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The Mitochondrion-Targeted PENTATRICOPEPTIDE REPEAT78 Protein Is Required for *nad5* Mature mRNA Stability and Seed Development in Maize

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ABSTRACT

Pentatricopepetide repeat (PPR) proteins are a large family of RNA-binding proteins involved in RNA metabolism in plant organelles. Although many PPR proteins have been functionally studied, few of them are identified with a function in mitochondrial RNA stability. By using a reverse genetic approach, we characterized the role of the mitochondrion-targeted PPR78 protein in *nad5* mature mRNA stability and maize (*Zea mays*) seed development. Loss of PPR78 function leads to a dramatic reduction in the steady-state level of mitochondrial *nad5* mature mRNA, blocks the assembly of complex I in the electron transport chain, and causes an arrest in embryogenesis and endosperm development. Characterization of a second strong allele confirms the function of PPR78 in *nad5* mRNA accumulation and maize seed development. The generation of mature *nad5* requires the assembly of three distinct precursor RNAs via *trans*-splicing reactions, and the accumulation of *nad5*T1 precursor is reduced in the *ppr78* mutants. However, it is the instability of mature *nad5* rather than *nad5*T1 causing loss of the full-length *nad5* transcript, and degradation of *nad5* losing both translation start and stop codons is enriched in the mutant. Our data imply the assembly of mature *nad5* mRNA precedes the protection of PPR78.

Key words: PPR, *nad5*, mitochondrial RNA stability, complex I, seed development, maize

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INTRODUCTION

Pentatricopeptide repeat (PPR) proteins are a large family of RNA-binding proteins in land plants, with more than 400 members in *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), and maize (*Zea mays*) (Lurin et al., 2004; O'Toole et al., 2008). PPR proteins are characterized by degenerate 31–36 amino acid motifs arranged in tandem array (Lurin et al., 2004). They are classified into two subclasses: P subclass, which has only canonical 35-amino acid motifs; and PLS subclass, containing a combination of shorter, canonical, and longer motifs. The PLS subclass is further classified into PLS, PPR-E, and PPR-DYW groups, and the last two groups contain an additional E or E-DYW domain at the C terminus. Crystal structure analysis reveals that PPR motifs adapt an antiparallel helix-loop-helix fold whose repetition forms a solenoid-like structure (Ban et al., 2013; Yin et al., 2013; Gully et al., 2015). The RNA-binding activity of PPR proteins has been demonstrated biochemically (Delannoy et al., 2007). The code for RNA recognition by PPR proteins is elucidated, and combinations involving amino acids at positions 5 and 35 in the PPR repeats correlate strongly with the identity of the RNA base to be bound (Barkan et al., 2012; Yin et al., 2013; Cheng et al., 2016).

PPR proteins localize primarily in chloroplasts and mitochondria, where they are involved in multiple aspects of organellar gene expression. The members of the PLS subclass are mainly required for RNA editing (Sosso et al., 2012; Liu et al., 2013; Li

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et al., 2014; Sun et al., 2015; Yang et al., 2017), while those in the P subclass function largely in intron splicing, RNA maturation, RNA stability, and translation initiation (Pfalz et al., 2009; Zhelyazkova et al., 2012; Haïli et al., 2013; Wu et al., 2016; Xiu et al., 2016; Wang et al., 2017). PPR proteins are essential for the normal activities of chloroplasts and mitochondria, and loss of PPR functions may cause defects in pollen viability, leaf pigmentation, seed development, and plant growth (Wang et al., 2006; Pfalz et al., 2009; Sosso et al., 2012; Haïli et al., 2013; Liu et al., 2013; Li et al., 2014; Xiu et al., 2016).

In chloroplasts, the roles of a PPR protein in RNA maturation and stability are usually interlinked (Pfalz et al., 2009; Prikryl et al., 2011; Zhelyazkova et al., 2012; Hammani et al., 2016). Most chloroplast genes in higher plants are transcribed as large polycistronic precursors, and generation of the mature RNAs requires processing at the intercistronic regions between coding regions (Stern et al., 2010). The process of RNA maturation is proposed to be initiated by cleavages of endonucleases such as RNase P and RNase Z at specific sites, or RNase J and RNase E at multiple sites with low similarity, which generate intermediates bearing variable transcript ends (Pfalz et al., 2009; Stern et al., 2010; Luro et al., 2013). Exonucleases such as RNase J, PNPase, and RNase R would trim the heterogeneous termini of the intermediate transcripts to form homogeneous ends, which are observed in most mature mRNAs. In this process, a PPR protein binds to the 5′ or 3′ border of its RNA target and serves as a barrier to exonucleolytic cleavage in either the 5′ or 3′ direction. IN this way, the PPR protein defines mature transcript end(s) and protects the RNA target(s) from degradation. Evidence from PPR10 confirms the dual roles of PPR proteins in RNA maturation and stability (Pfalz et al., 2009; Prikryl et al., 2011). PPR10 protein binds to a segment mapped in the intergenic region between *atpI* and *atpH* coding regions, determines the mature 3′ terminus of *atpI* and the 5′ terminus of *atpH*, and stabilizes them by blocking against the exonucleolytic cleavages 3′-to-5′ and 5′-to-3′, respectively (Prikryl et al., 2011). In the *ppr10* mutant, exonucleases trim beyond the transcript termini defined by PPR10 protein and degrade the *atpI* and *atpH* mRNAs. Zhelyazkova et al. (2012) show that this mechanism underlying RNA maturation and stability is predominant in chloroplasts.

