT | Disruptant | Ratio of disruptant to WT |
|-----------|----|------------|-------------------------|
| φPSII     | 0.158 ± 0.008 | 0.113 ± 0.009 | 0.713                  |
| q_p       | 0.592 ± 0.023 | 0.598 ± 0.025 | 1.011                  |
| \( F_{m}'/F_{m} \) | 0.589 ± 0.046 | 0.358 ± 0.055 | 0.607                  |
| \( F_{o}'/F_{o} \) | 0.269 ± 0.017 | 0.193 ± 0.023 | 0.718                  |

*φPSII, quantum yield of PSII photochemistry; q_p, photochemical quenching; \( F_{m}'/F_{m} \), variable chlorophyll fluorescence yield (\( F_{m}' \), at the closed PSII center); \( F_{o}'/F_{o} \), maximum chlorophyll fluorescence yield (\( F_{o}' \), at the closed PSI center). Experiments were repeated four times, and results are expressed as a mean ± S.E.
higher levels in the disruptant (Fig. 6B, fragment g), probably due to aberrant accumulation of the 3.2-kb primary transcript containing rpl20. As a control we also performed RT-PCR analysis of petD pre-mRNA and confirmed that petD pre-mRNA splicing was not affected in the disruptant (Fig. 6C). We further quantified the amount of uncleaved transcripts between clpP and 5′-rps12 in the wild-type and disruptant (Fig. 6A, fragment d). Uncleaved transcripts accumulated to two to three times higher levels in the disruptant than in the wild-type. These observations clearly indicated that disruption of PPR531-11 resulted in significantly reduced efficiencies of both clpP pre-mRNA splicing and the intergenic cleavage between clpP and 5′-rps12.

Chloroplast Proteins Are Reduced in the PPR531-11 Disruptant—Chloroplast proteins were subjected to Western blot analysis because of abnormal thylakoid membranes stacking in the chloroplasts and the impairing of clpP mRNA maturation in the disruptant. As shown in Fig. 7, the ClpP protein was reduced to ~30% of the wild-type level. The PSII reaction center protein D1 and PSII 33-kDa extrinsic protein PsbO, ATPase β subunit, and Rubisco large (Fig. 7, LS) and small subunit (SS) were also reduced to 10–30% of the wild-type level. By contrast, the nuclear-encoded plastid proteins, RNA-binding protein cp28 (37) and light-harvesting chlorophyll-binding protein (LHCII apoprotein), were not reduced in the disruptant.

**DISCUSSION**

We present a phenotypic and biochemical characterization of a knock-out mutant of the gene encoding a PPR protein in the moss *P. patens*, designated PPR531-11. The cognate gene was highly expressed in the light but not in the dark, suggesting its essential roles in chloroplast functions (3). In this study, disruption of PPR531-11 caused significantly retarded growth of the protonemal colony, probably due to blocking the transition of chloronemal to caulonemal filaments. Caulonema formation in the disruptant was not restored by the addition of glucose to the BCD medium, which is known to induce caulonemal filaments (34). In addition, despite higher amounts of chlorophyll in the protonemata, the quantum yield of photosystem II was reduced in the disruptant (Table 1). Unlike the wild-
type chloroplasts, consisting of two distinct membrane regions, most thylakoid membranes were not stacked and did not form distinct membrane regions in the disruptant chloroplasts (Fig. 4). A similar thylakoid membrane has been observed in the partial clpP disruptant of tobacco (38). Thus, disruption of PPR531-11 largely affected the structure and function of the moss chloroplasts.

Several loss-of-function mutations of PPR protein genes have been isolated, and they are mostly involved in RNA processing in chloroplasts (8, 14, 16). In this study, disruption of the PPR531-11 gene resulted in aberrant accumulation of the primary transcript from clpP-5'-rps12-rpl20 genes and unspliced clpP pre-mRNA. The observed accumulation of the primary transcript can be explained due to a high transcription rate, decreased turnover, or impaired endonucleolytic cleavage of this transcript. As shown in Fig. 6A, the efficiencies of splicing and intergenic RNA cleavage of clpP pre-mRNA were severely reduced in the disruptant, and subsequently the primary transcript and unspliced clpP transcript accumulated. This is likely the reason why the primary transcript highly accumulated in the disruptant. By contrast, rps12 pre-mRNA was normally trans-spliced in the disruptant and accumulated at similar levels of the wild type. This indicates that PPR531-11 is involved in the two RNA processing steps, site-specific cleavage and splicing, for maturation of clpP pre-mRNA. Arabidopsis HCF152 also functions in intergenic RNA processing between psbH and petB (14). The HCF152 forms a homodimer in vivo and may facilitate to interact with both intron-exon splicing junctions of the petB pre-mRNA (15). At present, whether the moss PPR531-11 has a binding ability to a target RNA molecule and makes a dimer or larger complexes with other factors remains to be addressed.

