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RADICLELESS 1 (RL1)-mediated *nad4* intron 1 splicing is crucial for embryo and endosperm development in rice (*Oryza sativa* L.)

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ABSTRACT

Pentatricopeptide repeat (PPR) proteins are one of the largest protein families in land plants. PPR proteins exhibit sequence-specific RNA-binding activity and are implicated in plant growth and development related processes. In this study, we report that the *radicleless 1* (*rl1*) mutant in rice (*Oryza sativa* L.) exhibited defective radicle emergence in embryos and compromised grain filling in endosperms. Gene cloning and confirmation via genetic complementation analyses showed that *RL1* encodes a P-type PPR protein, which is localized to mitochondria. The RL1 protein was specifically involved in the splicing of intron 1 of the mitochondrial *nad4* transcript, which encodes a subunit of the mitochondrial NADH dehydrogenase complex. Consistent with this observation, the *rl1* mutant exhibited altered mitochondrial morphology and lower ATP accumulation compared with the wild type. Thus, our findings suggest that RL1-mediated *nad4* splicing is crucial for embryo and endosperm development in rice.

1. Introduction

Pentatricopeptide repeat (PPR) proteins constitute one of the largest protein families in eukaryotes, with over 400 members in flowering plants [1,2]. Studies over the past few decades reveal that most of these PPRs are nucleus-encoded RNA processing factors that are delivered to the mitochondria or plastids, where they play important roles in the editing, splicing, stabilization, cleavage and translational regulation of mRNA [2]. PPR proteins are characterized by the presence of tandem repeats of 35-amino-acid (aa) motifs (P motifs), which form a pair of antiparallel α-helices [3]. These PPR proteins are categorized into two subtypes: P-type and PLS-type. The P-type PPR proteins contain an array of canonical P motifs, whereas the PLS-type PPRs contain triplets of P, L (>35aa) and S (<35aa) motifs and an additional C terminal that consists of 1–3 non-PPR motifs (E, E+, and DYW) [2,4,5].

In flowering plants, primary transcripts of most mitochondrial genes contain introns that need to be removed to form functional transcripts. These introns are classified into two groups, group I and group II, depending on their secondary structure and splicing mechanism [6]. According to secondary structure, group I introns contain 10 domains, each of which plays a specific role in mRNA folding to enhance splicing and ligation, while group II introns contain 6 domains [6,7]. More than 20 group II RNA introns have been identified in plant mitochondria, and most of those have been identified in *nad1, nad2, nad4, nad5*, and *nad7* genes, which encode different subunits of the NADH complex I [8]. In *Arabidopsis thaliana* and maize (*Zea mays* L.), splicing of *nad* transcripts is regulated via P-type mitochondria-targeted PPR proteins. For example, *nad1* transcripts are spliced by AtOTP43 and ZmEMP11 [9,10], *nad2* transcripts by AtABO5 and ZmEMP10 [11,12], *nad4* transcripts by ZmDEK35 and ZmEMP602 [13,14], *nad5* transcripts by AtOTP439 and AtTANG [15], *nad7* transcripts by AtSLO3 and AtBIR6 [16,17]. Although the splicing of certain introns by PPR proteins has been established, the molecular mechanism underlying this process is not clear. The rice genome encodes 246 P-types PPR proteins [18], however, only one of them, OsFLO10, has been reported to regulate the splicing of *nad1* transcripts [19]. Thus, the correlation between PPR proteins and *nad* transcripts is more obscure in rice than in

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other plant species.

In this study, we report the identification and molecular genetic characterization of the *rl1* mutant in rice. The *rl1* mutant exhibited defective radicle emergence, and chalky starchy endosperm. Bulk sequencing analyses performed of homozygous *rl1* plants identified in a segregating F_2 population showed that *RL1* encodes a P-type PPR protein. Additionally, Cell imaging analyses showed that RL1 is localized to mitochondria, and quantitative Real Time PCR (qRT-PCR) results showed that mutation of *RL1* compromised the splicing of intron 1 of mitochondrial *nad4* transcripts.