The mitochondrial genome is more complicated than the chloroplast genome and the dynamic rearrangement of the mitochondrial genome leads to a loss of long polycistronic precursors (Maier et al., 1995; Clifton et al., 2004). As in chloroplasts, most mitochondrial transcripts are generated by post-transcriptional processing (Forner et al., 2007), and the 3′ end maturation of mitochondrial transcript is linked to RNA stability (Haïli et al., 2013; Wang et al., 2017). *Arabidopsis* MITOCHONDRIAL STABILITY FACTOR 1 (AtMTSF1) and AtMTSF2 are two PPR proteins reported to confer RNA stability in mitochondria. AtMTSF1 binds to the 3′ end of *nad4* mRNA, defines the mature 3′ end, and protects it from exonucleolytic degradation 3′-to-5′ (Haïli et al., 2013). In the absence of AtMTSF1, the steady-state level of *nad4* mRNA is dramatically decreased, and *nad4* transcripts losing the translation stop codon are enriched. AtMTSF2 binds to the 3′ end of *nad1* exons 2–3 precursor, determines the transcript terminus, and stabilizes it by blocking the progression of 3′-to-5′ exonuclease (Wang et al., 2017). Unlike in chloroplasts, the 5′ end maturation of mitochondrial transcripts is not linked with RNA stability. *Arabidopsis* RNA PROCESSING FACTOR 2 (AtRPF2) is a PPR protein required for 5′ end maturation of the mitochondrial *nad9* and *cox3* (Jonietz et al., 2010). In the *atrpf2* mutant, *nad9* and *cox3* precursor RNAs with longer 5′ UTRs are overaccumulated. Similarly, AtRPF1, AtRPF5, AtRPF7, and ZmMPPR6 are another four PPR proteins required for 5′ end maturation of *nad4*, *nad6*, *nad2*, and *rps3*, respectively (Holzle et al., 2011; Manavski et al., 2012; Hauler et al., 2013; Stoll et al., 2014). In the four *ppr* mutants, the defects in 5′ end maturation of the mitochondrial targets are accompanied by an increased accumulation of the precursors, not degradation of the downstream coding region. These studies indicate the mechanism of RNA maturation and stability in mitochondria may be different from chloroplasts. One explanation for this difference is the lack of 5′-to-3′ exonuclease in plant mitochondria (Binder et al., 2013; Ruwe et al., 2016), and the 5′ end maturation of mitochondrial transcripts may be not associated with exonucleolytic trimming.

To further unravel the mechanism underlying mitochondrial RNA maturation and stabilization, we focus on the functional studies of a subset of P-subclass PPR proteins that have a potential mitochondrial transit peptide in maize. In this report, we provide evidence that mitochondrion-targeted PPR78 is required for *nad5* mature mRNA stability. In the *ppr78* null mutant, the instability of *nad5* mature mRNA dramatically decreases the steady state of *nad5* transcripts, severely compromises the formation of complex I (NADH-dehydrogenase) in the electron transfer chain, and partially arrests embryogenesis and endosperm development in maize.

RESULTS

PPR78 Is a P-Subclass PPR Protein Targeted to Mitochondria

The maize gene GRMZM2G070381 encodes a PPR protein, named PPR78 (Figure 1A and 1B). The *PPR78* gene harbors one intron 99 bp upstream of the translation initiation codon ATG, and cDNA comprises an open reading frame predicted a 722-amino acid polypeptide. PPR78 protein has 14 canonical PPR motifs, thus a P-subclass PPR protein. It shares high similarity with the putative orthologs in monocots, such as sorghum (*Sorghum bicolor*, 89%), millet (*Setaria italica*, 77%), and rice (66%) (Supplemental Figure 1). Notably, no proteins with strong similarity were found in dicots.

Based on TargetP analysis (http://www.cbs.dtu.dk/services/TargetP), PPR78 was predicted to have a putative mitochondrial localization signal at the N terminus (Figure 1B). To study the subcellular localization experimentally, a truncated form of *PPR78* including the sequences encoding the potential mitochondrial transit peptide was fused with the *GFP* (*Green Fluorescent Protein*) gene, and the resulting construct was transiently expressed in tobacco (*Nicotiana tabacum*) leaf epidermal cells. MitoTracker was used as a mitochondrion marker. Confocal laser scanning microscopy revealed that GFP fluorescence overlapped with the red signals from MitoTracker (Figure 1C). The result confirms the mitochondrial localization of PPR78.

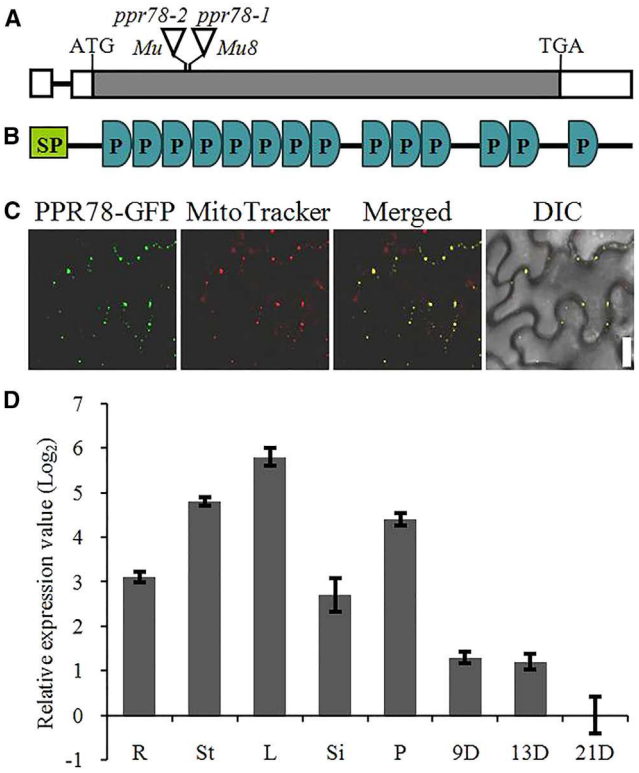

Figure 1. The Maize (*Zea mays*) *PPR78* Gene and Protein Structure, Protein Subcellular Localization, and Gene Expression Pattern.

(A) *PPR78* gene structure and the *Mutator* insertion sites in *ppr78-1* and *ppr78-2* alleles. Exons and introns are shown as boxes and solid lines, respectively.

