BRIEF COMMUNICATION

Chloroplast ribonucleoprotein-like proteins of the moss *Physcomitrella patens* are not involved in RNA stability and RNA editing

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Abstract

Many RNA recognition motif (RRM)-containing proteins are known to exist in chloroplasts. Major members of the RRM protein family, which are chloroplast ribonucleoproteins (cpRNPs), have been investigated in seed plants, including tobacco and *Arabidopsis thaliana*, but never in early land plants, such as bryophytes. In this study, we surveyed RRM proteins encoded in the moss *Physcomitrella patens* genome and predicted 25 putative chloroplast RRM proteins. Among them, two RRM-containing proteins, PpRBP2a and PpRBP2b, resembled cpRNPs and were thus referred to as cpRNP-like proteins. However, knockout mutants of either one or two *PpRBP2* genes exhibited a wild type-like phenotype. Unlike *Arabidopsis* cpRNPs, the levels of mRNA accumulation in chloroplasts were not affected in the *PpRBP2* knockout mutants. In addition, the efficiency of RNA editing was also not altered in the mutants. This suggests that PpRBP2a and 2b may be functionally distinct from *Arabidopsis* cpRNPs.

Additional key words: pentatricopeptide repeat protein; photosynthesis gene; posttranscriptional regulation; RNA-binding protein; RNA processing.

The components of the photosynthetic apparatus, as well as the transcriptional and translational machinery, are encoded by the chloroplast and nuclear genomes in algae and plants (Allen *et al.* 2011). The nucleus-encoded components are synthesized as precursor polypeptides that are imported into the chloroplasts and subsequently assembled with chloroplast-encoded components. The expression of many chloroplast genes is posttranscriptionally regulated by nuclear-encoded RNA binding proteins (del Campo 2009, Stern *et al.* 2010). Among them, RNA recognition motif (RRM)-containing proteins are considered to be major regulators in various types of RNA metabolism in chloroplasts (Nickelsen 2003, Maris *et al.* 2005, Tillich *et al.* 2010). The RRM is about 80 amino acids long and contains two consensus motifs, RNP1 and RNP2. RNP1 is highly conserved with 8 amino acids located near its center, while RNP2 is not as well conserved with 6 amino acids found near its N-terminus. RRM folds into a $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\beta 3$ - $\alpha 2$ - $\beta 4$ structure and forms

an anti-parallel four-stranded β -sheet, $\beta 2\beta 3\beta 1\beta 4$, in this order. RNP1 and RNP2 are juxtaposed on the two central β strands, $\beta 3$ and $\beta 1$, respectively (Nagai *et al.* 1990). This arrangement is responsible for RNA binding.

Chloroplast RRM proteins were first identified in tobacco as approximately 30-kDa single-stranded DNA-binding proteins (Li and Sugiura 1990). They were named ‘chloroplast ribonucleoprotein’ (cpRNP) as they featured a high abundance of chloroplasts and their amino acid sequences were similar to yeast and animal RRM-containing ribonucleoproteins, such as heterogeneous nuclear ribonucleoprotein (hnRNP) and U1 small nuclear ribonucleoprotein (snRNP). Nuclear-encoded cpRNPs are 250–340 amino acids long and have a characteristic domain organization: an N-terminal transit peptide, which is removed after import into chloroplasts, is followed by an acidic region at the N-terminus of the mature cpRNP and two consecutive RRM. The cpRNPs, which associate with ribosome-free mRNAs and pre-tRNAs and form

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Abbreviations: 5-CLF – 5-formyltetrahydrofolate cyclo-ligase; cpRNP – chloroplast ribonucleoprotein; GFP – green fluorescence protein; GUS – β -glucuronidase; hnRNP – heterogeneous nuclear ribonucleoprotein; KO – knockout; PABP – poly(A)-binding protein; PPR – pentatricopeptide repeat; RRM – RNA recognition motif; snRNP – small nuclear ribonucleoprotein.