2. Materials and methods

2.1. Plant materials and growth conditions

Plants of rice (*Oryza sativa* L. ssp. *Japonica*) cultivar Zhonghua 11 (ZH11, wild type) were grown in the experimental field of the Institute of Botany, Chinese Academy of Sciences in Beijing, China, or in growth rooms maintained at 30 ± 2 °C during the day, and 22 ± 2 °C during the night, 12 h light (410 μmol/s)/12 h dark photoperiod with 60–90% humidity. The *rl1* mutant was identified by screening of an ethyl methane sulfonate (EMS)-mutagenized M2 population of rice cultivar ZH11.

2.2. Histological analysis and transmission electron microscopy

ZH11 and *rl1* mutant caryopses were collected at 9, 12, and 14 days after pollination (DAP) and sectioned transversely into two halves using a razor blade. each half with embryo was immediately fixed in a modified FAA solution (50% ethanol, 6% acetic acid, 5% formaldehyde, 5% glycerol) for 24 h. Mature ZH11 and *rl1* caryopses were sectioned transversely into approximately 2-mm thick slices, soaked in water at 4 °C for 4 h, then fixed and embedded, as described previously [20]. The embedded embryo slices were further cut into 1 μm thick sections with a glass knife using a Leica Ultra-Cut microtome (EM UC7, Leica Microsystems). The semi-thin sections were stained with periodic acid-Schiff's (PAS) reagent and washed first with tap water, and then with double distilled water. Photographs were captured using a microscope (Eclipse 80i, Nikon).

For transmission electron microscopy (TEM), ZH11 and *rl1* caryopses at 9 DAP were cut along the horizontal axis into small pieces (1 mm thick) and fixed in 100 mM phosphate buffer (pH 7.2) with 2.5% (v/v) glutaraldehyde. The samples were further treated and stained with osmic acid at Chinese Academy of Agricultural Sciences.

2.3. Map-based cloning and complementation analysis

To identify the *RL1* gene, an F_2 population was generated from a cross between the *rl1* mutant and an *indica* rice variety Nanjing 6 (NJ6) for map-based cloning. For complementation analysis, a genomic fragment spanning the 1,386 bp coding sequence (CDS), and the 1,364 bp and 2,094 bp sequence upstream and downstream of RL1 start and stop codons, respectively, was amplified from ZH11 genomic DNA and cloned into the *pPLV15* vector thus generating the *pRL1:gRL1* construct. Additionally, the *RL1* CDS (1,383 bp) minus the stop codon was fused with a *β-Glucuronidase* (*GUS*) reporter gene in the *pPLV15* vector under the control of the *GLUTEIN C* (*GluC*) to generate the *pGluC:RL1-GUS:NOSt*. To determine the expression pattern of *RL1*, The 1,364 bp *RL1* promoter region was cloned upstream of the *GUS* reported gene in the *pPLV15* vector, thus generating the *pRL1:GUS* construct. Primers used are listed in Table S1.

2.4. qRT-PCR analyses

Total RNA was extracted using the TRIzol Reagent (Invitrogen), and cDNA synthesis were performed using the FastQuant RT Kit (Tiangen) according to manufacturers' instructions. qRT-PCR was performed using Bio-Rad iTaq™ Universal SYBR® Green reagent and CFX-Connect™ PCR program, with rice *Ubiquitin* gene used as an internal control. Primers used for qRT-PCR analyses are listed in Table S1.

2.5. GUS staining assay

The GUS assay was performed as described previously [21]. These samples were then cleared using a destaining solution (75% ethanol, 25% acetic acid), and photographs of GUS staining were captured under a microscope (SMZ800, Nikon).