(B) PPR78 protein structure. SP, mitochondrion signal peptide predicted by TargetP; P, PPR motif predicted by PROSITE.

(C) The transient expression of the *PPR78-GFP* fusion gene in tobacco (*Nicotiana tabacum*) leaf epidermal cells. MitoTracker is a mitochondrial marker; GFP, green fluorescent protein. Scale bar, 0.05 mm.

(D) Quantitative RT–PCR analyses of *PPR78* gene expression. Values represent the mean and SD of three biological replicates; normalization was performed against the maize *actin* gene, and 21D was regarded as 1; R, root; St, stem; L, seedling leaf; Si, silk; P, pollen; the developing kernels are at 9, 13, and 21 days after pollination.

To study whether *PPR78* is expressed, quantitative RT–PCR (qRT–PCR) was performed on major organs of the maize plant as well as the developing kernels (Figure 1D). *PPR78* transcripts were detected in all tissues tested. Relatively, it was expressed low in the mature kernel (21 days after pollination [DAP]), but high in young kernels (9 and 13 DAP) and other tissues, including root, stem, leaf, silk, and pollen.

Phenotypic Characterization of the *ppr78-1* Mutant

To study the role of *PPR78* in maize development, we searched the Uniform*Mu* library documented in the Maize Genetics and Genomics Database (www.maizegdb.org) and identified a stock in which a *Mutator* (*Mu*) element was inserted in *PPR78*, i.e., UFMu-00247 (Figure 1A). This mutant was named *ppr78-1*. The *Mu* insertion site in *ppr78-1* is 338 bp downstream of ATG. PCR amplification and DNA sequencing results confirmed the insertion site and showed the *Mu* element was *Mu8*.

Previous studies showed a *Mu* element inserted into a functional gene could act as introns and be spliced (Zhang et al., 2013). RT–PCR using primers flanking the *Mu8* insertion site (PPR78-F1 and PPR78-R1, −347 and +622 relative to the *Mu8* insertion site, respectively) generated a product with the expected size in the wild-type (WT), whereas no such product or any *Mu8* spliced transcript was amplified in the mutant (Supplemental Figure 2A and 2B). In contrast, two sets of primers anchored on both the *PPR78* coding region and *Mu* TIR (i.e., PPR78-F1+TIR8 and TIR8+PPR78-R1) detected two novel transcripts (Supplemental Figure 2A and 2C), and sequencing of the PCR products showed both of them contained *Mu* TIR and PPR78 sequences (Supplemental Figure 2D). The product amplified by PPR78-F1+TIR8 contained a new stop codon in the TIR, while an in-frame start codon appeared in the *Mu8* TIR of TIR8+PPR78-R1 product. The former transcript could be translated into a 117-amino acid polypeptide, and the latter was predicted to be a truncated PPR78 protein losing the potential mitochondrial targeting peptide. These results show that the *Mu8* is transcribed in the *ppr78-1* mutant, where it interrupts the open reading frame of *PPR78*. Therefore, *ppr78-1* should be a null allele.

The selfed progeny of *ppr78-1*/+ segregated a *small kernel* (*smk*) phenotype, with kernel weight reduced by about 40% at seed maturity (Figure 2A and 2B). Longitudinal sectioning of the *smk* kernels revealed that the endosperm seemed normal but the embryo did not (Figure 2C). The seed germination test showed the *smk* kernels were largely embryo lethal (Figure 2D). Only about 15% of *smk* kernels germinated, of which 1.4% produced a seedling having coleoptile, leaves, and roots, 4.6% developed coleoptile and roots but no leaf, and 9.2% produced roots only. The few seedlings that developed leaves grew slowly and died before flowering (Figure 2E). Of the kernel set in selfed *ppr78-1*/+, 1315 were normal and 411 were *smk*, a ratio consistent with the action of one gene recessive mutation (normal:*smk*, 3:1, $\chi^2 = 1.3$, $p > 0.25$). PCR genotyping analysis of more than 200 plants from selfed *ppr78-1*/+ showed the *smk* phenotype was tightly linked with the presence of the *Mu8* insertion in *PPR78* gene. These results suggest *ppr78* mutation may cause the *smk* phenotype.

Histological Analysis of the *ppr78-1* Mutant

Embryo and endosperm development in the *ppr78-1* mutant was further characterized using light microscopy. At 9 DAP, the scutellum of WT embryo had been formed, and the coleoptile had been initiated (Figure 3A). In the WT endosperm, the aleurone cell layer, the starchy endosperm, and the basal endosperm transfer cell layer (BETL) had all been differentiated (Figure 3A; Supplemental Figures 3A and 4A). At the same developmental stage, the mutant embryo established an apical-basal axis, but did not develop further (Figure 3D). Although the size of the mutant endosperm was much smaller, it had been fully differentiated as well, comprising the aleurone cell layer, starchy endosperm, and BETL (Figure 3D; Supplemental Figures 3D and 4D). By 13 DAP, the full set of embryonic structures (scutellum, coleoptile, leaf primordium, and shoot and root meristems) had been formed in the WT (Figure 3B). But in the mutant embryo, only scutellum was differentiated (Figure 3E). By 18 DAP, the first five leaf primordia were visible in the WT embryo (Figure 3C and 3G), but in the mutant, embryos with variable morphologies were observed: some contained only scutellum (Figure 3F and 3H); some contained scutellum and root meristem (Figure 3I); some

