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OsPPR19, a rice pentatricopeptide repeat protein, is essential for mitochondrial biogenesis and seed development

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Abstract

Despite the accumulating evidence showing the essential role of pentatricopeptide repeat (PPR) proteins in organellar biogenesis and plant development in *Arabidopsis thaliana* and maize (*Zea mays*), the functions of most PPR proteins in rice (*Oryza sativa*) are still unknown. A former study demonstrated that the mitochondria-localized *Arabidopsis* PPR19 is crucial for mitochondrial function and normal plant growth and development. In this study, we characterized the functional role of a rice ortholog (LOC_Os12g04110) of *Arabidopsis* PPR19 protein. The loss-of-function *osppr19* mutant displayed delayed seed germination and stunted root and seedling growth compared with wild-type. The height of the *osppr19* mutant was significantly shorter, and the grain mass of the mutant was lower than that of the wild-type. The *osppr19* mutant carried few filled grains and a higher number of aborted seeds than the wild-type. The structures of mitochondria in the *osppr19* mutant were abnormal, and more reactive oxygen species were accumulated in the mutant, suggesting defective mitochondrial biogenesis and function in the *osppr19* mutant. Importantly, the amount of mature mitochondrial transcripts was significantly decreased in the mutant. Taken together, these results suggest that the mitochondrial OsPPR19 is essential for mitochondrial biogenesis and function, which is crucial for plant growth and development of rice grain.

Keywords: intron splicing, mitochondria, pentatricopeptide repeat, rice, seed development.

Introduction

The plant mitochondrial genome encodes only 30 to 40 proteins (Andersson et al., 2003). However, more than 2 000 nuclear proteins are transported to the mitochondrion, which play an essential role in gene expression, translation, as well as assembly of respiratory complexes (Millar et al., 2005, 2006; Hammani and Giegé, 2014). The regulation of mitochondrial gene expression is achieved mainly *via* modulation of the fate of RNAs, including pre-RNA processing, splicing of mRNAs and tRNAs, C-to-U base editing, and RNA decay. During these cellular processes, diverse nucleus-encoded RNA-binding proteins (RBPs), such as pentatricopeptide repeat (PPR) proteins, plant organelle RNA recognition domain proteins, and chloroplast RNA splicing and ribosome maturation domain proteins are transported to mitochondria and play crucial roles in organellar RNA metabolism (Barkan and Small, 2014; Hammani and Giegé, 2014).

PPR proteins are characterized by tandem repeats of 35-amino acid sequence motifs and are particularly abundant in land plants. Hundreds of PPR proteins are encoded by the plant genome (Cheng et al., 2016), and most PPR proteins are transported to mitochondria or chloroplasts (Lurin et al., 2004; Schmitz-Linneweber and Small, 2008). Despite the potential roles of PPR proteins

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Abbreviations: DAB - 3,3'-diaminobenzidine; PPR - pentatricopeptide repeat; RBP - RNA-binding protein.
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in organellar function, the roles of many P-type PPR proteins are required to be demonstrated in *Arabidopsis thaliana*, *Zea mays*, and *Oryza sativa* mitochondria. In *Arabidopsis* mitochondria, the ABO5 is involved in the *cis*-splicing of *nad2* intron 3 (Liu et al., 2010), and BIR6 (Koprivova et al., 2010) and SLO3 (Hsieh et al., 2015) mediate the *cis*-splicing of the *nad7* intron 1 and intron 2. OTP43 (Falcon de Longevialle et al., 2007) and OTP439 (Colas des Francs-Small et al., 2014) are involved in the *trans*-splicing of *nad1* and *nad5* introns, respectively. *Arabidopsis* PPR proteins, mitochondrial stability factor 2 (MTSF2) was shown to stabilize *nad4* transcripts (Wang et al., 2017). In addition, recent studies have determined that MTSF4 and MSP1 are involved in a 3' terminus stabilization of *rpl5-cob* transcripts (Jung et al., 2023) and *nad1* intron 1.1 (Best et al., 2023), respectively. In maize mitochondria, several PPR proteins such as EMPTY PERICARP10 (EMP10), EMP12, EMP16, and defective kernel 37 (dek37) are involved in the splicing of mitochondrial *nad2* introns, and are required for mitochondrial function and seed development (Xiu et al., 2016; Cai et al., 2017; Sun et al., 2019). Dek35 and PPR18 affect the splicing of mitochondrial *nad4* intron and EMP11 and EMP603 are crucial for the splicing of *nad1* introns, which is crucial for seed development in maize (Chen et al., 2017; Ren et al., 2017; Liu et al., 2020; Fan et al., 2021). In rice mitochondria, a few of P-type PPR proteins including rice OsNBL3 and OsPPR939 are involved in the splicing of mitochondrial *nad5* introns, which is essential for mitochondrial function and plant development (Qiu et al., 2021; Zheng et al., 2021). All loss-of-function mutants lacking these PPR proteins displayed growth- and developmental-defect phenotypes, such as retarded seed germination and seedling growth, abnormal leaf formation, low fertility, and reduced seed yield, suggesting the essential role of PPR proteins in plant growth, development, and seed production.

