A pentatricopeptide repeat gene of rice is required for splicing of chloroplast transcripts and RNA editing of *ndhA*

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Abstract The pentatricopeptide repeat (PPR) family has been reported to be involved in many post-transcriptional processes, including RNA editing and RNA splicing in both chloroplasts and mitochondria. There are a large number of PPR proteins in plant genome, however, the biological functions of most PPR proteins have not yet been determined. We describe the involvement of a new PPR member (OsPPR4) for the splicing and RNA editing of chloroplast transcripts. The rice P-class PPR protein, OsPPR4, possesses an RNA recognition motif (RRM) and 15 PPR motifs. A retrotransposon (*Tos17*)-insertion mutant, *osppr4*, showed an albino phenotype with early seedling lethality. The same results were obtained from plants transformed with an *OsPPR4* RNA interference (RNAi) construct. Disruption of *OsPPR4* expression led to a strong defect in the splicing of *atpF*, *ndhA*, *rpl2*, and *rps12-2* introns and influences the splicing of *petB* and *rps16* introns. In addition, RNA editing of unspliced *ndhA* transcripts was decreased in the *osppr4* line, whereas both spliced and unspliced *ndhA* were completely edited in WT plants. These results suggest that OsPPR4 is an essential factor for greening in plants and plays a non-redundant role in post-transcriptional regulation of chloroplast genes.

Key words: Albino, chloroplast, pentatricopeptide repeat, rice, RNA editing, splicing.

Arabidopsis and rice genome sequencing projects have revealed the presence of some large gene families (Goff et al. 2002; The Arabidopsis Genome Initiative 2000; Yu et al. 2002). Pentatricopeptide repeat (PPR) genes constitute a large multigene family consisting of 450 genes in Arabidopsis, 477 genes in Oryza sativa (rice), and 103 genes in moss Physcomitrella patens (Lurin et al. 2004; O'Toole et al. 2008). On the other hand, the genomes of the parasitic protozoan Trypanosoma brucei, yeast, Drosophila, and humans are predicted to contain only 28, 5, 2, and 6 PPR genes, respectively (Lurin et al. 2004; O'Toole et al. 2008). The PPR proteins have tandem 35-amino-acid motif, and can be divided into 2 subfamilies and 4 subclasses based on their C-terminal domain structure (O'Toole et al. 2008). Most PPR proteins are predicted to localize to either mitochondria or chloroplasts with subcellular localization prediction programs (Lurin et al. 2004).

PPR proteins are thought to bind DNA or RNA molecules or to mediate protein-protein interactions (Schmitz-Linneweber and Small 2008). PPR proteins are involved in many post-transcriptional processes in both chloroplasts and mitochondria. PPR proteins function in RNA editing (Cai et al. 2009; ChateignerBoutin et al. 2008; Fujii and Small 2011; Hammani et al. 2009; Ichinose et al. 2012; Kim et al. 2009; Kotera et al. 2005; Okuda et al. 2007, 2009, 2010; Robbins et al. 2009; Rudinger et al. 2011, 2012; Sosso et al. 2012; Takenaka 2010; Takenaka et al. 2010; Tang et al. 2010; Tasaki et al. 2010; Toda et al. 2012; Verbitskiy et al. 2010a, 2010b; Zehrmann et al. 2009; Zhou et al. 2009), RNA processing (Hattori et al. 2007; Meierhoff et al. 2003), RNA splicing (Falcon de Longevialle et al. 2007, 2008; Ichinose et al. 2012; Meierhoff et al. 2003; Schmitz-Linneweber et al. 2006), RNA stability (Beick et al. 2008), and translational activation (Schmitz-Linneweber et al. 2005; Tavares-Carreon et al. 2008). In contrast to Arabidopsis PPRs, very little is known about the functions of rice PPRs. Rice PPR genes, Rf-1, RF1A and RF1B, are identified as a fertility restorer gene, (Kazama and Toriyama 2003; Komori et al. 2004; Wang et al. 2006). Rf-1 promotes the processing of atp6-orf79 transcripts of cytoplasmic male-sterile rice (Kazama et al. 2008). The rice DYW-class PPR protein, OGR1, is essential for RNA editing in rice mitochondria and is required for normal growth and development (Kim et al. 2009). The biological functions and biochemical properties of most PPR genes are largely unknown.