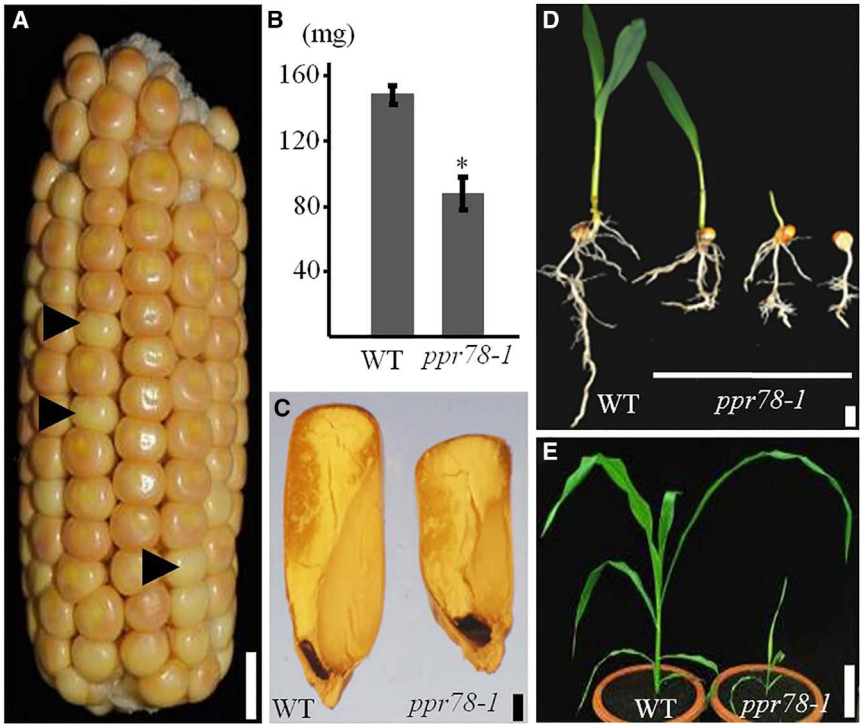

Figure 2. The Phenotype of the *ppr78-1* Mutant.
(A) A selfed ear segregating *small kernel* (*smk*) mutants (arrowed).
(B) Mean kernel weight of *ppr78-1* mutant at seed maturity. Error bars indicate SD from a minimum of three biological replicates (Student's *t*-test, $^{*}p < 0.01$).
(C) Longitudinal section of a *ppr78-1* kernel.
(D) Seed germination of the *ppr78-1* mutant.
(E) Slow growth of *ppr78-1* seedlings. Pictures were taken 33 days after germination.
Scale bars: **(A)** 1 cm, **(C)** 1 mm, **(D and E)** 10 cm.
WT, wild-type; *ppr78-1*, *ppr78-1* mutant.

contained scutellum, coleoptile, and root meristem (Figure 3J); some contained scutellum, coleoptile, and shoot and root meristems (Figure 3K); some contained scutellum, coleoptile, shoot and root meristem, and the first two leaf primordia (Figure 3L). These results show that *ppr78-1* mutants have partially arrested embryos with variable morphologies and reduced size endosperms.

Although BETL in the *ppr78-1* mutant was differentiated, we noticed it was not fully developed (Figure 3D–3F; Supplemental Figure 4D–4F). From 9 to 18 DAP, the BETL in the WT endosperm was recognized as elongated transfer cell layers in the basal region (Supplemental Figure 4A–4C), but in the mutant, these elongated cells did not develop (Supplemental Figure 4D–4F). *BETL2* is a BETL-specific gene in maize, and it was used as a marker to study BETL formation (Liu et al., 2013). Immunohistology using the BETL2 antibody showed that three to four layers of BETL were identified in the basal region of the WT kernels, but only one to two in the mutant (Supplemental Figure 5), indicating a less-developed BETL. Together, both embryogenesis and endosperm development are partially arrested in the *ppr78-1* mutant.

nad5 Mature mRNA Accumulation Is Dramatically Reduced in the *ppr78-1* Mutant

P-subclass PPR proteins are implicated in plant organelle RNA metabolism, including intron splicing, cleavage, maturation, and stabilization (Barkan and Small, 2014). To uncover the molecular function of PPR78 protein, we first studied whether the accumulation of mitochondrial transcripts was affected in the *ppr78-1* mutant using RT–PCR (Figure 4A and 4B). Total RNAs were extracted from 12 DAP kernel tissues in which the pericarp was removed to eliminate contamination from maternal tissues. For semi-quantitative RT–PCR, the primers were anchored on 5′ and 3′ UTRs of each gene to cover the full-length coding region (Xiu et al., 2016); for qRT–PCR, the primers were designed in the protein coding regions (Supplemental Table 2). Among the 35 mitochondrial genes analyzed, most were either increased or unchanged in the *ppr78-1* mutant. By contrast, the steady-state level of *nad5* was dramatically decreased, although not absent. The residual amount of *nad5* was not contamination from the maternal tissue, because analysis of the *ppr78-1* mutant seedling gave similar results (Supplemental Figure 6).

The maize *nad5* gene is composed of three independent precursors (Figure 4C): one comprises exon 1, intron 1, exon 2, and intron 2a (*nad5*T1); the second intron 2b, exon 3, and intron 3a (*nad5*T2); and the third intron 3b, exon 4, intron 4, and exon 5 (*nad5*T3). Generation of mature *nad5* requires two *trans*-splicing reactions among the three distinct transcripts and two *cis*-splicing reactions in *nad5*T1 and *nad5*T3 (Clifton et al., 2004). To examine whether the reduction in *nad5* full-length transcript is due to defective intron splicing, we analyzed the abundance of intron spliced products using RT–PCR (Supplemental Figure 6; Figure 4C and 4D). As shown in Supplemental Figure 6A, the primers were designed on exons to cross each intron. The results showed that the intron 3 and (or) 4 spliced products were accumulated at a comparable level between WT and *ppr78-1* mutant, and the abundance of intron 1 and (or) 2 spliced transcripts was dramatically decreased in the mutant (Supplemental Figure 6B). However, the reduction in intron 1 spliced transcripts was not accompanied by an increased accumulation of the unspliced products. qRT–PCR using primers anchoring on exons and introns showed the abundance of intron 1 or 2a unspliced transcripts was also decreased in the mutant, but intron 2b, intron 3, or intron 4 unspliced products were either increased or almost unchanged (Figure 4C and 4D). These analyses suggest the loss of mature *nad5* may be caused by *nad5*T1 instability rather than intron splicing deficiency.

