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Identification of a pentatricopeptide repeat RNA editing factor in *Physcomitrella patens* chloroplasts

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ABSTRACT

The moss *Physcomitrella patens* has two RNA editing sites in the chloroplasts. Here we identified a novel DYW-subclass pentatricopeptide repeat (PPR) protein, PpPPR_45, as a chloroplast RNA editing factor in *P. patens*. Knockdown of the *PpPPR_45* gene reduced the extent of RNA editing at the chloroplast *rps14*-C2 site, whereas over-expression of *PpPPR_45* increased the levels of RNA editing at both the *rps14*-C2 site and its neighboring C site. This indicates that the expression level of PpPPR_45 affects the extent of RNA editing at the two neighboring sites.

1. Introduction

In plants, RNA editing frequently occurs at specific cytidines (C) to convert uridines (U) in organelle transcripts. Thirty to 40 RNA editing sites have been identified in chloroplasts and over 500 sites in mitochondria of flowering plants [1,2]. Some editing events create translation initiation codons and therefore RNA editing seems to be an essential process for organelle biogenesis [3,4]. However, the molecular mechanism of RNA editing is not completely understood.

Genetics and biochemical studies over the last decade have revealed that nuclear-encoded pentatricopeptide repeat (PPR) proteins are involved in RNA editing in plant organelles [4,5]. PPR proteins are widely distributed among protists, yeasts, animals and plants [6] and play a central role in the post-transcriptional and translational regulation in mitochondria and chloroplasts [7,8]. Plant-specific PPR proteins with a C-terminal E or E and DYW domains site-specifically recognize target RNA editing sites and perform RNA editing [5]. In addition, general editing factors such as RNA binding proteins [9,10] MORF/RIP proteins [11,12], and protoporphyrinogen IX oxidase 1 (PPO1) [13] participate in RNA editing in *Arabidopsis* organelles.

In contrast to flowering plants, the moss *Physcomitrella patens* has only 11 editing sites in the mitochondria [14,15] and eight DYW-subclass PPR proteins have been identified as editing site specific recognition factors at all 11 sites [15–20]. On the other hand, two editing sites have been identified in the *P. patens* chloroplasts [21]. Editing at the *rps14*-C2 site occurs at a high efficiency and creates a translation initiation codon AUG. In addition, the *rps14*- -1C site in the 5′ untranslated region (UTR) is edited at a low efficiency (~5%) [21]. These editing sites also exist in the related moss *Funaria hygrometrica* [17], but not found in the chloroplasts of higher plants. However, no editing factors for these sites have been identified yet.

Here, we report that a DYW-subclass PPR protein, PpPPR_45, is required for RNA editing at the two sites in the chloroplast *rps14* transcript.

2. Materials and methods

2.1. Subcellular localization of PpPPR_45 fused to green fluorescent protein (GFP)

Isolation of RNA from *P. patens* protonemata, preparation of RNA-free cDNA and amplification of cDNA fragments by polymerase chain reaction (PCR) were carried out as described previously [22]. The amplified cDNA encoding the N-terminal 118 amino acids of PpPPR_45 was cloned in-frame into the *Sma*I site in pKSPGFP9

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[15]. The resultant plasmid p45N-GFP was introduced into the transgenic Mt-RFP OX moss line, which expresses the mitochondria-localized red fluorescent protein (RFP) [20]. GFP fluorescence was monitored using a confocal microscope FLUOVIEW FV10i (Olympus).

2.2. Selection of RNA interference (RNAi) and over-expression lines

RNAi moss line selection was performed according to [23]. A target 0.6 kb DNA region encoding the 16th to the 22nd PPR motifs of PpPPR_45 was introduced into pGG626 RNAi vector by the Gateway LR reaction and p45RNAi was generated. To construct the over-expression p45OX plasmid, the *PpPPR_45* coding region was amplified from cDNA using the primers listed in Supplementary Table 1. The PCR product was cloned into the *Swa*I site of the modified pOX7WH1, which was derived from the over-expression vector pPpMADS2 7113 containing the E7113 promoter (containing seven 35S promoters and additional enhancers [24]), a NOS terminator and an *nptII* cassette. The *nptII* cassette was replaced with the hygromycin resistance gene (*hpt*) cassette in pOX7WH1. The linearized plasmids, p45RNAi and p45OX, were introduced into the GFP-tubulin/histone-RFP moss line (GH line, [23]) or the wild type, respectively, and hygromycin-resistant lines were selected. To analyze RNAi mosses, the protonemata were cultured in the presence of 1 μM β-estradiol.