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heterogeneous complexes in the stroma (Nakamura *et al.* 1999), are involved in mRNA stabilization (Nakamura *et al.* 2001), mRNA 3'-end formation (Schuster and Grissem 1991), and RNA editing (Hirose and Sugiura 2001, Tillich *et al.* 2009). *Arabidopsis thaliana* has 10 members of the cpRNP family. In addition to 10 cpRNPs, at least 13 non-cpRNP RRM proteins have been identified in *A. thaliana*, but their function in chloroplasts remains unknown (Ruwe *et al.* 2011). It is possible that cpRNPs and non-cpRNP RRM proteins are widely distributed among land plant species, including early land plants, such as bryophytes.

To identify chloroplast RRM proteins in the moss *P. patens*, we surveyed the *P. patens* genome for RRM proteins that were predicted to be localized in chloroplasts. To this end, various RRM proteins were used as queries and *BLAST* searched against the plant genomics resource *Phytozome v. 11.0* (<https://phytozome.jgi.doe.gov/pz/portal.html>). The localization of chloroplasts was predicted by *TargetP* and *Predotar* programs. This analysis revealed over 220 RRM proteins in *P. patens*, 11% of which were predicted to be chloroplast RRM proteins.

Two chloroplast pentatricopeptide repeat (PPR) proteins, PpPPR_3 (Pp3c5_10110) and PpPPR_76 (Pp3c16_280), which consist of an N-terminal RRM and 15 PPR motifs, were previously identified in *P. patens* (Sugita *et al.* 2013). These two PPR proteins show 59% amino acid identity and are 87% similar to each other. In this study, we found 25 additional chloroplastic RRM proteins, including their paralogs (Table 1). These could be further divided into several groups based on their amino acid length and identities, position of the RRM, and the presence of additional domains (Fig. 1S, *supplement available online*). Pp3c9_17530 (named PpRBP1) is 674 amino acids long and contains a C-terminal RRM. This seems to be unique to *P. patens* because of its absence in *Arabidopsis*. Pp3c25_3310 contains a C-terminal PWI domain (Pfam ID: PF01480), which is commonly found in splicing factors. *Arabidopsis* has two proteins with a PWI domain (At2g29210 and At1g60200). Pp3c25_3310 is likely the homolog of At1g60200. Pp3c18_17520 consists of a domain of the 5-formyltetrahydrofolate cyclo-ligase (5-FCL) family (Pfam ID: PF01812) and a C-terminal RRM. 5-FCL, which catalyzes the interchange of

Table 1. Putative chloroplastic RRM proteins in *Physcomitrella patens*. ^a Locus name of RRM protein represents the gene model (version 3.3) assigned in *COSMOSS* The *Physcomitrella patens* resource database (www.cosmoss.org). ^b Length (aa) includes a transit peptide that can be removed after import into the chloroplasts. ^c Location of RRM protein was predicted by *TargetP 1.1* (<http://www.cbs.dtu.dk/services/TargetP/>) and *Predotar* (<http://urgi.versailles.inra.fr/predotar/predotar.html>). Prediction scores by *TargetP* are indicated in parenthesis. Reliability class, from 1 (score, >0.8), 2 (0.8–0.6), 3 (0.6–0.4), and 4 (0.4–0.2), where C1 indicates the strongest chloroplast prediction. C, M, N, and n.d. indicate chloroplasts, mitochondria, nucleus, and 'not determined', respectively. ^d According to Mueller *et al.* (2014), Fesenko *et al.* (2016).