Maize PPR protein, CRP1, was reported to be required for the translation of the petA mRNA and for the accumulation of processed petB and petD mRNAs (8). Recently, RNA sequences associated with CRP1 were identified (39). This indicates that some PPR protein targets to distinct mRNA molecules. We cannot exclude the possibility that PPR531-11 interacts with other target RNA species. At present, we have no experimental evidence to support this possibility for PPR531-11.

It is interesting to note that splicing of clpP was not completely blocked in the disruptant. Such a partial inhibition of splicing was also observed for petB RNA in the Arabidopsis HCF152 knock-out mutant (14, 15). PPR531-11 may facilitate splicing of clpP RNA by interacting with central major splicing factors, such as maize CRS1 and CRS2 (40). Similarly,
PPR531-11 may be indirectly involved in site-specific cleavage. If PPR531-11 were a major determinant for site-specific cleavage and recruited a RNA cleavage enzyme, multiple cleavage sites different from −53 and −52 sites might have been detected in the PPR531-11 disruptant. However, such aberrant cleavage sites were not detected. Thus, PPR531-11 might interact with other factors and facilitate clpP mRNA maturation. Because PPR531-11 consists of a rather simple array of PPR motifs, it is unlikely that PPR531-11 itself has enzymatic activities of RNA cleavage and splicing.

ClpP is a proteolytic subunit of the ATP-dependent Clp protease, which locates in the chloroplast stroma, but its exact role remains unclear (41). Complete disruption of the chloroplast-encoded clpP gene was not successful in tobacco (38) and Chlamydomonas (42). Nonphotosynthetic maize BMS suspension cells lack chloroplast clpP gene from the chloroplast genome (43). This suggests that clpP has an indispensable function for cell survival and photosynthesis. Complete loss of the clpP gene from the chloroplast genome results in ablation of the shoot system of tobacco, suggesting that ClpP-mediated protein degradation is essential for shoot development (44). ClpP protein was shown to accumulate at a higher level in young plant than in matured in A. thaliana (45). The moss PPR531-11 disruptant displayed severe phenotypes in the protonemata as did normal phenotypes in the adult gametophores. This suggests that function of ClpP protease is more important in the vegetatively growing protonemata.

Recently, Zheng et al. (45) reported that an antisense repression mutant of the nuclear clpP4 gene contains smaller chloroplasts without a definable thylakoid membrane in A. thaliana. This indicates that clpP disruption affects chloroplast development. Taken together, we speculate that the reduced level of ClpP leads to an abnormal thylakoid membrane formation and the reduction of chloroplast proteins, D1, PsbO, H⁺-ATPase, and Rubisco. In A. thaliana, antisense transgenic lines of the nuclear clpP6 gene also showed the reduced levels of other Clp protease, D1, ATPase β subunit, and Rubisco (46). The ClpP protein was reduced to ~30% of the wild-type level (Fig. 7). This correlates to reduction of mature clpP mRNA level (Figs. 5 and 6). By contrast, the steady-state levels of psbA mRNA encoding D1 and rbcL mRNA for the Rubisco large subunit accumulated at similar levels in the wild-type and the disruptant (Fig. 5A). Accordingly, we suggest that translational or post-translational control of the accumulation of D1 and LS proteins is impaired in the disruptant. This supports the hypothesis that chloroplast-encoded ClpP primarily contributes to the quality control of chloroplast proteolysis (41). Our study may provide a clue to the role of ClpP in the chloroplasts. A certain level of accumulation of ClpP protein may be required for normal organization of thylakoid membranes and maintenance of Rubisco level (Fig. 8). An alternative explanation is that the chloroplast-encoded proteins were reduced by a possible reduction in the chloroplast ribosomal proteins. As shown in Fig. 6B, the steady-state levels of rps12 and rpl20 mRNAs were not decreased in the disruptant. Therefore, the entire phenotype of the mutant might be caused by via the reduction in ClpP than via a reduction in the ribosomal proteins. However,
we cannot exclude the possibility that the levels of ribosomal proteins S12 and L20 are decreased and hence functional ribosomes are limited in this disruptant.