Many plant chloroplast genes contain introns of the

Abbreviations: PPR, pentatricopeptide repeat; RRM, RNA recognition motif.

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group-II family (Bonen and Vogel 2001). Previously, some nucleus-encoded proteins, such as PPR proteins, CRM proteins, and domain of unknown function 860 (DUF860) proteins, have been identified as being required for splicing in most plastid introns in plants (Falcon de Longevialle et al. 2010). The complexes CAF1/ CRS2 and CAF2/CRS2 are required for the splicing of overlapping subsets of nine plastid transcripts in maize (Ostheimer et al. 2003), and maize RNC1 is required for the splicing of ten introns (Watkins et al. 2007). Arabidopsis CFM2 with four CRM domains is required for the splicing of four introns (Asakura and Barkan 2007). The Arabidopsis PPR protein, OTP51, is required for the splicing of plastid ycf2 transcripts (Falcon de Longevialle et al. 2008), and OPT70 is involved in splicing of the plastid rpoC1 transcripts (Chateigner-Boutin et al. 2011). Maize PPR4 promotes the transsplicing of rps12 introns (Schmitz-Linneweber et al. 2006). Each nucleus-encoded protein is involved in the splicing of different plastid introns. The mechanisms of RNA processing events are poorly understood. Here, we performed a functional analysis of the rice PPR gene, OsPPR4, using a retrotransposon (Tos17)-insertion mutant, osppr4, and transgenic rice plants expressing an OsPPR4 RNA interference (RNAi) construct. We find that OsPPR4 is an essential factor for rice growth and development in plants. In addition, OsPPR4 is involved not only in splicing of atpF, ndhA, rpl2, rps12-2, petB, and rps16 but also in RNA editing of ndhA. These results suggest that OsPPR4 plays a non-redundant role in posttranscriptional regulation of chloroplast genes.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* L. cv. Nipponbare) plants were grown in a growth chamber or in a greenhouse under natural light at 28°C. For expression analysis of *OsPPR4* in various tissues, roots and leaf blades were harvested from 2-week-old seedlings grown in a growth chamber. Spikelets (before flowering) and developing seeds (5–10 days after flowering) were harvested from plants grown in a greenhouse.

Screening of the osppr4 mutant

The *Tos17* insertion mutant was obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/ index.html). Homozygous *osppr4* mutant plants were identified from the segregated *Tos17* insertion mutant line (NE9048) by genomic PCR with the *OsPPR4*-L1, *OsPPR4*-R2 (Supplemental Table S1), and Tos-com-PA0131 (5'-TAG CTG AGA CCG ATG CTT CA-3') primers. PCR analysis was conducted using TaKaRa LA Taq (Takara Bio, Shiga, Japan) under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min and a final extension at 72°C for 2 min.

Generation of the OsPPR4 RNAi construct and rice transformation

Using the OsPPR4-entry and OsPPR4-R22 primers (Supplemental Table S1), an OsPPR4-specific fragment was amplified via RT-PCR to generate the double-stranded RNA interference construct. PCR was performed using KOD-Plus- (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The amplified fragment was cloned into the Gateway entry vector, pENTR//D-TOPO (Invitrogen, USA), and subsequently into a binary vector, pANDA (Miki et al. 2005). The cloned OsPPR4 fragment was confirmed by nucleotide sequence analysis. The plasmid was introduced into Agrobacterium tumefaciens strain EHA105 (Hood et al. 1993) by electroporation. Transgenic rice plants were produced by Agrobacterium-mediated transformation as described previously (Ozawa 2009).

Analysis of splicing

Isolation of RNA and RT-PCR assays were performed as described previously (Asano et al. 2011). Total RNA was extracted from roots, leaf blades, spikelets, and developing seeds of rice plants using an RNeasy Plant Mini Kit (Qiagen, Germany). The isolated RNA was treated with TURBO DNase (Applied Biosystems/Ambion, USA). cDNA synthesis was performed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen). RT-PCR analysis was performed using EX Taq Hot Start Version or TakaRa LA Taq (Takara Bio) under the following conditions: 94°C for 2 min, followed by 22–35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The primers used for RT-PCR are listed in Supplemental Table S2.