We performed RNA gel blot to confirm the RT–PCR results. If *nad5*T1 instability causes transcript loss of full-length *nad5*, over-accumulation of the precursor RNA containing intron 2b, exon 3–5, and 3′ UTR (*nad5*T2+3) should be detected in the *ppr78-1* mutant. Prior to northern blot, it is necessary to determine the

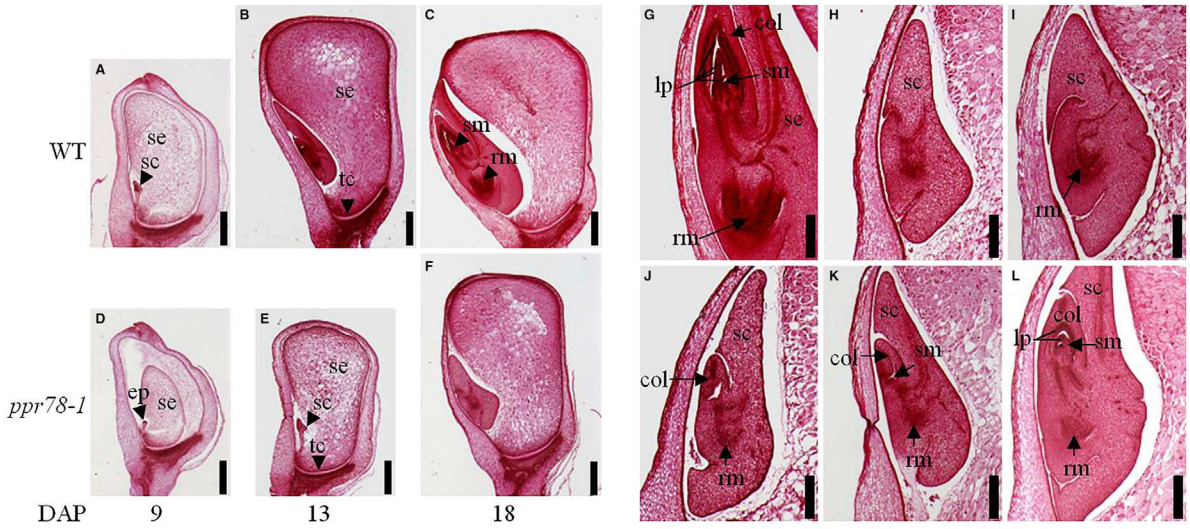

Figure 3. Seed Development in the *ppr78-1* Mutant.

(A–F) Histological analysis of seed development in *ppr78-1* mutant.

(**A**, **B** and **C**) and (**D**, **E** and **F**) Seed development in the wild type and *ppr78-1* mutant at 9, 13 and 18 DAPs, respectively.

(G–L) *ppr78-1* mutant embryos with variable structures at seed maturity. At 18 DAP, five leaf primordia have been differentiated in the wild-type embryo **(G)**, but the embryo structures of the *ppr78-1* mutant are variable with some developed scutellum only **(H)**, some developed scutellum and root meristem **(I)**, some developed scutellum, coleoptile, and root meristem **(J)**, some developed scutellum, coleoptile, and shoot and root meristems **(K)**, and some developed scutellum, coleoptile, two leaf primordia, and shoot and root meristems **(L)**. col, coleoptile; ep, embryo proper; lp, leaf primordia; rm, root meristem; sc, scutellum; se, starchy endosperm; sm, shoot meristem; tc, basal endosperm transfer cell layer. Scale bars: **(A–K)** 1 mm, **(G–L)** 0.5 mm. WT, wild-type; *ppr78-1*, *ppr78-1* mutant; DAP, days after pollination.

size of *nad5*T2+3. In another study, we improved a circular RT–PCR (cRT–PCR) method and mapped the termini of most maize mitochondrial transcripts, including *nad5*T2+3, *nad5*T1, and mature *nad5* (our unpublished results). cRT–PCR allows simultaneous mapping of the 5′ and 3′ ends of the target transcripts, thus determines the transcript size (Forner et al., 2007). Combined with RNA 5′ polyphosphatase treatment, cRT–PCR analysis could distinguish primary (derived from transcription initiation) and processed (derived from post-transcriptional processing) transcripts. Based on the analysis, one dominant form *nad5*T2+3 derived from post-transcriptional processing is present in W22 mitochondria: the 3′ end is about 92 nt downstream of exon 5 and the 5′ end is 56–152 nt upstream of exon 3, thus the calculated size is 715–811 nt.

Consistent with the RT–PCR results, RNA gel blot using probes specific to *nad5* exon 1 or 2 detected a trace amount of mature transcripts in the mutant, and intron 1 and 2a unspliced products were not detected (Figure 5A and 5B). However, the exon 4 or 5 probes recognized two strong bands of similar size and pattern as mature *nad5*. These transcripts were neither *nad5*T2+3 nor mature *nad5* because they were much longer (about 2400 nt), and *nad5* exon 1 and 2 probes could not recognize them. In maize genome, the *nad5* exon 3 is 358 bp downstream of *nad1* exon 5 (Clifton et al., 2004). We hypothesize that the two exons are transcribed as a dicistron and give rise to a fusion transcript containing *nad1* exon 1–5, *nad5* exon 3–5, and the 385-nt interspacer linking them (*nad1:nad5*). *nad1:nad5* may be the transcripts detected in the *ppr78-1* mutant by *nad5* exon 4 and 5 probes. RT–PCR using primers anchoring on the interspacer or *nad1* exon 1 and *nad5* exon 5, as well as RNA gel blots using probes specific to *nad1* exon 1 or 5 confirmed our hypothesis (Figure 5C–5E). The subsequent cRT–PCR results showed *nad1:nad5* was close to the full-length *nad5* in size. These results indicate (1) the reduction in mature *nad5* is not accompanied by increased accumulation of *nad5*T2+3 precursor, and (2) the bands recognized by *nad5* exon 1 or 2 probes in RNA gel blots are mature *nad5* only, but by exon 4 or 5 probes are a combination of *nad5* and *nad1:nad5*.