Although these studies point to the crucial roles of mitochondria-localized P-type PPR proteins in *Arabidopsis* and maize, the functions of the majority of PPR proteins in model plant species, as well as in rice remain unknown. Previously, we showed that *Arabidopsis* PPR19 (At1g52620) is essential for the splicing of mitochondrial *nad1* transcripts by stabilizing the *nad1* exon2-exon3 transcript, which is crucial for *Arabidopsis* growth and development (Lee et al., 2017). In this study, we characterized the rice ortholog (LOC_Os12g04110) of *Arabidopsis* PPR19 protein. By analyzing the T-DNA insertion mutant, we show that the mitochondrial OsPPR19 affects mitochondrial biogenesis and function, which is crucial for the growth and seed development of rice.

Materials and methods

Plants and growth conditions: The rice (*Oryza sativa* L.), a *japonica* Dongjin variety, was used in this study. The T-DNA insertion mutant, PFG_1B-07312, was obtained from the Rice Functional Genomic Express Database (Jeon et al., 2000; Jeong et al., 2006), Kyung Hee University, Korea. To validate a single insertion of the T-DNA in the mutant, the levels of *hygromycin phosphotransferase II* (*HptII*) gene were measured in the PFG_1B-07312 line and a reference line B1031 that was found to carry a single T-DNA (Jeon et al., 2000) by RT-qPCR using the primers listed in Table 1 Suppl. The absence of *OsPPR19* expression in the mutant was confirmed by RT-PCR using gene-specific primers listed in Table 1 Suppl. The plants were grown either in soil or on a half-strength Murashige and Skoog (MS) medium containing 1% sucrose at 23 ± 2°C under long-day conditions (16-h photoperiod) with relative humidity 50 - 60% in a growth room or greenhouse. For the measurement of plant height, panicle number, tiller number, fertility, and grain mass, the plants were grown in a rice farm.

Subcellular localization of OsPPR19: The cDNA encoding OsPPR19 was ligated in-frame of green fluorescence protein (GFP) coding sequence within CsvGFP vector under the control of a Cauliflower mosaic virus (CaMV) 35S promoter. The *Agrobacterium tumefaciens* (GV3101 strains) harboring the construct 35:OsPPR19-GFP, were infiltrated into 21-d-old tobacco leaves (*Nicotiana benthamiana*) and were further incubated for two days under long-day conditions as previously described (Lee et al., 2019; Lee, 2023). The mitochondria were stained with *MitoTracker Red CMXRos* (*Invitrogen*, Carlsbad, CA, USA). The GFP images and mitochondria were visualized using a *Zeiss LSM510* laser scanning confocal microscope as previously described (Lee et al., 2017).

RNA extraction, RT-PCR, and RT-qPCR: Total RNA was extracted using the *Plant RNeasy* extraction kit (*Qiagen*, Valencia, CA, USA), and the concentration of RNAs was quantified using *Nano Drop* spectrophotometer (*Thermo Scientific*, Waltham, MA, USA). Two hundred nanograms of total RNAs were used for RT-PCR with gene-specific primers listed in Table 1 Suppl. Real-time quantification of the RNAs was carried out in a *Rotor-Gene Q* real-time thermal cycling system (*Qiagen*) using a *QuantiTect SYBR Green* RT-PCR kit (*Qiagen*) with the gene-specific primers listed in Table 1 Suppl. as previously described (Kim et al., 2017; Dinh et al., 2019). The splicing efficiency of introns was measured by RT-qPCR on a *Rotor-Gene Q* thermal cycler (*Qiagen*) using the gene-specific primers listed in Table 1 Suppl. as previously described (Lee et al., 2017).

Alexander staining of pollen grains: Rice inflorescences were collected from adult plants and fixed in *FPA50* (37% formaldehyde, 5% propionic acid, and 50% ethanol) for 1 h. The anthers were isolated and treated with Alexander's stain as previously described (Alexander 1969). A light microscope equipped with differential interference contrast (DIC) was used. Aborted or non-aborted pollen grains stained blue and purple, respectively.