2.6. Subcellular localization

To determine the subcellular localization of RL1, the *RL1-green fluorescence protein* (*GFP*) fusion construct *p35S:RL1-GFP* was co-infiltrated into *Nicotiana benthamiana* leaves with the *p35S:ZmPPR78-mcherry* construct using *Agrobacterium* EHA105 strain. ZmPPR78, a mitochondria-localized protein, was used as a marker [22]. GFP and mCherry were detected in leaf epidermal cells at 2 days post infiltration using a confocal laser scanning microscope (FV1000MPE, Olympus; excitation wavelengths GFP 488 nm, mCherry 543 nm)

3. Results

3.1. Identification and phenotypic characterization of rice rl1 mutant

The *rl1* mutant was identified from an EMS-mutagenized M2 population of the rice cultivar ZH11, based on its defects in endosperm development and seed germination. Compared with ZH11, seeds of homozygous *rl1* plants showed delayed germination and did not produce primary root (called radicle hereafter) (Fig. 1A); instead, *rl1* seedlings were supported by the growth of the adventitious roots. We therefore named this mutant *radicleless 1* (*rl1*). Additionally, *rl1* grains also exhibited chalky starchy endosperm, especially in the central region (Fig. 1B and C), indicating grain filling defects.

Histological analyses of mature embryos showed that, in ZH11 embryos, the radicle was well-separated from the surrounding epiblast cells (Fig. 1D). Sampling at different developmental stages showed that a clear gap was formed between the radicle and epiblast cells from 12 DAP onwards (Fig. S1). However, in *rl1* embryos, the radicle was tightly fused to the surrounding epiblast tissue, leaving no gap between the radicle and epiblast (Fig. 1E; Fig. S1), which could be the primary defect responsible for the failure of radicle emergence.

3.2. Mapping and validation of the RL1 gene and phylogenetic analysis

To get rid of undesirable traits resulting from EMS mutagenesis, the *rl1* mutant was backcrossed to ZH11 for two generations. The BC_2 generation plants (*RL1/rl1*) were self-pollinated. Genetic analyses of BC_2F_1 progeny showed a 3:1 segregation ratio of that the wild type and *rl1* phenotype (534:186, $\chi 2 = 0.13$, $P > 0.05$), suggesting that *rl1* is a single recessive mutation. To clone the *RL1* gene, the *rl1* was crossed to the *indica* rice cultivar NJ6, and the progeny plants were self-pollinated to obtain an F_2 mapping population.

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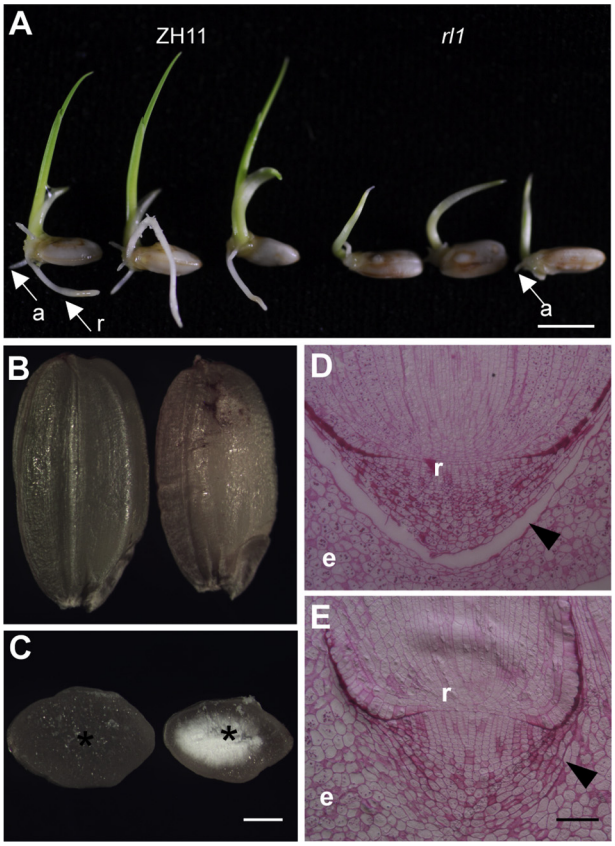

Fig. 1. Phenotypes of the ZH11 and *rl1* mutant. (A) 4-d-old seedlings of ZH11 and *rl1*. Scale bar = 1 cm. (B, C) The mature caryopsis of ZH11 (L) and *rl1* (R). R, right; L, left. Scale bar = 1 mm. (D, E) Transverse sectioned mature radicles of ZH11 (D) and *rl1* (E). The black triangles indicate the gap between radicle and epiblast, asterisks indicate starchy endosperm. Scale bar = 50 μm. a, adventitious root; r, radicle; e, epiblast.