nad1:nad5 contains the *nad5* intron 2b sequences, and its accumulation level was increased in the *ppr78-1* mutant (Figure 5C–5E). It is still possible that the loss of mature *nad5* transcripts is due to the instability of *nad5*T1 because intron 2a may directly interact with the intron 2b region in *nad1:nad5* to facilitate intron 2 *trans*-splicing. However, this possibility could be ruled out. If the intron 2b sequences in *nad1:nad5* interact with *nad5*T1 intron 2a to facilitate intron 2 splicing, it would generate an equal amount of full-length *nad1* and *nad5*. The reduction of *nad5*T1 would decrease the transcript level of mature *nad1*, which we did not observe in both RT–PCR and northern blot experiments (Figures 4A, 4B, and 5E). It is more likely that the instability of *nad5* mature mRNA itself rather than *nad5*T1 precursor causes the transcript loss of mature *nad5*, and PPR78 protein may function in protection of mature *nad5* from degradation.

To further exclude the possibility that *nad5*T1 instability caused mature *nad5* transcript loss, we performed cRT–PCR to analyze *nad5*T1 transcript termini in the *ppr78-1* mutant (Figure 5F and 5G). If *nad5*T1 is unstable, degradation of *nad5*T1 losing transcript termini should be detected. In the maize W22

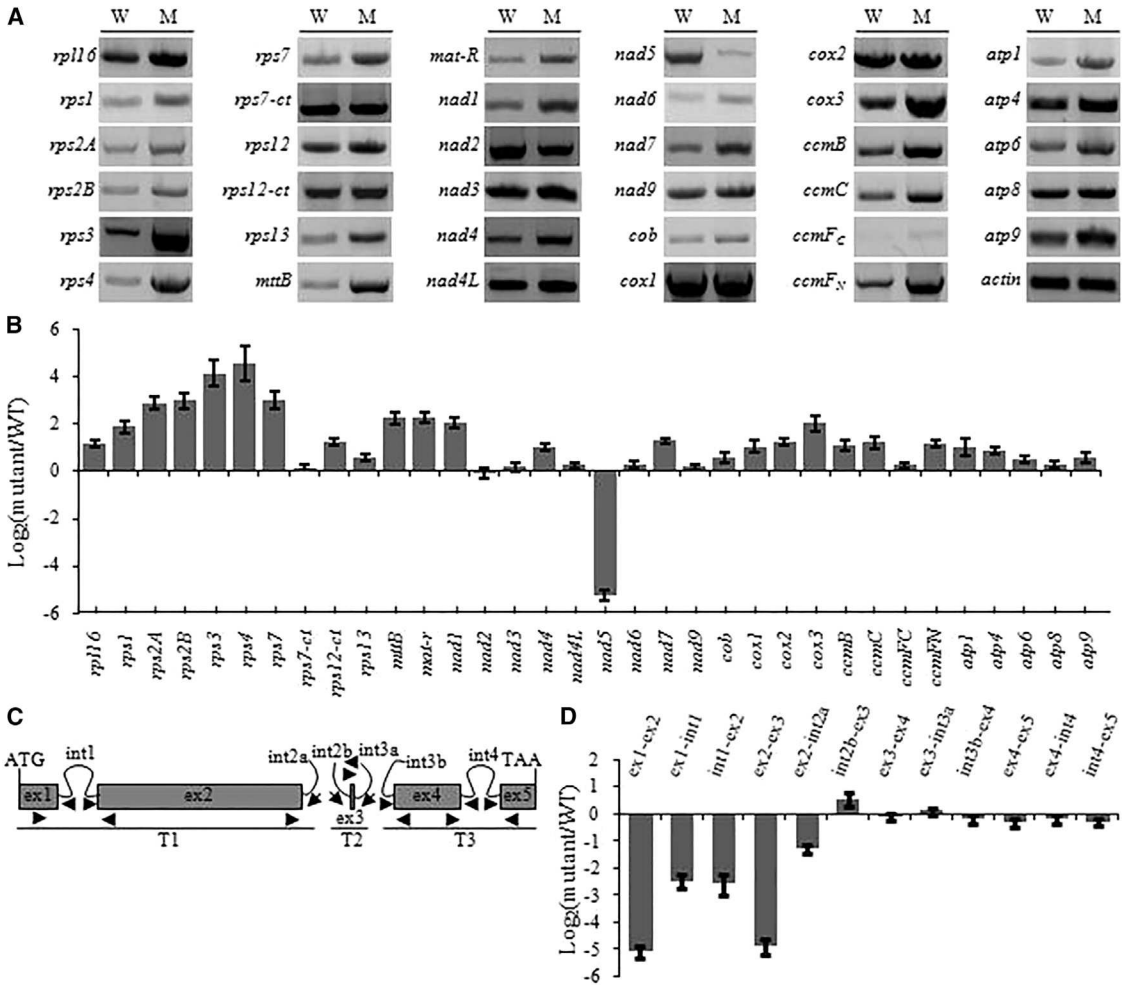

Figure 4. The Accumulation of *nad5* Transcripts Was Impaired in the *ppr78-1* Mutant.

(A and B) Semi-quantitative and quantitative RT–PCR analyses on the expression of 35 mitochondrion-encoded genes in the *ppr78-1* mutant, respectively. W, wild-type; M, *ppr78-1* mutant.

(C) Gene structure of maize *nad5.* Exons and introns are shown as gray boxes and curved lines, respectively. Two *trans*-splicing introns divide the *nad5* gene into three distinct transcripts: T1, T2, and T3. Arrows indicate the position of the primers used for quantitative RT–PCR analysis of *nad5* transcript abundance.