Transmission electron microscopy: Sample preparation and TEM observations were conducted essentially as described previously (Lee et al., 2017). Briefly, 3-week-old

wild-type and mutant seedlings were fixed and embedded in *LR White* (*London Resin Co.*, London, UK). Approximately 80 to 100 nm thin sections were prepared using an ultra-microtome and stained with uranyl acetate and lead citrate. The specimens were examined using a transmission electron microscope *JEM-1400* (*Jeol*, Tokyo, Japan).

DAB (3,3'-diaminobenzidine) staining: The content of H_2O_2 was evaluated by DAB staining as previously described (Ramel et al., 2009; Lee et al., 2017). Briefly, leaves were submerged in DAB staining solution (0.01 M Na_2HPO_4, pH 3.8, 0.1% diaminobenzidine, 0.05% *Tween-20*), subjected to vacuum-infiltration for 10 min, and further incubated overnight at room temperatures in the dark. Chlorophylls were removed by incubating the leaves in 80% ethanol solution at 55°C.

Results

Subcellular localization and expression analysis of OsPPR19: The rice OsPPR19 protein contains 19 PPR motifs and a mitochondrial transit peptide at the N-terminus (Fig. 1*A*), the domain structures which are similar to the *Arabidopsis* PPR19 protein which is targeted to mitochondria (Lee et al., 2017). To compare the similarity between the PPR19 proteins of dicotyledonous *Arabidopsis* and monocotyledonous rice, the amino acid sequences of PPR motifs were compared and phylogenetic tree was constructed with putative orthologous groups (POG) of *Arabidopsis* PPR19 (http://cas-pogs.uoregon.edu/#/). The results showed that *Arabidopsis* and OsPPR19 proteins share approximately 40.8% amino acid sequence homology and OsPPR19 (Os12g4110) is orthologous with *Arabidopsis* PPR19 (Figs. 1 Suppl. and 3 Suppl.).