Locus name ^a (Paralogous loci)	RRM protein name	RRM No.	Length ^b (aa)	Predicted location ^c TargetP	Location Predotar	Location experimentally determined	Detected in chloroplasts ^d	<i>Arabidopsis</i> homologs
Pp3c9_17530	PpRBP1	1	674	C1 (0.83)		C		
Pp3c18_16140 (Pp3c19_7020, Pp3c1_27480, Pp3c21_4170)		1	275	C1 (0.91)		N		At4g35785
Pp3c12_22790 (Pp3c8_3970, Pp3c3_28550)		1	399	C1 (0.92)	C	N		At1g16610 (SR45)
Pp3c25_3310		1	817	C3 (0.52)		n.d.		At1g60200
Pp3s152_10 (Pp3s5_15600)	SCL30A-like	1	370	C2 (0.61)		C		At3g13570 (SCL30A)
Pp3c8_3240		1	267	C2 (0.53)		N		At5g04600
Pp3c18_17520	COG0212-like	1	456	C2 (0.65)	C	C		At1g76730 (COG0212)
Pp3c1_41900	RBD1-like	1	523	C1 (0.88)	C	C		At1g70200 (RBD1)
Pp3c1_20440		1	561	C1 (0.89)	C	n.d.		At3g27700
Pp3c12_15180		1	533	C4 (0.35)	C	n.d.		
Pp3c12_9890	PpRBP2a	2	287	C1 (0.87)	C	C	Yes	cpRNPs
Pp3c24_17170	PpRBP2b	2	281	C1 (0.91)	C	C		cpRNPs
Pp3c5_17010		2	475	C2 (0.61)	C	n.d.		At1g09230 (U11/U12-65K)
Pp3c8_16740	PpRBP3a	3	418	C1 (0.84)	C	C	Yes	
Pp3c24_17150	PpRBP3b	3	478	M3 (0.41)		C and M	Yes	
Pp3c17_14650 (Pp3c14_9430)		3	446	C3 (0.51)	C	N		
Pp3c10_4970 (Pp3c14_4340)	PABP-like	4	643	C3 (0.47)		N		At2g36660 (PAB7)

5-formyltetrahydrofolate to 5,10-methenyltetrahydrofolate, is likely a homolog of *Arabidopsis* COG0212 that is targeted to chloroplasts and is required for embryo viability (Pribat *et al.* 2011). However, *Arabidopsis* COG0212 lacks an RRM. Recently, chloroplast RNA-binding protein RBD1 (At1g70200) and organelle RRM protein 1 (ORRM1, At3g2093) have been characterized in *Arabidopsis*: RBD1 is involved in chloroplast 23S rRNA processing (Wang *et al.* 2016), while ORRM1 is the chloroplast RNA editing factor required for several editing sites (Sun *et al.* 2013). *P. patens* has a homolog of RBD1 (Pp3c1_41900), but does not possess ORRM1.

Since two RRM-containing proteins, Pp3c12_9890 and Pp3c24_17170, showed 53% amino acid identity with each other, we named them PpRBP2a and PpRBP2b, respectively. Proteomic analysis detected PpRBP2a in chloroplasts as a highly abundant protein (Mueller *et al.* 2014, Fesenko *et al.* 2016). Expression data deposited in *Phytozome v. 11.0* indicates that PpRBP2b mRNA is highly expressed. Two PpRBP2 proteins showed high amino acid identity (>40%) with *Arabidopsis* cpRNPs, CP28A, CP29B, and CP31B, and may thus be cpRNP-like proteins. The other member containing two RRM, Pp3c5_17010 is expressed at low levels. Proteins containing three RRM, Pp3c8_16740 (PpRBP3a) and Pp3c24_17150 (PpRBP3b), were detected in the *P. patens* chloroplasts by proteomics analyses (Mueller *et al.* 2014, Fesenko *et al.* 2016), although their homologs are not found in *Arabidopsis*. Rather, At2g42890 (AML2, ARABIDOPSIS-MEI12-LIKE 2) was identified in *Arabidopsis* as a chloroplast protein containing three RRM (Ruwe *et al.* 2011), but this protein was not found in *P. patens*. Two poly(A)-binding protein (PABP)-like proteins were identified as chloroplastic RRM proteins. *Arabidopsis* has eight PABPs, two of which, PAB4 (At2g23350) and PAB7 (At2g36660), were predicted to be chloroplast PABPs (Belostotsky 2003). However, *P. patens* PABP-like proteins were targeted to the nucleus but not to chloroplasts (data not shown).