(D) The steady-state levels of *nad5* transcripts in the *ppr78-1* mutant. Values are means and SD of three biological replicates; RNAs used for RT–PCR in (**A, B, and D)** were isolated from 12 DAP kernel tissues, and cDNAs were normalized by amplification of the maize *actin* gene.

mitochondria, there are two major forms of *nad5*T1: the primary one is located 374 nt upstream of AUG, and the processed one is ended 51–334 nt upstream of AUG; the 3′ ends of both forms are about 732 nt downstream of exon 2 (our unpublished results). In the *ppr78-1* mutant, these two types of *nad5*T1 were easily amplified, and sequencing of 30 molecules failed to detect any truncated *nad5*T1 transcript. These results also argue against the instability of *nad5*T1 in *ppr78-1* mutant. The decreased transcript abundance of *nad5*T1 in the mutant might be a secondary effect to the loss of mature *nad5*.

Moreover, these data exclude the possibility that PPR78 protein functions in *nad1:nad5* processing. If PPR78 functions in the cleavage of *nad1:nad5* at the 385-nt interspacer to generate full-length *nad1* and precursor *nad5*T2+3, loss of PPR78 should lead to an overaccumulation of *nad1:nad5* and *nad5*T1, but reduction of mature *nad1*. Actually, the abundance of *nad5*T1 was decreased while the steady-state level of mature *nad1* was increased in the mutant (Figures 4 and 5E).

PPR78 Is Required for Seed Development and *nad5* Mature mRNA Accumulation

To confirm the role of PPR78 protein in seed development and *nad5* mature mRNA accumulation, we identified a second *ppr78* allele from the Uniform*Mu* library, named *ppr78-2*. *Mu* insertion in *ppr78-2* is 336 bp downstream of ATG, i.e., 2 bp upstream of the *Mu8* insertion site in *ppr78-1* (Figure 1A). The self-pollinated *ppr78-2*/+ plants segregated *smk* kernels, most of which were embryo lethal (Figure 6A). Although some *smk* kernels germinated, the emerging mutant seedlings were abnormal: some had roots only and some had coleoptile and roots (Figure 6B and 6C). Crosses between *ppr78-1*/+ and *ppr78-2*/+ produced about 25% *smk* kernels (Figure 6A). These

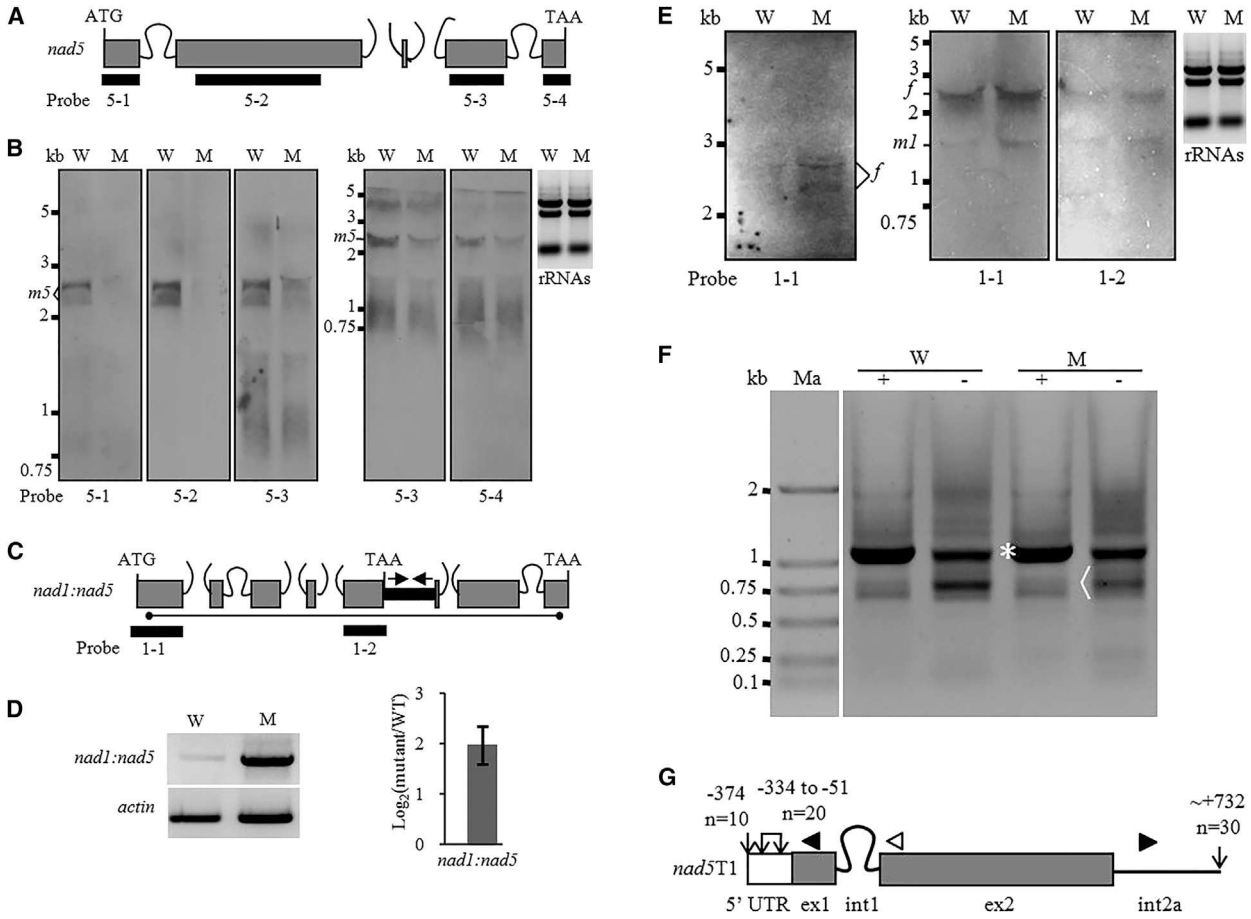

Figure 5. *nad5* Mature mRNA Accumulation Is Dramatically Reduced in the *ppr78-1* Mutant.