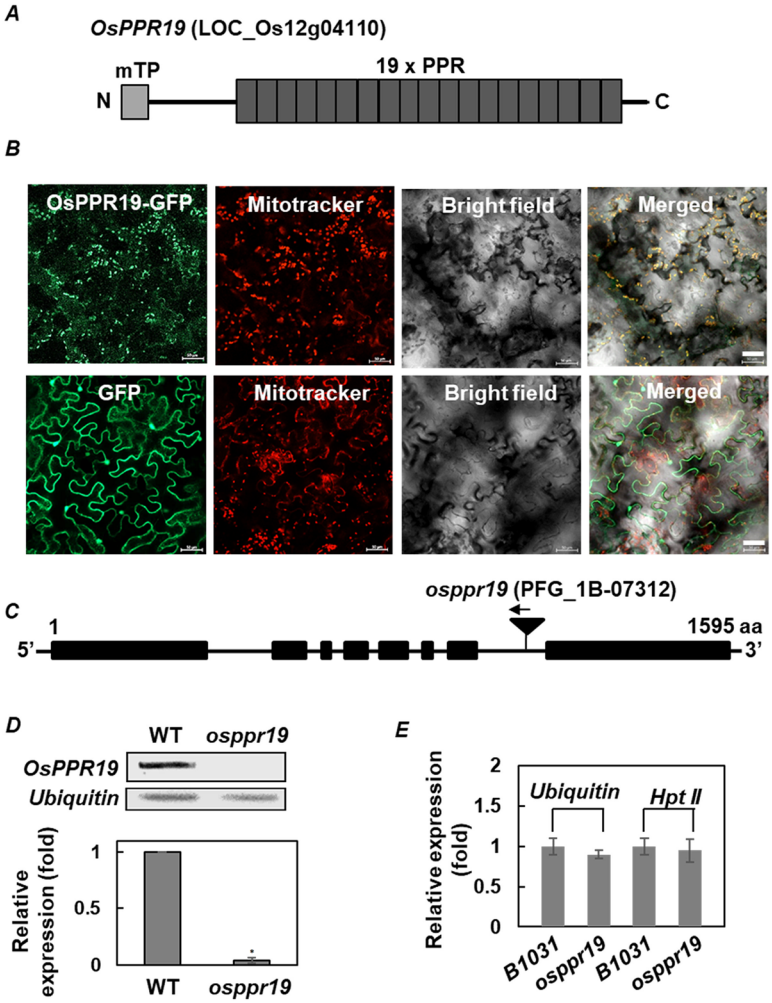

Fig. 1. The motif structure and identification of OsPPR19 mutant. *A* - Schematic representation of the motif structures of OsPPR19 protein. The mitochondrial transit peptide (mTP) and predicted PPR motifs are shown. *B* - GFP signals in OsPPR19-GFP- or GFP-expressing tobacco leaves were observed using a confocal microscope. *Mitotracker* was used to stain mitochondria. *Scale bar* = 50 µm. *C* - Genomic structure of *OsPPR19*. *Black boxes* and thick lines represent exons and introns, respectively. The T-DNA insertion site in the *osppr19* mutant is shown; aa - amino acid. *D* - Confirmation of a single T-DNA insertion in the mutant. Means ± SEs of three biological replicates (n = 10 plants per replicate), and *asterisk* indicates statistically significant difference between wild-type and mutant (Student's t-test, $P \leq 0.05$). *E* - Absence of *OsPPR19* expression in the mutant was confirmed by RT-PCR and RT-qPCR. *Ubiquitin* was used as a loading control. *HptII* - *hygromycin phosphotransferase II*, B1031 - the rice line containing a single T-DNA.

In addition, the PPR codes of rice PPR19 protein were compared to *Arabidopsis* PPR19 protein and the essential PPR codes and predicted target RNA sequences were highly similar (Fig. 2 Suppl.). These results suggest that the PPR19 protein is functionally conserved in dicot and monocot.

To examine whether the OsPPR19 protein is transported into mitochondria, the subcellular localization of a OsPPR19-green fluorescent protein (GFP) fusion protein was investigated in tobacco leaves. The GFP signals were overlapped with the *Mito-tracker*-stained mitochondria (Fig. 1*B* and Fig. 5 Suppl.), indicating that the nucleus-encoded OsPPR19 protein is mainly targeted to the mitochondria. To investigate the function of OsPPR19 in rice growth and development, a T-DNA insertion mutant *osppr19* (PFG_1B-07312), in which the T-DNA is inserted into the last intron of *OsPPR19* gene (Fig. 1*C*), was obtained from the Rice Functional Genomic Express Database, Kyung Hee University, Korea. The absence of *OsPPR19* expression in the mutant was confirmed by RT-PCR and quantitative RT-PCR analyses (Fig. 1*D*). Because we analyzed only one mutant line, to rule out the possibility that the observed phenotypes were due to the disruption of other genes in the rice genome, a single T-DNA insertion in the mutant was verified by RT-qPCR (Fig. 1*E*). The level of *hygromycin phosphotransferase II* (*hptII*) gene in the *osppr19* mutant was almost similar to that in the B1031 rice line that contains a single T-DNA (Jeon et al., 2000), confirming a single T-DNA insertion in our mutant.

OsPPR19 is essential for normal growth and development of rice: We next assessed the function of OsPPR19 in rice growth and development in the *osppr19* mutant. The mutant displayed delayed seed germination and defective root growth compared with wild-type (Fig. 2*A*). The defective growth phenotypes of the *osppr19* mutant were largely evident when grown in soil. The height of the *osppr19* mutant was significantly shorter than that of the wild-type during vegetative growth and at