The moss *P. patens* nuclear genome size is estimated to be 511 Mb (19), which is similar to the 430-Mb rice genome. The whole genome sequence has been finished this year and is already available at DOE Joint Genome Institute and PHYSCObase. Our blast search revealed that 103 PPR protein genes were identified. The moss PPR gene family is four times smaller than those of flowering plants. This suggests that the PPR protein gene family expanded widely and diverged significantly in concert with the development of advanced, multicellular forms of plants during evolution. Flowering plants are composed of multiple and more complicated tissues and organs that contain different types of plastids (47). Although all types of plastids contain identical plastid genomes, their expression could be differentially regulated in a tissue- or organ-specific manner. Accordingly, multiple sets of regulatory factors might be required for plastid type-specific gene expression at the transcriptional, post-transcriptional, or translational levels (48). By contrast, the moss *P. patens* is a nonvascular plant and has a simple organization. No apparent plastid differentiation occurs, and only chloroplasts exist in the cells (49). Thus, plastid ontogeny in mosses is distinctly different from that in vascular plants. In addition, the size and gene organization of the chloroplast genome is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52). By contrast, the moss *P. patens* is highly conserved, but RNA editing frequency differs greatly between the mosses and vascular plants. For instance, only two RNA editing sites are found in *P. patens* (50). *Arabidopsis* CRR4 with 11 PPR motifs is essential for RNA editing of *ndhD* mRNA (51, 52).

Acknowledgments—We thank Dr. Takahiro Nakamura for valuable discussion and Dr. Mitsuhito Matsuo for technical guidance of analytical PSII fluorescence measurements. We also thank Dr. Masahiko Ikeuchi, Dr. Tohru Hitabori, Dr. Fumihiro Sato, and the late Prof. Akira Watanabe for kindly providing us with antibodies and Dr. Jesse Machuka for pMBL6 encoding nptII gene cassette.

REFERENCES

1. Small, I. D., and Peeters, N. (2000) *Trends Biochem. Sci.* **25**, 46 – 47
2. Blatch, G. L., and Lassle, M. (1999) *BioEssays* **21**, 932 – 939
3. M. Hattori and M. Sugita, unpublished observation.

A Novel PPR Protein in Moss
A Novel PPR Protein in Moss

137–150
43. Cahoon, A. B., Cunningham, K. A., and Stern, D. B. (2003) *Plant Cell Physiol.* **44**, 93–95
44. Kuroda, H., and Maliga, P. (2003) *Nature* **425**, 86–89
45. Zheng, B., Macdonald, T. M., Sutinen, S., Hurry, V., and Clarke, A. K. (2006) *Planta* **224**, 1103–1105
46. Sjögen, L. L. E., Stanne, T. M., Zheng, B., Sutinen, B., and Clarke, A. K. (2006) *Plant Cell* **18**, 2635–2649
47. Herrmann, R. G., Westhoff, P., and Link, G. (1992) *Cell Organelles* (Herrmann, R. G., ed) pp. 275–349, Springer-Verlag, Vienna
48. Yamaguchi, K., Mayfield, S. P., and Sugita, M. (2005) *Photosystem II: The Light-Driven Water Plastoquinone Oxidoreductase* (Wydrynski, T., and Satoh, K., eds) pp. 650–668, Kluwer Academic Publishers, Netherlands
49. Duckett, J. G., and Renzaglia, K. S. (1988) *Adv. Bryol.* **3**, 33–93
50. Shikanai, T. (2006) *Cell Mol. Life Sci.* **63**, 698–708
51. Kotera, E., Tasaka, M., and Shikanai, T. (2005) *Nature* **433**, 326–330
52. Okuda, K., Nakamura, T., Sugita, M., Shimizu, T., and Shikanai, T. (2006) *J. Biol. Chem.* **281**, 37661–37667