Analysis of RNA editing

The cDNAs for *ndhA* and *rpl2* were amplified by PCR with the appropriate gene-specific primers listed in Supplemental Table S2. The RT-PCR products were purified by QIAquick Spin Kit (Qiagen) and sequenced using the *ndhA*-1N (5'-GGT AGA AGC TAT CAA TTCTT-3'), *ndhA*-L3 (5'-CAA ACC GAA TAT TCT GGT ATC-3'), and *rpl2*-R8 (5'-GGG CAT TTC CCA TTG ATA TAG G-3') primers.

Results

Gene structure of OsPPR4

To determine the biological function of rice *PPR* genes, we screened a collection of *Tos17* insertion lines with disrupted rice *PPR* genes in the Rice *Tos17* Insertion Mutant Database (http://tos.nias.affrc.go.jp/index.html. en) (Miyao et al. 2003). We found several *Tos17* insertion mutants for rice *PPR* genes and evaluated the correlation between gene disruption and phenotype. In this study, we investigated the line NE9048, in which *Tos17* was inserted in a *PPR* gene (Os04g0684500), because this line showed significant morphological change. *Tos17* insertion in the PPR gene co-segregated with the albino phenotype. The *Os04g0684500* locus consists of 12 exons



Figure 1. Structure of *OsPPR4* and its expression pattern. (A) Motif organization of OsPPR4. RRM: RNA recognition motif. PPR: Pentatricopeptide repeat. (B) Four alternatively spliced forms (*OsPPR4-a*, *OsPPR4-b*, *OsPPR4-y*, and *OsPPR4-b*) for *OsPPR4*. Solid boxes and lines indicate exons and introns, respectively. No splicing of intron 7 occurred in *OsPPR4-b*. The splicing site of *OsPPR4-y* and *OsPPR4-b* is different from that of *OsPPR4* at intron 3-exon 4 and intron 4-exon 5, respectively. The position of the *Tos17* insertion is indicated by a triangle. (C) Expression of *OsPPR4* in four different tissues of WT plants. Total RNA was isolated from various tissues [roots, leaf blades (2 weeks after germination), spikelets after heading, and developing seeds (5–10 days after flowering)]. RT-PCR analysis (35 cycles for *OsPPR4* or 25 cycles for *IsS rRNA*) was performed with *OsPPR4*- and *IsS rRNA*-specific primers.

and 11 introns and is predicted to encode a protein of 901 amino acids with a molecular mass of 101.1 kDa. The *Os04g0684500* gene product, OsPPR4, contains an RNA recognition motif (RRM) and 15 PPR motifs (Figure 1A). OsPPR4 belongs to the P-class and shares a high degree of sequence similarity with maize PPR4 (84% identity) and Arabidopsis At5g04810 (59% identity). OsPPR4 possesses a long N-terminal region with no PPR motifs and is predicted to localize to chloroplasts by the TARGETP program (Emanuelsson et al. 2000).

We found four alternatively spliced forms of OsPPR4in the database and designated them as $OsPPR4-\alpha$, $OsPPR4-\beta$, $OsPPR4-\gamma$, and $OsPPR4-\delta$ (Figure 1B). $OsPPR4-\alpha$ is the only transcript that encodes the putative full-length PPR protein; $OsPPR4-\beta$ is an unspliced form at intron 7; $OsPPR4-\gamma$ is alternatively processed at intron 3 with excision of a 20 bp sequence of exon 4; and $OsPPR4-\delta$ is alternatively spliced at intron 4 with excision of an 8 bp sequence of exon 5. These alternative splicing events generated a stop codon in the amino acid sequence deduced from $OsPPR4-\beta$, $OsPPR4-\gamma$, and $OsPPR4-\delta$. RT-PCR analysis showed that OsPPR4 was predominantly expressed in green tissues, such as leaf blades and spikelets (Figure 1C).

Osppr4 is an albino mutant

To elucidate the biological function of OsPPR4 in planta,



Figure 2. Loss-of-function lines of *OsPPR4*. (A) Phenotype of *Tos17* insertion mutant, line NE9048. Upper panel: ten-day-old homozygous *OsPPR4/OsPPR4* and *osppr4/osppr4* seedlings; lower panel: Fifteen-day-old heterozygous *OsPPR4/osppr4*, homozygous *osppr4/osppr4*, and *OsPPR4/OsPPR4* seedlings. (B) Ten-day-old etiolated seedlings. *Tos17* insertion plants of the NE9048 line were grown in darkness. (C) Expression of *OsPPR4* in *osppr4* and *OsPPR4* RNAi lines. *OsPPR4* RNAi lines 1, 2, 6, 7, and 9 were randomly selected for gene expression analysis. RT-PCR (35 cycles for *OsPPR4* and 22 cycles for *18S rRNA*) was performed with *OsPPR4-* and *18S rRNA-*specific primers. (D) Phenotype of regenerated vector control plant (VC). A transgenic plant transformed with the empty vector pANDA was used as a negative control. A red arrow indicates a green spot on calli. (E) Phenotype of calli.