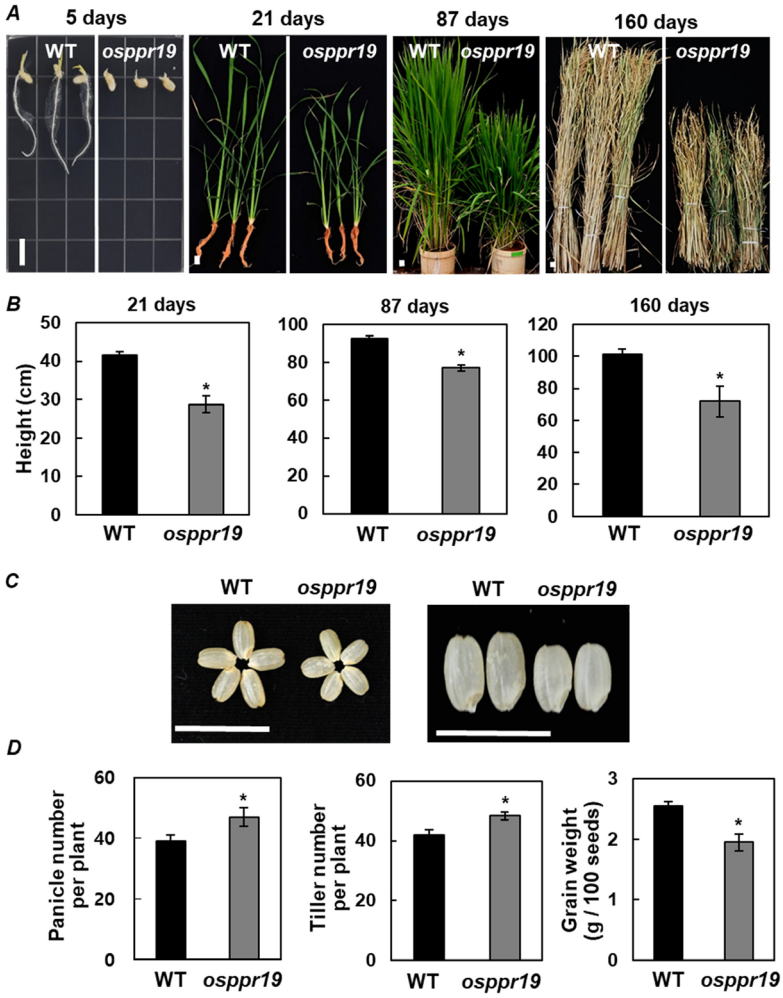

Fig. 2. OsPPR19 is essential for rice growth and development. *A* - Photographs of wild-type (WT) and *osppr19* mutant were taken at the indicated days; *bar* = 1 cm. *B* - Heights of wild- type (WT) and *osppr19* mutants were measured on the indicated days. *C* - Photographs of wild-type and *osppr19* mutant seeds; *bar* = 1 cm. *D* - Panicle number, tiller number, and grain mass of wild-type and *osppr19* mutant were measured; means ± SEs of three biological replicates (n = 10 plants per replicate), and *asterisks* indicate statistically significant differences between wild-type and mutant (Student's t-test, $P \leq 0.05$).

maturity (Fig. 2*A*,*B*). The size of the *osppr19* mutant seeds was smaller than that of the wild-type seeds (Fig. 2*C*). Interestingly, the panicle and tiller numbers in each *osppr19* mutant were higher than those in wild-type. However, the grain mass of the *osppr19* mutant was significantly lower than that of wild-type (Fig. 2*D*). All progenies of the *osppr19* mutant displayed the same growth defect, whereas those of wild-type showed normal growth. Taken together, these results suggest that mitochondria-targeted OsPPR19 is crucial for normal growth and development of rice during vegetative and reproductive growth stage.

OsPPR19 is crucial for pollen fertility and seed development: Because grain mass of the *osppr19* mutant was lower than that of the wild-type, we next evaluated whether OsPPR19 affected pollen fertility and seed development. At maturity, the *osppr19* mutant carried many aborted seeds (Fig. 3*A*), and seed-setting rate per panicle in the *osppr19* mutant was significantly lower than that in wild-type (Fig. 3*B*). To understand why the *osppr19* mutant carried aborted seeds, Alexander's stain was used to evaluate the viability of pollen grains. Results showed that many pollen grains in the *osppr19* mutant were stained blue (Fig. 3*C*), suggesting that the mutant pollen grains are less viable than the wild-type. Clearly, OsPPR19 plays a crucial role in pollen fertility and seed development.

OsPPR19 is involved in mitochondrial biogenesis and function: As it is evident that the mitochondria-localized OsPPR19 is essential for the normal development and seed setting of rice, we reasoned that OsPPR19 displays its role by influencing mitochondrial biogenesis and function. To test this possibility, mitochondrial structures in the *osppr19* mutant were examined *via* transmission electron microscopy. Results showed that the mitochondria of the *osppr19* mutant contain poorly-organized cristae, whereas the wild-type mitochondria contain normally folded inner membranes and well-organized cristae (Fig. 4*A* and Fig. 4 Suppl.). We next measured the content

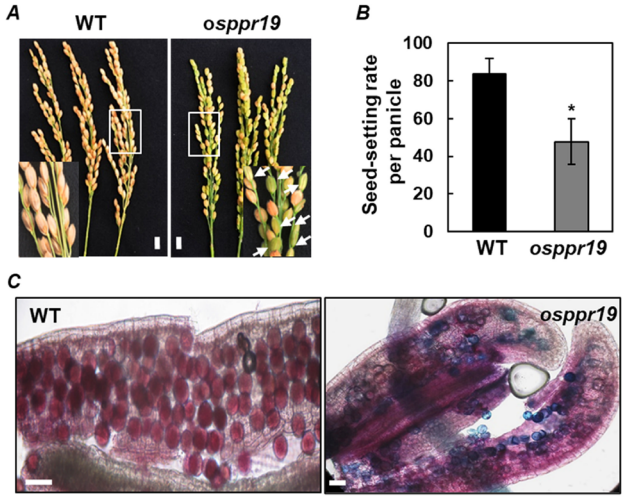

Fig. 3. OsPPR19 is essential for pollen grain viability. *A* - Photographs of wild-type (WT) and *osppr19* mutant at maturity. Inserts represent enlarged pictures of the boxed region. *Bar* = 1 cm. *Arrows* - abnormal seeds. *B* - Seed-setting rate per panicle. Values are mean ± SE of three biological replicates (n = 10 plants per replicate), and asterisks indicate statistically significant differences between wild-type and mutant (Student's t-test, $P \leq 0.05$). *C* - Alexander's stain was used to detect pollen grain viability (n = 3). *The blue color* represents aborted grains. *Bar* = 50 μm.

of H_2O_2 by DAB staining as an indicator of mitochondria function. It was evident that H_2O_2 content was significantly higher in the leaves of the *osppr19* mutant than in wild-type (Fig. 4*B*), suggesting that mitochondrial activity and function is lower in the mutant than in wild-type. Collectively, these results suggest that OsPPR19 is important for mitochondria biogenesis and function.