To confirm the localization of chloroplasts, N-terminal sequences of RRM proteins were fused to green fluorescence protein (GFP) or β -glucuronidase (GUS) and then expressed in *P. patens* protonema cells (Ichinose *et al.* 2013). Among the 13 RRM proteins tested, eight were targeted to chloroplasts and five to the nucleus (Table 1; Fig. 2S, *supplement available online*). It is interesting to observe that PpRBP3b was targeted to both chloroplasts and mitochondria. Thus, at least 10 RRM proteins, including PPR proteins, have been confirmed to exist in *P. patens* chloroplasts. Cellular localization of the remaining RRM proteins has yet to be determined.

Proteomics analysis revealed that some cpRNPs (CP29, CP31, and CP33) were cold-inducible proteins in *Arabidopsis* and wheat (Amme *et al.* 2006, Sarhadi *et al.* 2010). To examine the effect of cold temperature on the expression of five *PpRBP* genes, moss protonemata grown at 25°C were transferred to 4°C and grown for 4 d, and

Fig. 1. Northern blot analysis of chloroplast genes. Total RNA from wild type (WT) and knockout (KO) mutant mosses grown at 25°C or 4°C was separated on a 1% agarose gel and subjected to Northern blot analysis using chloroplast gene-specific DNA probes (Goto *et al.* 2016). Analyzed mutants indicate single ($\Delta 2a-1$, $\Delta 2b-9$) or double ($\Delta 2a/2b-21$, $\Delta 2a/2b-26$) mutants of *PpRBP2* genes. Ten μ g of total RNA was loaded on the gel to detect mRNAs (5 μ g of total RNA to detect processed 23S rRNAs). The positions of RNA size markers are indicated as 0.2 to 8 kb.

quantitative RT-PCR was performed on these genes. The transcript levels of *PpRBP2a* and *PpRBP2b* increased 3- and 6-fold, respectively, at 4°C compared to levels at 25°C. In contrast, *PpRBP1*, *PpRBP3a* and *PpRBP3b* transcripts were detected at similar levels in moss grown at 25 and 4°C. This suggests that PpRBP2a and PpRBP2b can be classified as cpRNPs. To further investigate their function, we constructed single and double knockout (KO) mutants of *PpRBP2* genes. Null KO mutants were verified by RT-PCR using gene-specific primers (Fig. 3SA, *supplement available online*). Surprisingly, all KO mutants exhibited the wild type (WT)-like phenotype, even on different growth media (BCD or BCDATG) and at 25°C or at 4°C (Fig. 3SB, *supplement available online*). Single and double mutants of *Arabidopsis* CP31A and CP29A exhibited the WT-like phenotype at 23°C, while the *cp29a* single and *cp29a/31a* double mutants showed bleaching of newly emerged leaves at 8°C (Kupsch *et al.* 2012). The loss of *Arabidopsis* cpRNP CP33A yielded

albino seedlings that exhibited aberrant leaf development (Teubner *et al.* 2017). A global loss of the accumulation and processing of chloroplast transcripts was observed in *cp33a* null mutants (Teubner *et al.* 2017). Similarly, CP31A and CP29A are required for the accumulation of target mRNAs and for processing by a hidden break in 23S rRNA under cold stress (Kupsch *et al.* 2012). They are thought to confer cold stress tolerance by influencing various chloroplast RNA processing steps (Kupsch *et al.* 2012). In contrast, no single and double KO mutants of *PpRBP2* genes affected the levels of accumulation of the examined *ndhF*, *petB*, *atpB*, and *psaB*, and processed 23S rRNA transcripts (Fig. 1).

Tobacco cpRNP CP31 was shown by an *in vitro* assay to play a role in C-to-U RNA editing of *psbL* and *ndhB* transcripts as a common editing factor (Hirose and Sugiura 2001). In *Arabidopsis*, the loss of CP31A influences the efficiency of editing at 13 out of 34 examined editing sites in chloroplasts (Tillich *et al.* 2009). In this mutant, the efficiency of RNA editing at an editing site in *ndhF* and at three sites in *ndhD* transcripts was reduced to 60% of the level in WT transcripts (Tillich *et al.* 2009). In *P. patens* (moss) chloroplasts, two editing sites have been identified in chloroplasts (Miyata and Sugita 2004). Editing at the *rps14*-C2 site occurs at 70–80% efficiency and creates a translation initiation codon AUG. In addition, the *rps14*-1C site in the 5'-untranslated region of the *rps14* transcript is edited at a very low efficiency (<10%). This editing is