Bulk genome sequencing of 36 F_2 homozygous *rl1* individuals mapped the *RL1* gene to a 210 kb genomic region between the insertion-deletion (InDel) markers RM23464 and RM23477 on chromosome 8. Using 96 individuals with the *rl1* phenotype, the *RL1* locus was further narrowed down to an 80 kb region, which contained 11 putative open reading frames (ORFs), between the simple sequence repeat (SSR) markers of M1 and M2. Comparison of the sequence of these ORFs between the *rl1* mutant and ZH11 revealed a single nucleotide substitution (G^{354} to A) in the ORF of *LOC_Os08g41380* in the *rl1* mutant, resulting in a premature stop codon (Fig. 2A). Analysis of the *RL1* gene sequence showed that it is an intron-less gene, and encodes a canonical P-type PPR protein with nine P motifs, as predicted by the PPR Code Prediction webserver (http://yinlab.hzau.edu.cn/pprcode/; Fig. 2B). Phylogenetic analyses showed that RL1 shares high sequence similarity with maize ZmEMP8 (88.2% identity at the protein level; Fig. S3) [24].

To validate the identity of *RL1* gene, a genomic fragment of *LOC_Os08g41380*, containing the 1,386 bp *RL1* CDS, and 1,364 and 2,094 bp upstream and downstream sequences, was amplified and cloned into a binary vector, and the resulting construct was introduced into the *rl1* mutant via *Agrobacterium*-mediated transformation. To determine if the transgene complemented the mutant phenotype, we conducted phenotypic analyses of three independent transgenic lines (C7, C8 and C11).Transgenic seed showed radicle emergence (Fig. 2D and E) and a healthy endosperms, as opposed to the chalky phenotype of *rl1* mutant grains (Fig. 2C), and plants derived from these transgenic grains were indistinguishable from the ZH11 (Fig. S2D).

In addition, we expressed the *RL1* ORF as a fusion with the GUS reporter gene in the *rl1* mutant, under the control of the endosperm-specific promoter *GluC* (Fig. S2A) [23]. Grains of three independent transgenic lines (L1, L3, and L5) showed a normal endosperm (Fig. S2B), indicating rescue of the grain filling defect observed in *rl1* grains. However, the height of *rl1* plants expressing *pGluC:RL1-GUS:NOSt* construct was not restored (Fig. S2D). Interestingly, the germination of transgenic *rl1* seeds was not indistinguishable from ZH11 (Fig. S2C), indicating endosperm development possibly plays a role in embryo development. Together, these results confirm that RL1 is encoded by *LOC_Os08g41380.*

3.3. Expression analysis of RL1

We examined the expression of *RL1* in various plant organs by qRT-PCR. The results showed that *RL1* is constitutively expressed in all tissues examined in this study, including roots, stems, leaves, seedlings, calli, panicles, anthers, ovaries, caryopses, endosperms and embryos. The expression level of *RL1* in caryopsis and embryo continued to increase from 4 to 7 DAP onward, respectively (Fig. 3A). To further examine the *RL1* expression in caryopsis, we expressed the GUS reporter gene under the control of *RL1* promoter (*pRL1:GUS:NOSt*) in ZH11. GUS staining of developing caryopses of representative transgenic lines revealed that *RL1* was constitutively expressed in caryopses, with higher levels in embryos, pericarp and testa than in starchy endosperms (Fig. 3B).

3.4. RL1 is a mitochondria-localized PPR protein

MitoFates analysis (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi) predicted that RL1 harbors a mitochondria-targeting sequence at its N-terminus (Fig. 2B). To verify the mitochondrial localization of RL1, we co-expressed the *RL1-GFP* fusion construct containing the cauliflower mosaic virus (CaMV) *35S* promoter (*p35S:RL1-GFP:NOSt*) with a mitochondria marker construct of *p35S:ZmPPR78-mCherry* in tobacco leaf epidermal cells by *Agrobacterium*-mediated infiltration [22]. Confocal microscopy analyses showed that the GFP signal detected as small green patches overlapped with mCherry-labeled mitochondria. The GFP signal did not overlap with the auto-fluorescence of chloroplasts (Fig. 3C). These results indicate RL1 is localized to the mitochondria.