OsPPR19 affects the splicing of mitochondrial transcripts: A previous study revealed that *Arabidopsis* PPR19 stabilizes *nad1* transcripts by specifically binding to

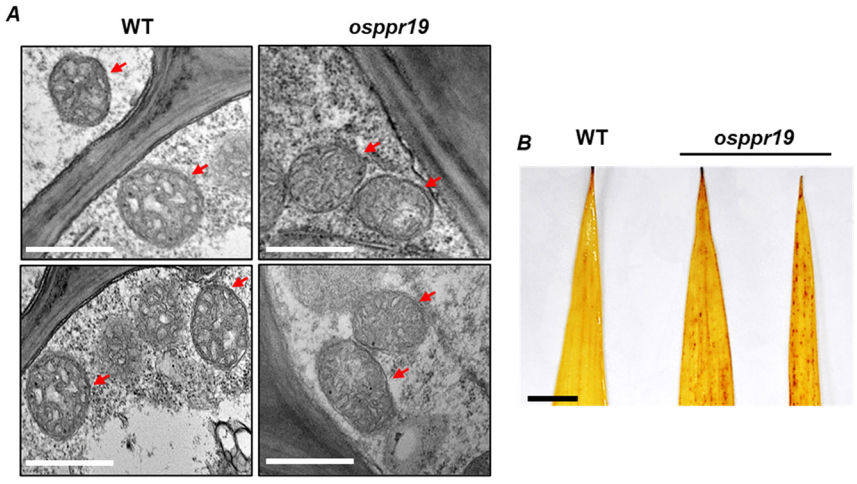

Fig. 4. OsPPR19 affects mitochondrial biogenesis and function. *A* - The structures of mitochondria in wild-type (WT) and *osppr19* mutants were analyzed using transmission electron micrograph; *bar* = 500 nm; *red arrows* represent mitochondria. *Left and right columns* - the mitochondria in WT and *osppr19* mutant, respectively. *B* - DAB staining for the detection of hydrogen peroxide in the leaves of wild-type and mutant plants; *bar* = 1 cm.

the specific sequence in the 3'-terminus of *nad1* transcript, which affects the accumulation and splicing of many mitochondrial transcripts such as *nad1* intron 1, intron 2, intron 3 and intron 4, and *nad2* intron 1 (Lee et al., 2017). To evaluate whether rice OsPPR19 is also involved in the accumulation and intron splicing of mitochondrial genes, amounts of mitochondrial intron-containing transcripts were determined by RT-qPCR analysis. Results showed that amounts of several mitochondrial transcripts including *nad1*, *nad2*, *nad4*, *nad5*, and *nad7* were significantly decreased in the *osppr19* mutant compared to wild-type. However, transcripts such as *rpl2*, *rps3*, and *cox2* showed only a marginal decrease in the mutant (Fig. 5*A*). We next analyzed the splicing efficiency of mitochondrial introns. Levels of the spliced and un-spliced transcript of each gene were measured using RT-qPCR, and the ratio of spliced to un-spliced transcripts was calculated in the wild-type *versus* the *osppr19* mutant. Clearly, the ratio of spliced to un-spliced transcripts of many intron-containing mitochondrial genes, including *nad1* intron 2 and intron 3, *nad2* intron 1 and intron 4, *nad5* intron 1, and *nad7* intron 1 and intron 2, was significantly reduced in the mutant compared with the wild-type (Fig. 5*B*). Our current and previous results clearly show that PPR19 is an essential factor involved in the accumulation and splicing of many mitochondrial genes.