2.3. Chlorophyll fluorescence analysis

Chlorophyll fluorescence was measured with a kinetics multispectral fluorescence imaging FluorCam 800 MF (Photon System Instruments) according to the manufacturer's instructions. Thirty-day-old protonemal colonies were dark-adapted for 10 min before measurement.

2.4. RNA analysis

Quantitative (q) reverse transcription (RT)-PCR was carried out as described previously [20] using the primers indicated in Supplementary Table 1. *TUA1* (α-tubulin) was used as the internal control. RNA editing efficiency was calculated from the number of edited cDNA clones. For RNA gel blot analysis, gene-specific DNA of the chloroplast *rps14* (311 bp) was amplified using the primers in Supplementary Table 1, and was internally labeled with digoxigenin as described previously [22].

3. Results

3.1. PpPPR_45 is localized in the chloroplasts

DYW-subclass PpPPR_45, deduced from the gene (Pp1s543_6V6.1, http://www.cosmoss.org [25]) and cDNA (AB979874), was predicted to be localized in the chloroplasts and therefore was expected to be a candidate of the RNA editing factor for the chloroplast *rps14* transcript [26]. PpPPR_45 consists of 23 PLS-type PPR motifs and C-terminal E/E+ and DYW domains (Fig. 1A). To verify the chloroplast localization, we performed a transient expression assay of PpPPR_45-GFP protein. GFP fluorescence was observed in the chloroplasts but not in the mitochondria nor in the nucleus (Supplementary Fig. 1), indicating that PpPPR_45 is a chloroplast-localized protein.

3.2. Knockdown of PpPPR_45 resulted in growth retardation and a reduction of photosynthetic activity

To investigate whether PpPPR_45 is involved in RNA editing of the chloroplast *rps14* transcript, we initially tried to generate *PpPPR_45* knockout mosses by insertion of an *nptII* or *hpt* gene cassette into the second exon of the gene. However, no knockout mosses were obtained from the genotyping of 251 moss plants. Instead, we generated *PpPPR_45* RNAi mosses by introducing an inducible RNAi construct into the transgenic GH moss line [23]. Among several RNAi candidate lines selected, two lines (45RNAi #3 and 45RNAi #5) reduced the level of *PpPPR_45* mRNA as measured by qRT-PCR (Fig. 1B). The two independent RNAi lines exhibited retarded growth when treated with β-estradiol. In addition, the pale-green phenotype was observed on the surface

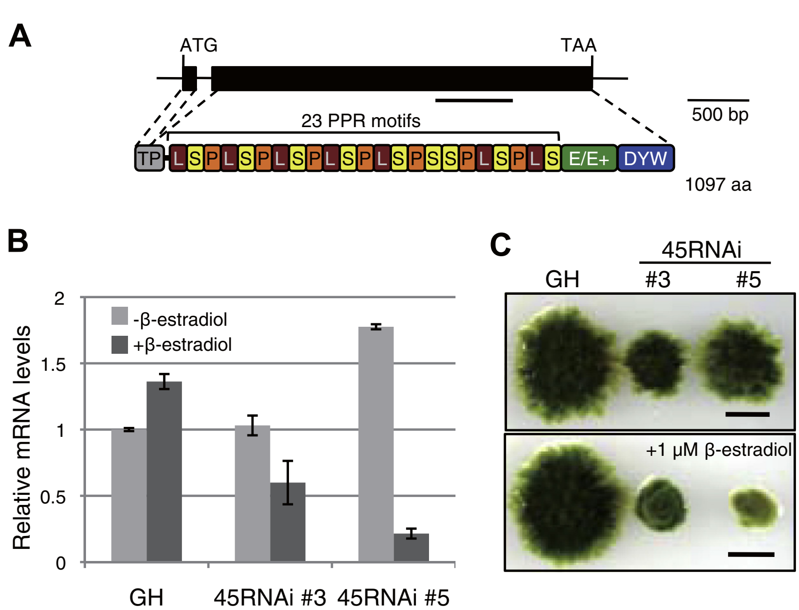

Fig. 1. Generation and characterization of the *PpPPR_45* RNAi lines. (A) Schematic structure of the *PpPPR_45* gene and the encoded protein. Black boxes indicate the translated regions. The amplified region using RNAi is underlined. TP indicates a putative transit peptide. (B) qRT-PCR analysis to quantify *PpPPR_45* mRNA levels in GH and 45RNAi lines with or without β-estradiol. SDs are indicated ($n = 3$). (C) Protonemata colonies of GH and 45RNAi lines. The mosses were grown for 30 days on BCDATG medium plates with or without 1 μM β-estradiol. Bars = 10 mm.