unique to *P. patens* and has not been found in other plant species (Miyata and Sugita 2004). To investigate the effect of RNA editing at these sites in the KO mutants of *PpRBP2* genes, we cloned and sequenced 20 *rps14* cDNAs. This analysis showed that the efficiency of RNA editing at the *rps14*-C2 site was 65% in WT and 75% in the *PpRBP2a/2b* double KO mutant. The efficiency of RNA editing at the *rps14*-1C site was 10% in WT and 5% in the double KO mutant. This suggests that cpRNP-like PpRBP2a and PpRBP2b might not be primarily involved in RNA editing.

Our results showed that various types of RRM proteins exist in the moss *P. patens* chloroplasts. Half of the members of the chloroplast RRM family are cpRNPs in *Arabidopsis* (Ruwe *et al.* 2011), while only two cpRNP-like RRM proteins exist in *P. patens*. The *P. patens* cpRNP-like proteins, PpRBP2a and PpRBP2b, are structurally similar to *Arabidopsis* cpRNPs, especially CP28, CP29, and CP31, but their function does not seem to be identical. Chloroplast RRM-type RNA-binding proteins might have expanded during the evolution of plants. The RRM protein family is thought to govern chloroplast gene expression by regulating RNA metabolism. To understand the specialized posttranscriptional mechanism in chloroplasts, functional characterization of RRM proteins and other related RNA-binding proteins needs to be urgently performed.

References

- Allen J.F., de Paula W.B.M., Puthiyaveetil S. *et al.*: A structural phylogenetic map for chloroplast photosynthesis. – *Trends Plant Sci.* **16**: 645-655, 2011.
- Amme S., Matros A., Schlesier B. *et al.*: Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. – *J. Exp. Bot.* **57**: 1537-1546, 2006.
- Belostotsky D.A.: Unexpected complexity of poly(A)-binding protein gene families in flowering plants: Three conserved lineages that are at least 200 million years old and possible auto- and cross-regulation. – *Genetics* **163**: 311-319, 2003.
- del Campo E.M.: Post-transcriptional control of chloroplast gene expression. – *Gene Reg. Syst. Biol.* **3**: 31-47, 2009.
- Fesenko I., Seredina A., Arapidi G. *et al.*: The *Physcomitrella patens* chloroplast proteome changes in response to protoplastation. – *Front. Plant Sci.* **7**: 1661, 2016.
- Goto S., Kawaguchi Y., Sugita C. *et al.*: P-class pentatricopeptide repeat protein PTSF1 is required for splicing of the plastid pre-tRNA^{Ile} in *Physcomitrella patens*. – *Plant J.* **86**: 493-503, 2016.
- Hirose T., Sugiura M.: Involvement of a site-specific *trans*-acting factor and a common RNA-binding protein in the editing of chloroplast mRNAs: development of a chloroplast *in vitro* RNA editing system. – *EMBO J.* **20**: 1144-1152, 2001.
- Ichinose M., Sugita C., Yagi Y. *et al.*: Two DYW subclass PPR proteins are involved in RNA editing of *ccmF*c and *atp9* transcripts in the moss *Physcomitrella patens*: First complete set of PPR editing factors in plant mitochondria. – *Plant Cell Physiol.* **54**: 1907-1916, 2013.
- Kupsch C., Ruwe H., Gusewski S. *et al.*: *Arabidopsis* chloroplast RNA binding proteins CP31A and CP29A associate with large transcript pools and confer cold stress tolerance by influencing multiple chloroplast RNA processing steps. – *Plant Cell* **24**: 4266-4280, 2012.
- Li Y., Sugiura M.: Three distinct ribonucleoproteins from tobacco chloroplasts: each contains a unique amino terminal acidic domain and two ribonucleoprotein consensus motifs. – *EMBO J.* **9**: 3059-3066, 1990.
- Maris C., Dominguez C., Allain F.H.T.: The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. – *FEBS J.* **272**: 2118-2131, 2005.
- Miyata Y., Sugita M.: Tissue- and stage-specific RNA editing of *rps14* transcripts in moss (*Physcomitrella patens*) chloroplasts. – *J. Plant Physiol.* **161**: 113-115, 2004.
- Mueller S.J., Lang D., Hoernstein S.N.W. *et al.*: Quantitative analysis of the mitochondrial and plastid proteomes of the moss *Physcomitrella patens* reveals protein macrocompartmentation and microcompartmentation. – *Plant Physiol.* **164**: 2081-2095, 2014.
- Nagai K., Oubridge C., Jessen T.H. *et al.*: Crystal structure of the RNA-binding domain of the U1 small nuclear ribonucleoprotein A. – *Nature* **348**: 515-520, 1990.
- Nakamura T., Ohta M., Sugiura M. *et al.*: Chloroplast ribonucleoproteins are associated with both mRNAs and intron-containing precursor tRNAs. – *FEBS Lett.* **460**: 437-441, 1999.
- Nakamura T., Ohta M., Sugiura M. *et al.*: Chloroplast