Discussion

Approximately 650 PPR proteins are distributed in rice (Lurin et al., 2004). Although the understanding of the mitochondria-localized PPR proteins has been increased in higher plants, the cellular and molecular roles of only some PPR proteins have been determined in rice. In this study, we have demonstrated that OsPPR19 is an essential protein that is localized to mitochondria and is involved in mitochondrial intron splicing, which is a crucial role in normal mitochondrial biogenesis and function. Previous studies have demonstrated that mutations in *Arabidopsis* OTP43, OTP439, MID1, and SLO3 affect splicing of *nad* introns (Falcon de Longevialle et al., 2007; Colas des Francs-Small et al., 2014; Hsieh et al., 2015; Zhao et al., 2020). Interestingly, the loss-of-function mutants exhibited severe phenotypic defects such as abnormal mitochondrial structure, delayed germination, and embryo development. Our results show that a loss-of-function mutant of OsPPR19 harboring T-DNA inserted in seventh intron (Fig. 1) displayed defective growth and development phenotype during vegetative and reproductive growth stage (Figs. 2, 3). These results are also comparable to those observed in the *Arabidopsis ppr19* mutant showing delayed germination, poorer root and seedling growth, shorter height, and decreased seed yield compared with

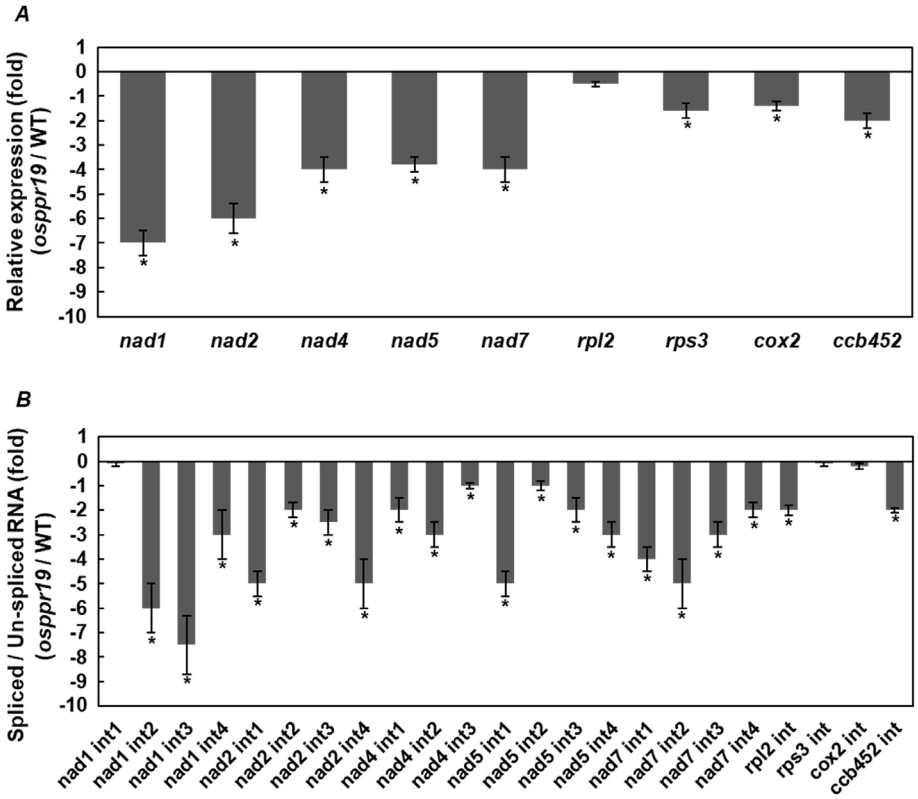

Fig. 5. Transcript levels and splicing efficiency of intron-containing mitochondrial genes. *A* - Total RNA was extracted from 4-week-old wild-type (WT) and *osppr19* mutants, and transcript levels of mitochondrial genes were determined by RT-qPCR. The levels of mature transcripts in the mutant were calculated by comparison with the wild-type level. *B* - The splicing efficiency of intron-containing mitochondrial transcripts was calculated as a ratio of spliced to un-spliced transcripts between the mutant and wild-type; means ± SEs of three biological replicates (n = 10 plants per replicate), and *asterisks* indicate statistically significant differences between wild-type and mutant (Student's t-test, $P \leq 0.05$).

the wild-type plants (Lee et al., 2017). Although it is clear that a nucleus-encoded mitochondrial PPR19 is essential for normal growth and development in monocot and dicot plants, we cannot exclude the functional redundancy of OsPPR19 in the plant growth and development based on our phylogenetic tree analysis (Fig. 3 Suppl.). Further studies are required to determine the functional roles of another orthologous Os11g04295 of *Arabidopsis* PPR19 in mitochondrial biogenesis and seed development.