we investigated the phenotypes of loss-of-function lines. The *Tos17* insertion was located within exon 7 of *OsPPR4* (Figure 1B). Therefore, the function of OsPPR4 was assumed to be incomplete in the homozygous *Tos17* mutant line. Because the progeny seeds of line NE9048 were distributed as a mixed population of heterozygotes and homozygotes for the *OsPPR4* mutation, PCR-based screening was performed. Furthermore, the relationship between genotype and phenotype in the progenies of line NE9048 was examined. All homozygous *osppr4*



Figure 3. Splicing defects in *osppr4* and *OsPPR4* RNAi lines. (A) RT-PCR assay for splicing of chloroplast transcripts. Total RNA was extracted from leaf blades of *osppr4*, *OsPPR4* RNAi (lines 6 and 7, as described Figure 2), and WT plants. Genomic DNA (gDNA) was extracted from leaf blades of WT plants. RT-PCR was performed with the appropriate gene-specific primers (U, unspliced transcripts; S, spliced transcripts). (B) Schematic map of the split *rps12* gene and RT-PCR assay for *rps12*. PCR primers are indicated with arrowheads.

seedlings (17 plants) showed a chlorophyll-deficient phenotype and were lethal at the fourth leaf stage; as a result, no eventual greening occurred. In contrast, the remaining 36 heterozygous OsPPR4/osppr4 and 24 homozygous OsPPR4/OsPPR4 lines underwent normal seedlings development (Figure 2A). We next investigated the phenotype of shoots generated from etiolated *osppr4* seedlings. Whereas shoots of wild-type (WT) plants displayed a pale green phenotype when grown in darkness for ten days, osppr4 line showed a pale yellow phenotype (Figure 2B). RT-PCR analysis showed that OsPPR4 expression in the osppr4 line was abolished by the insertion of Tos17 (Figure 2C). These results suggest that the chlorophyll-deficient phenotype results from the disruption of OsPPR4. Furthermore, OsPPR4 probably plays an important role in the early stage of chloroplast biogenesis. Because we could find only one Tos17 insertion mutant, OsPPR4 RNA interference (RNAi) plants were generated by introducing an RNAi construct. We performed three independent experiments of rice transformation, which yielded OsPPR4 RNAi plants. OsPPR4 RNAi lines, as well as osppr4 line, showed a chlorophyll-deficient phenotype. The albino phenotype was observed at early growth stages for OsPPR4 RNAi lines. Green spots were observed on hygromycin-resistant calli from control cells expressing an empty vector (Figure 2D). In contrast to

vector control calli, white spots were found in *OsPPR4* RNAi calli supplemented with regeneration medium, indicating that the chloroplast defect occurs at an early growth stage (Figure 2E). Twenty-five albino seedlings were generated from hygromycin-resistant calli, whereas six green seedlings were produced from *OsPPR4* RNAi calli. We randomly selected five lines from *OsPPR4* RNAi plants indicating pale yellow phenotype and examined the expression levels of *OsPPR4* in respective transgenic plants. RT-PCR analysis showed that the expression of *OsPPR4* was reduced in albino *OsPPR4* RNAi lines with early seedling lethality (Figure 2C). These results suggest that the albino phenotype results from the disruption of *OsPPR4*.

OsPPR4 is required for splicing of multiple plastid transcripts

As PPR proteins are reported to be involved in RNA processing, we analyzed the splicing of several chloroplast transcripts (*atpF*, *ndhA*, *ndhB*, *petB*, *petD*, *rpl2*, *rpl16*, *rps12*, and *rps16*) in *osppr4* and *OsPPR4* RNAi lines 6 and 7. No spliced transcripts for *atpF*, *ndhA*, and *rpl2* found in *osppr4* and *OsPPR4* RNAi lines (Figure 3A). Spliced transcript levels of *petB* and *rps16* were reduced in *osppr4* and *OsPPR4* RNAi lines relative to WT plants; in contrast, the unspliced transcript levels of *petB* and *rps16* were increased in *osppr4* and *OsPPR4* RNAi lines