3.5. Mutation of RL1 affected the splicing of mitochondrial nad4 intron 1 and altered mitochondrial morphology and energy supply

Previous studies showed that P-type PPR proteins are mainly involved in RNA splicing in either mitochondria and/or chloroplasts [2]. Because RL1 is a mitochondria-localized P-type PPR protein, we analyzed the splicing efficiency of 23 introns that distribute in 9 mitochondrial genes [8,19] at 6 DAP caryopses by qRT-PCR. The results showed that the splicing efficiency of *nad4* intron1 was significantly reduced in *rl1*, and that of *nad1* intron 1 and *nad2* intron 1 was slightly reduced in *rl1* compared with the ZH11 (Fig. 4A). Splicing of *nad4* intron 1 was recovered in caryopses of complementary line (Fig. S4).

Since *nad4* encodes a subunit of the mitochondrial NADH dehydrogenase complex, and maize genotype with defective *nad4* splicing exhibits abnormal mitochondrial morphology [17,18,20], we examined the mitochondria in ZH11 and *rl1* endosperms at 9 DAP using an electron microscope. Mitochondria in *rl1* showed obscure cristae and large internal spaces (Fig. 4B), whereas those in ZH11 showed normal morphology and regular cristae, indicating that mutation in *RL1* gene altered mitochondrial structure. Because mitochondria are the primary source for ATP, we measured the ATP contents of ZH11 and *rl1* caryopses at 9 DAP by a luciferin-luciferase

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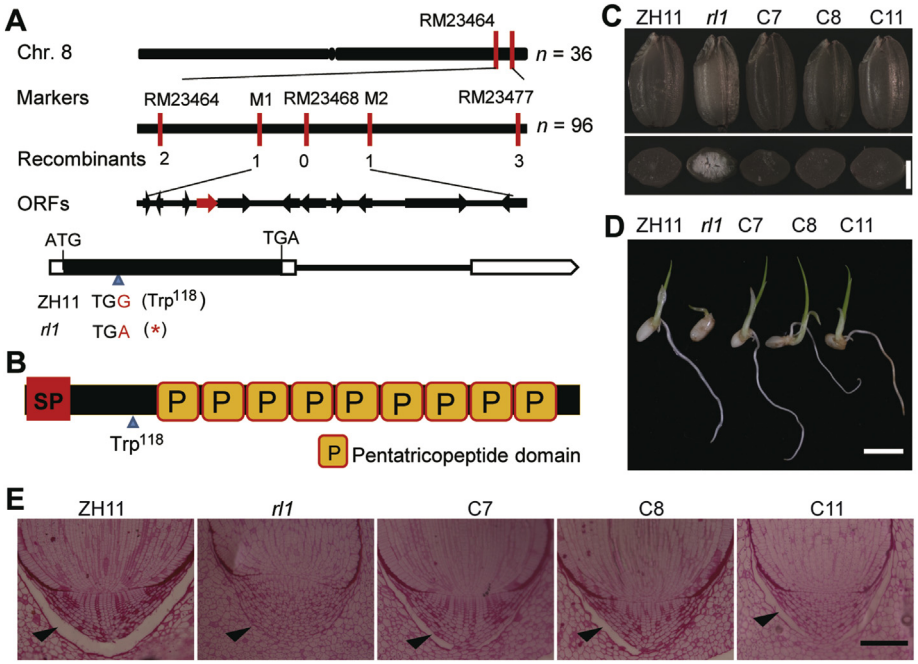

Fig. 2. *RL1* encodes a P-type PPR protein. (A) Mapping of the *RL1* locus. The primers of markers are listed in Table S1. (B) Schematic structure of *LOC_Os08g41380*. SP, signal peptide. (C) Phenotype of mature caryopsis of ZH11, *rl1* mutant and complementation ($rl1^{pRL1:gRL1}$) lines (C7, C8, C11), $n = 30$. Scale bar = 1 mm. (D) Phenotype of 4-d-old seedlings of ZH11, *rl1* and three complementation lines, $n = 30$. Scale bar = 1 cm. (E) Transverse sectioned mature endosperm and radicle of ZH11, *rl1* and three complementation lines, $n = 3$. Scale bar = 100 μm. The black triangles indicate the gap between radicle and epiblast.