Cytoplasmic male sterility (CMS) is a widespread phenomenon found in nearly 200 flowering plant species (Bentolila et al., 2002; Hu et al., 2012). CMS is a maternally inherited trait, and CMS-associated genes are located in the mitochondrial genome (Hu et al., 2014). In many instances, the restorer of fertility (*Rf*) genes are encoded by the nuclear genome and are responsible for fertility restoration (Budar et al., 2003). Importantly, many *Rf* genes encode PPR proteins such as Rf1a, Rf1b, and Rf5 in rice (Wang et al., 2006; Hu et al., 2012), Rfo and Rfk in *Brassica* cv. Ogura and radish cv. Kosena (Brown et al., 2003; Koizuka et al., 2003), and PPR592 in *Petunia* (Bentolila et al., 2002). Previous results showed that *Arabidopsis ppr19* mutant also carried aborted and empty seeds in siliques (Lee et al., 2017). Our current results showing the role of OsPPR19 in pollen grain viability suggest that OsPPR19 is another *Rf* gene involved in CMS (Fig. 3).

Mitochondrial complex I serves as the inaugural enzyme complex in the mitochondrial respiratory chain. The defect in the splicing of mitochondrial *nad* introns exhibits abnormal mitochondrial structure and dysfunction in assembly and activity of the mitochondrial complex I, thereby leading to electron leakage and subsequent accumulation of ROS (Koprivova et al., 2010; Lee et al., 2017, 2019; Qiu et al., 2021). In line with previous results, our results show abnormal mitochondrial structure and higher ROS accumulation with the defect splicing of intron in *osppr19* mutant, clearly demonstrating that OsPPR19 is pivotal for mitochondria biogenesis and activity (Fig. 4). Moreover, the *Arabidopsis ppr19* mutant exhibits changes in alternative splicing patterns in nucleus, mediated by retrograde signaling (RS) pathways from mitochondria activated by the accumulation of ROS (Lee et al., 2017), which indicate that the growth and developmental defects in *osppr19* mutant could solely arise from the alteration of nuclear gene expression *via* RS pathways as well as the mitochondrial splicing deficiency. Next, it is of our interest to support the concept that the accumulation of ROS from the mitochondrial splicing defect in the *osppr19* mutant affects the nuclear gene expression and splicing patterns by RNA-seq analysis.

Many P-type PPR proteins have been shown to play an essential role in the splicing of mitochondrial introns and plant growth and development in land plants (Hsieh et al., 2015; Wang et al., 2017; Sun et al., 2019). Maize PPR mutations in DEK37, EMP10, and EMP12 show the reduction in splicing efficiencies of *nad2* introns, which are essential for mitochondrial function and seed development (Cai et al., 2017; Sun et al., 2019). ZmSMK9 and EMP32 are crucial for the splicing of *nad5* introns and *nad7* intron2, respectively, influencing the plant architecture, the kernel development, and the seed development (Pan et al., 2019; Yang et al., 2021). In rice, it has been demonstrated that PPR FLO10 is required for the *trans*-splicing of *nad1* introns and endosperm and seed development (Wu et al., 2019). Moreover, OsPPR939 displays the importance of splicing of *nad5* introns and plant growth and pollen development by the reduction in mitochondrial complex I activity (Zheng et al., 2021). Our results show severe defect in the splicing of several mitochondrial introns, including *nad1*, *nad2*, *nad5*, and *nad7* introns, in *osppr19* mutant, which disrupts mitochondria biogenesis and seed development (Fig. 5), suggesting OsPPR19 is a cellular factor essential for the splicing of mitochondrial introns and is critical for mitochondrial biogenesis and seed development.

The *Arabidopsis ppr19* mutant mainly displays a defect of splicing of mitochondrial *nad1* introns (Lee et al., 2017), whereas our result shows that the splicing patterns in *osppr19* mutant are inconsistent with those observed in *Arabidopsis ppr19* mutant (Fig. 5). Notably, recent studies have demonstrated that a P-type PPR protein, maize EMB603, can associate with other splicing factors including PMH2-5140, ODB1-0814, and ODB1-5061 *via* N-terminal before the PPR motifs (Fan et al., 2021). *Arabidopsis* EMB1270 and rice OsPPR11 are also involved in the interaction with CFM2 (Zhang et al., 2021) and OsCAF2 (Zhang et al., 2023), respectively. Indeed, OsPPR19 includes more additional N-terminal amino acids before PPR motifs compared to that in *Arabidopsis* PPR19, suggesting that the functional discrepancy of dicotyledonous and monocotyledonous PPR19 proteins could result from the involvement of different splicing factors. Future analysis is necessary to assess whether the OsPPR19 can affect mitochondrial RNA metabolism *via* interactions with other splicing factors.

In conclusion, our results demonstrate that OsPPR19 is essential for mitochondrial biogenesis and function, which is crucial for rice growth, fertilization, and seed development. Considering the crucial role of PPR proteins in the splicing of mitochondrial introns, mitochondrial biogenesis and function, and pollen and seed development, molecular functions of as yet uncharacterized mitochondria-localized PPR proteins should be determined to elucidate their significance in plant organelles.

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