Figure 4. Altered RNA editing of *ndhA* transcripts in *osppr4* line. Products of *ndhA* and *rpl2* transcripts from *osppr4* and WT plants were sequenced. The regions of the chromatograms encompassing the editing site are shown. Arrowheads indicate the editing sites. Green, black, red and blue color represent A, G, T and C, respectively.

relative to WT plants (Figure 3A). This result suggests that OsPPR4 is involved in splicing, rather than in stabilization, of the spliced transcripts. Splicing defects of *petD*, *ycf3-1*, and *ycf3-2* transcripts were detected in *osppr4* but not in *OsPPR4* RNAi lines (Figure 3A). No significant difference in the expression of *ndhB* and *rpl16* was observed between the loss-of-function and WT lines (Figure 3A). As for *rps12*, no *cis*-spliced transcript (*rps12-2*) was observed in *osppr4* or *OsPPR4* RNA lines. In contrast, a *trans*-spliced transcript (*rps12-1*) was found in *OsPPR4* RNAi lines but not in *osppr4* (Figure 3B). This result indicates that OsPPR4 promotes *cis*-splicing of the *rps12-2* intron.

Among abovementioned transcripts, *ndhA*, *ndhB*, and rpl2 transcripts were known to be edited in wildtype chloroplasts. Furthermore, splicing defects of ndhA and rpl2 transcripts were observed in osppr4 and OsPPR4 RNAi lines. In this study, we examined whether disruption of OsPPR4 affected editing of ndhA and rpl2 transcripts. The level of editing of intron-containing transcripts, ndhA (site I at position 112234 and site II at position 110650) and rpl2, was evaluated using unspliced and spliced transcripts from WT plants and unspliced transcripts from *osppr4* line. The editing of *ndhA* at sites I and II led to amino acid alteration, while the editing of rpl2 generated as start codon (ATG). Both unspliced and spliced ndhA transcripts were almost completely edited in WT plants, whereas the degree of editing for unspliced transcripts in *osppr4* line was relatively low (Figure 4). Spliced *rpl2* transcripts were edited more extensively than the unspliced transcripts in WT plants. The level of *rpl2* editing in *osppr4* line was similar to that of WT

plants (Figure 4), suggesting that OsPPR4 is unaffected in *rpl2* editing.

Discussion

PPR genes constitute a large multigene family in higher plants, such as Arabidopsis and rice. Most members of the PPR family are predicted to localize to either mitochondria or chloroplasts (Lurin et al. 2004). Genetic studies on PPR genes reveal that most proteins belonging to the PPR family are involved in editing, splicing, and regulating the stability of various organellar transcripts (Schmitz-Linneweber and Small 2008). In this study, we performed a functional analysis of the rice P-class OsPPR4 and found that it post-transcriptionally regulates chloroplast transcripts. OsPPR4 proteins have been predicted to localize to chloroplasts, and OsPPR4 was found to be predominantly expressed in chloroplastcontaining tissues, such as leaf blades and spikelets. Collectively, these results indicate that OsPPR4 functions in the chloroplast. In fact, we demonstrated that a loss of OsPPR4 function in rice led to an albino phenotype and early seedling lethality. OsPPR4 RNAi and osppr4 lines showed a strong defect in the splicing of *atpF*, *ndhA*, *rpl2*, and rps12-2 introns. In addition, the extent of editing for *ndhA* transcripts was decreased in *osppr4* line. These results indicate that OsPPR4 plays a non-redundant role in post-transcriptional regulation of chloroplast genes.

We obtained several *OsPPR4* RNAi lines with reduced expression of *OsPPR4* relative to WT plants. These *OsPPR4* RNAi lines as well as *osppr4* plants showed pale yellow-colored shoots and result in severe retardation at early stage of growth (Figure 2). This result indicates that a certain level expression of *OsPPR4* is required for chloroplast biogenesis. We found four alternatively spliced *OsPPR4* transcripts. Alternative splicing is a common mechanism for regulating gene expression in higher eukaryotes (Adams et al. 1996; Smith and Valcarcel 2000). Some plant genes are known to encode multiple proteins with different functions and/ or different subcellular localizations (Reddy 2007). However, the *OsPPR4* splicing variants exhibit no functional distinctions because a stop codon exists in each of the amino acid sequence encoded by each respective transcript. We were unable to determine why OsPPR4 was regulated at the transcriptional level by alternative splicing.