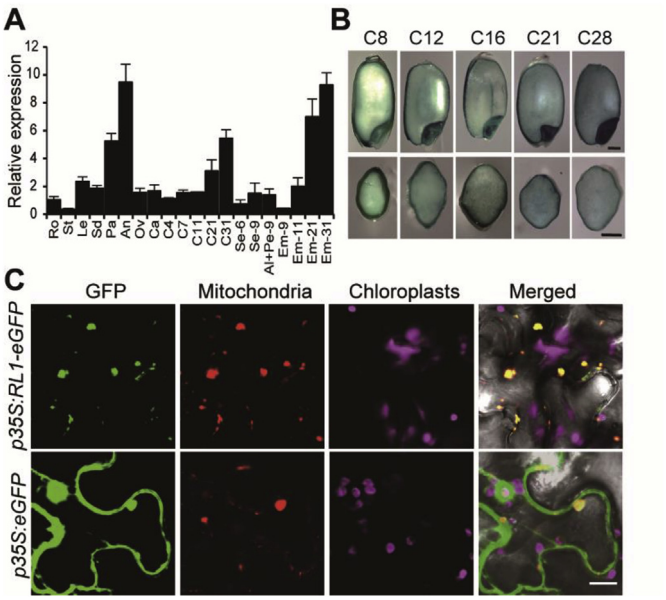

Fig. 3. Expression pattern of *RL1* and its subcellular localization. (A) qRT-PCR analysis of *RL1* mRNA levels in the root (Ro), stem (St), leaf (Le), seedling (Sd), panica (Pa), anther (An), ovary (Ov), callus (Ca), caryopsis from 4 DAP to 31 DAP (C4, C7, C11, C21 and C31), 6 DAP endosperm (Se-6), 9 DAP endosperm (Se-9), 9 DAP aleurone layer and pericarp (Al + Pe-9), embryo from 9 DAP to 31 DAP (EM-9, EM-11, EM-21 and EM-31). Data were shown as means ± SD, $n = 3$. Rice *Ubiquitin* gene was used as an internal control. The primers used were listed in Table S1. (B) Detection of *RL1* expression in different developmental stages of caryopses by GUS staining, the transgenic plants harboring the *pRL1:GUS:NOSt*. Scale bar = 1 mm. (C) Subcellular localization of RL1. GFP (Green), mitochondrial (Red), auto-fluorescent of chloroplasts (Magenta) and merged signals were showed form left to right. *35S:GFP* as control. Scale bar = 100 μm.