- ribonucleoproteins function as a stabilizing factor of ribosome-free mRNAs in the stroma. – *J. Biol. Chem.* **276**: 147-152, 2001.
- Nickelsen J.: Chloroplast RNA-binding proteins. – *Curr. Genet.* **43**: 392-399, 2003.
- Pribat A., Blaby I.K., Lara-Núñez A. *et al.*: A 5-formyltetrahydrofolate cycloligase paralog from all domains of life: comparative genomic and experimental evidence for a cryptic role in thiamin metabolism. – *Funct. Integr. Genomic.* **11**: 467-478, 2011.
- Ruwe H., Kupsch C., Teubner M. *et al.*: The RNA-recognition motif in chloroplasts. – *J. Plant Physiol.* **168**: 1361-1371, 2011.
- Sarhadi E., Mahfoozi S., Hosseini S.A. *et al.*: Cold acclimation proteome analysis reveals close link between the up-regulation of low-temperature associated proteins and vernalization fulfillment. – *J. Proteome Res.* **9**: 5658-5667, 2010.
- Schuster G., Gruissem W.: Chloroplast mRNA 3' end processing requires a nuclear-encoded RNA-binding protein. – *EMBO J.* **10**: 1493-1502, 1991.
- Stern D., Goldschmidt-Clermont M., Hanson M.R.: Chloroplast RNA metabolism. – *Annu. Rev. Plant Biol.* **61**: 125-155, 2010.
- Sugita M., Ichinose M., Ide M. *et al.*: Architecture of the PPR gene family in the moss *Physcomitrella patens*. – *RNA Biol.* **10**: 1439-1445, 2013.
- Sun T., Germain A., Giloteaux L. *et al.*: An RNA recognition motif-containing protein is required for plastid RNA editing in *Arabidopsis* and maize. – *P. Natl. Acad. Sci. USA* **110**: E1169-E1178, 2013.
- Teubner M., Fuß J., Kühn K. *et al.*: The RNA recognition motif protein CP33A is a global ligand of chloroplast mRNAs and is essential for plastid biogenesis and plant development. – *Plant J.* **89**: 472-485, 2017.
- Tillich M., Hardel S.L., Kupsch C. *et al.*: Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs. – *P. Natl. Acad. Sci. USA* **106**: 6002-6007, 2009.
- Tillich M., Beick S., Schmitz-Linneweber C.: Chloroplast RNA-binding proteins. Repair and regulation of chloroplast transcripts. – *RNA Biol.* **7**: 172-178, 2010.
- Wang S., Bai G., Wang S. *et al.*: Chloroplast RNA-binding protein RBD1 promotes chilling tolerance through 23S rRNA processing in *Arabidopsis*. – *PLoS Genet.* **12**: e1006027, 2016.