Nucleus-encoded PPR, CRM, and PORR (plant organelle RNA recognition)/DUF860 proteins have been determined to be essential for RNA splicing of chloroplast transcripts in land plants, and they involved in splicing a different subset of introns. PORR/DUF860 and CRM serve as RNA-binding modules specifically in plants. Arabidopsis CFM2 contains four CRM domains that are required for splicing of *clpP* intron 2, *ndhA*, ycf3 intron 1, and trnL (Asakura and Barkan 2007). The complexes CAF1/CRS2 (peptidyl-tRNA hydrolase) and CAF2/CRS2 are required for the splicing of overlapping subsets of nine plastid transcripts in maize (Ostheimer et al. 2003). CRS1 is required for the splicing of *atpF* (Till et al. 2001), and rice CFM3 is required for the splicing of ndhB, petB, petD, rpl16, rps16, and trnG transcripts (Asakura et al. 2008). The maize PORR/DUF860 protein WTF1 functions in concert with RNC1, a ribonuclease III domain protein, to promote the splicing of group II introns (Watkins et al. 2007). On the contrary, little is known regarding the functions and mechanisms of action of PPR proteins in splicing. To date, a few PPR proteins have been reported to function in the splicing of chloroplast transcripts. Arabidopsis hcf152 mutant show reduced amounts of spliced petB RNAs and is affected in the accumulation of transcripts cleaved between the genes *psbH* and *petB* (Meierhoff et al. 2003). Arabidopsis OTP51 has two LAGLIDAGD endonuclease motifs and is required for splicing of the ycf3 intron 2 (Falcon de Longevialle et al. 2008). OTP70, an E-class Arabidopsis PPR, promotes splicing of the plastid transcript rpoC1 (Chateigner-Boutin et al. 2011), although most E-class PPR proteins have been reported to be essential for RNA editing in chloroplasts and mitochondria. Maize PPR4 has an additional RRM motif and is involved in trans-splicing of the rps12-1 introns in plastids. Maize PPR4 is associated in vivo with the first intron of the chloroplast rps12 pre-mRNA. Maize ppr4 mutants are defective in splicing of this intron, suggesting that maize PPR4 facilitates rps12 trans-splicing through direct interaction with the intron RNA (Schmitz-Linneweber et

al. 2006). The action of maize PPR4 in splicing suggests that OsPPR4 also interacts with chloroplast *rps12-2* transcripts.

Some PPR mutants show visible growth or fluorescence phenotypes. Arabidopsis LPA66 is required for editing the *psbF* transcripts and the defect of editing results in reduced growth rates and pale green coloration (Cai et al. 2009). Defects in RNA editing of ndhD in crr4 and crr21, ndhB, ndhD and rpoB in crr22 and ndhB and ndhD in crr28 indicate lack of post-illumination increase in chlorophyll fluorescence (Kotera et al. 2005; Okuda et al. 2009; Okuda et al. 2007). ys1 mutants are impaired in the RNA editing of *rpoB* and show a virescent phenotype, which disappears by 3 weeks after germination (Zhou et al. 2009). OTP51 is required for the splicing of ycf3 intron 2, and otp51 seedlings are pale straw yellow in normal light conditions. Homozygous plants die on soil, but can be rescued by in vitro culture under low light (Falcon de Longevialle et al. 2008). Disruption of the OTP70 leads to a strong defect in splicing of the plastid transcript *rpoC1*, leading to a virescent phenotype (Chateigner-Boutin et al. 2011). In contrast, otp80, otp84, otp85, and otp86 show normal growth under standard conditions. Defects in RNA editing of rpl23, ndhD, and rps14 are found in otp80, otp85, and otp86, respectively. Three defects in RNA editing (*ndhB*, *ndhF*, and *psbZ*) were observed in otp84 and two defects in RNA editing (ndhG and ndhB) were found in otp82 (Hammani et al. 2009). Thus, disruption of PPR genes produces different mutant phenotypes depending on defects of chloroplast transcripts. A difference in the amount of splicing of petD, rps12-1, ycf3-1, and ycf3-2 was observed between osppr4 and OsPPR4 RNAi lines. The former is likely to be a null mutant, while OsPPR4 RNAi plants are knockdown lines. Therefore, a difference in the degree of OsPPR4 disruption may influence splicing of the four chloroplast transcripts in each of the respective lossof-function lines. Splicing of petD, rps12-1, ycf3-1, and ycf3-2 appears to be weakly affected by OsPPR4. In contrast, OsPPR4 RNAi and osppr4 lines are both affected in defects of the splicing of atpF, ndhA, rpl2, and rps12-2 introns. Particularly, rps12 and rpl2 genes encode ribosomal proteins, and therefore, translation defects in the plastids are expected to occur in the loss-of-function lines. These results suggest that the albino phenotype with early seedling lethality in loss-of-function lines may result from splicing defects in rps12 and rpl2 transcripts, and OsPPR4 has an essential role in chloroplast biogenesis.