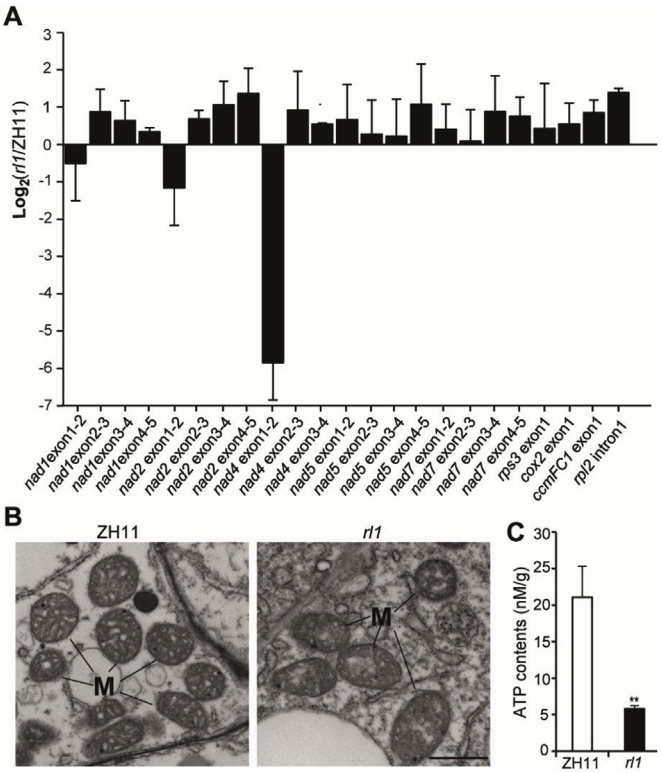

Fig. 4. *rl1* reduced splicing efficiency of *nad4* intron 1 and compromised mitochondrial morphology and ATP biosynthesis. (A) qRT-PCR analysis of mature mitochondrial transcripts. The primers used were listed in Table S1. Data were shown as means ± SD, $n = 3$. (B) Transmission electron microscope images of mitochondria in ZH11 and *rl1* endosperm at 9 DAP. M, mitochondria. Scale bar = 10 μm. (C) ATP contents in 9 DAP caryopsis of ZH11 and *rl1*. Data were shown as means ± SD, $n = 4$.

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ATP assay kit (Beyotime, Shanghai, China). Results showed that ATP content in *rl1* caryopses was only 27% of that in ZH11 (Fig. 4C). Additionally, the level of ATP was also lower in *rl1* calli than in ZH11 calli (Fig. S5). These results suggest that RL1 plays a crucial role in mitochondrial morphology and energy supply.

4. Discussion

In our study, we found that P-type PPR protein RL1 affected embryo and endosperm development. Although *RL1* was expressed in all tissues tested, elevated expression was observed in developing embryos. Consistent with this finding, the radicle of *rl1* failed to emerge during germination, and the primary roots were unable to grow properly possible because of defective separation between the radicle and its surrounding epiblast tissue. Many mutations in PPRs have been associated with defective embryo development in Arabidopsis [25] and maize [10,12–14,26], but defective separation of the radicle from its surrounding tissue has not been reported previously. Since the radicle in rice is formed internally during embryogenesis [26], it is highly likely that RL1 is essential for the formation of a gap between radicle and epiblast. Further investigation is needed to elucidate whether RL1 is involved in the regulation of programmed cell death in this region.

In this study, we showed that the mutation of the P-type PPR gene *RL1* in rice compromised the splicing of *nad4* intron 1 in mitochondria. The *nad4* gene encodes one subunit of the NADH dehydrogenase complex in plants, which is the first enzyme of mitochondrial respiratory chain and is responsible for electron transfer between NADH and quinone [27]. The *nad4* gene of flowering plants contains three introns [28]. Several P-type PPR proteins have been identified as splicing factors of *nad4* in maize [13,14,25,29]. Mutations in ZmDEK35 and ZmDERK41 compromised the splicing efficiencies of *nad4* intron 1 and intron 3, respectively, and altered the mitochondrial morphology [13,29]. The splicing of intron 4 of *nad1*, intron 1 of *nad2* and intron 1 of *nad4* were compromised in the *zmemp8* mutant [24], and that of *nad4* introns 1 and 3 in *zmemp602* mutant [14]. Although RL1 showed the highest sequence similarity with ZmEMP8 in maize, both proteins show distinct characteristics at the functional level, while RL1 seems to affect the splicing of only *nad4* intron 1, ZmEMP8 regulates the splicing of multiple introns including intron 1 of *nad4* transcripts. RL1 in rice functions as ZmDEK35 in maize, specifically processing the intron 1 of *nad4* transcripts, however, RL1 and ZmDEK35 share only 16.2% sequence similarity. Low sequence similarity (18.9%) has also been observed between RL1 and ZmEMP602, which is involved in splicing of introns 1 and 3 of *nad4*. Rice and maize are evolutionally closely related species, belonging to the gramineae family. However, the poorly conserved PPRs function on the same target, and highly conserved PPRs act on different targets. This suggests a rapid co-evolution between PPRs and their splicing targets. This is plausible since the processing of an intron in mitochondria relies on the tight binding of PPR protein with the transcript that needs to be processed, and the binding may rely on the conformation of the PPR protein.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.084.

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