P-class PPR proteins are involved in splicing and in regulating the stability of chloroplast transcripts (Schmitz-Linneweber and Small 2008). In addition to defects in splicing, *osppr4* line showed a decreased level of RNA editing of *ndhA* transcripts. A previous study reported that the editing sites, site I and site II, were edited in all transcripts analyzed (in the range 1.0–7.8 kb), and are probably edited early during transcription of the *ndhH–ndhD* operon of barley (del Campo et al. 2000). In WT plants, both unspliced and spliced *ndhA* transcripts were almost completely edited at sites I and II (Figure 4), indicating that editing also occurs during early processing of *ndhA* transcripts in WT plants. Contrary to WT plants, the level of editing was decreased in *osppr4* line (Figure 4). This result suggests that OsPPR4 is required for complete *ndhA* editing. The effect of OsPPR4 on RNA editing appears to be weak because *ndhA* editing was not completely impaired in *osppr4* line. Therefore, rather than exerting a direct influence, OsPPR4 may indirectly promote editing.

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Supplemental Table S1. PCR primers for OsPPR4.

Primer	Sequence
OsPPR4-L1	5'-CCATTTGCACGACTTTGCAA-3'
OsPPR4-R2	5'-TTCTAACTAGCCCGTGAATG-3'
OsPPR4-entry	5'-CACCAGCCACGGTCGCCGTGGCACGA-3'
OsPPR4-R22	5'-TCTTAGCCTCTTTGAACAAG-3'

Supplemental Table S2. PCR primers used in gene expression analysis.

Gene	Forward primer	Reverse primer
atpF	5'-ATGAAAAATGTAACCCATTCTTTCG-3'	5'-TCATTCCATGGCCCCGAGAAT-3'
ndhA	5'-GGTAGAAGCTATCAATTCTT-3'	5'-ATCTTATTTTATAGTGAAAC-3'
rpl2	5'-AGGAGAATACTTAATAAT-3'	5'-TTACTTACGGCGACGAAGAAT-3'
rps16	5'-ATGTTAAAACTTCGTTTAAAACG-3'	5'-TTAACTCAAAGTTCTTTCTTTTTC-3'
rpl16	5'-CTTTTAATAAAATTGCTATGCTTAG-3'	5'-TTATATTTCTAACCGAAGAAATTG-3'
petB	5'-GGTTTCGGTATCTCTGGAATATG-3'	5'-TTATAAAGGGCCCGAAATACC-3'
petD	5'-ACGAAAGAAAAGAAGGCGGATTATG-3'	5'-CTAAAAAAGACCTAAAGTTAAGG-3'
ndhB	5'-GGGGCAAGCTCTTCTATTCTGGTT-3'	5'-CTAAAAGAGGGTATCCTGAG-3'
ycf3-1	5'-TGCCTAGATCCCGTATAAAT-3'	5'-AATGACAGATCACGGCCATATT-3'
ycf3-2	5'-GTTGGCTCAATCCGAAGGAAA-3'	5'-CAAAGCGCTTCGTAATCTTCAA-3'
rps12-1	5'-ATGCCAACGGTTAAACAACT-3'	5'-TGGCAACTTTACGTAAGGCA-3'
rps12-2	5'-CCAACTCTGCCTTACGTAAAG-3'	5'-TTATTTTTTTGGCTTTTTTGA-3'
OsPPR4	5'-AGCCACGGTCGCCGTGGCACGA-3'	5'-TTCTAACTAGCCCGTGAATG-3'
rRNA	5'-CGTGGAGCCTGCGGCTTAATT-3'	5'-CGAGCTGATGACTCGCGCTTA-3'