(A) Gene structure of maize *nad5*. The positions of the *nad5* probes are indicated.

(B) RNA gel blot assay of *nad5* transcript accumulation. 20 μg of total RNAs from 12 DAP kernel tissues were loaded in each lane. Bands corresponding to mature *nad5* (*m5*) are marked.

(C) Structure of maize *nad1:nad5*. It contains *nad1* exon 1–5, *nad5* exon 3–5, and an interspacer linking them (bold black line). The primers used for semi-quantitative and quantitative RT–PCR in **(D)** are indicated as black dots and arrowheads, respectively; the *nad1* probes are indicated.

(D) RT–PCR analysis of *nad1:nad5* expression. The values represent the means and SD of three biological replicates.

(E) RNA gel blot assay on the transcript accumulation of *nad1:nad5* (*f*) and mature *nad1* (*m1*). The pictures on the right and left in **(B and E)** are different with regard to the duration of gel electrophoresis: the ones on the left ran for 6 h and those on the right ran for 3 h.

(F) Circular RT–PCR analysis of *nad5*T1 transcript termini in the *ppr78-1* mutant. + and −, mitochondrial RNAs treated or not by RNA 5′ polyphosphatase, respectively. The bands as indicated by the asterisk and curved line were recovered and sequenced by cloning into vectors.

(G) Transcript termini of maize *nad5*T1 in the *ppr78-1* mutant. Position of the 5′ or 3′ end relative to the translation start codon and *nad5* exon 2, and the number of clones obtained at that position are indicated.

The primers used for reverse transcription and PCR amplification of *nad5*T1 are indicated by open and closed arrows, respectively. W, wild-type; M, *ppr78-1* mutant; RNAs were normalized using cytosolic rRNAs, and cDNAs by amplification of the maize *actin* gene; Ma, DNA marker.

results confirm an essential role of PPR78 in maize seed development.

We performed RT–PCR to analyze the effect of *ppr78-2* mutation on *nad5* transcript accumulation (Figure 6D and 6E). Total RNAs were extracted from seedling roots, and the seedling genotype was determined by PCR using gene-specific and *Mu* TIR primers. In the *ppr78-2* homozygote, no WT *PPR78* transcript was detected, suggesting *ppr78-2* was likely a null allele (Figure 6D). In the previous experiments, we found the intron 1 and (or) 2 spliced products detected by RT–PCR were derived from *nad5* only, but the intron 3 and (or) 4 spliced transcripts observed were a combination of *nad5* and *nad1:nad5*. Semi-quantitative RT–PCR using primer pairs across intron 1 and 2 showed the abundance of the *nad5* transcript was dramatically decreased in the *ppr78-2* mutant (Figure 6D). The results from primer pair crossing *nad5* intron 3 and 4 showed a comparable transcript level between the mutant and WT. To quantify the steady-state level of *nad5* transcripts in *ppr78-2* mutant, we performed qRT–PCR using primers on *nad5* introns and exons (Figures 4C and 6E). The results showed that the transcripts with intron 1 or 2 spliced in *ppr78-2* mutant were reduced to about 3% and 2% of that assayed in the WT, respectively. However, the accumulation levels of transcripts with intron 3 or 4 spliced were slightly increased or almost unchanged in the mutant. These

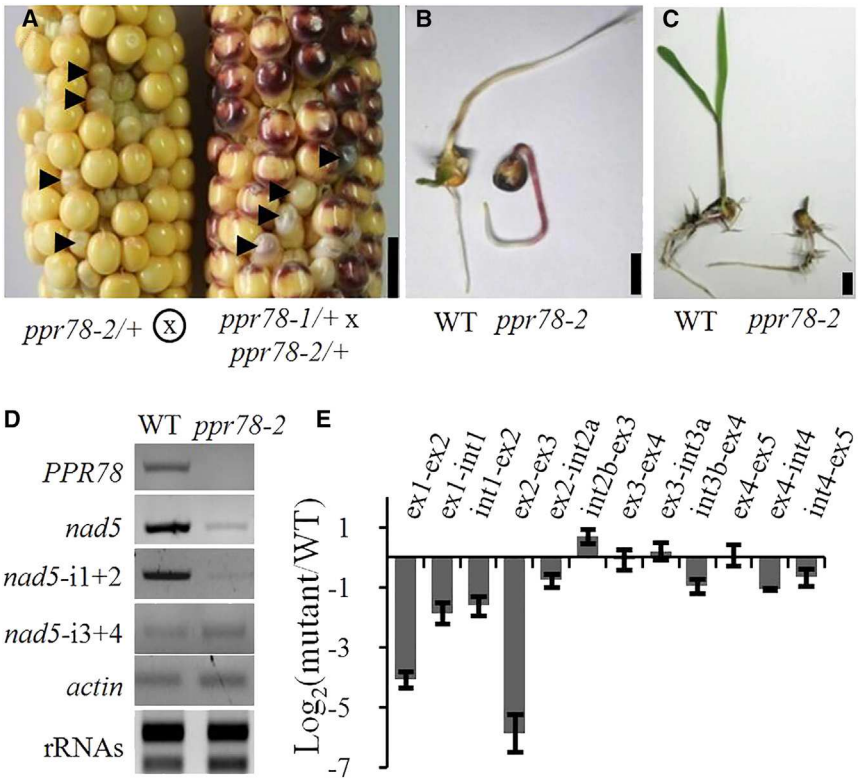

Figure 6. *PPR78* Is Essential for Seed Development and *nad5* Transcript Accumulation in Maize.

(A) *smk* kernels segregate in the ears of self-pollinated *ppr78-2/+* and the cross between *ppr78-1/+* and *ppr78-2/+*. Arrows point to the *smk* kernels.

(B and C) Seedling phenotype of *ppr78-2* mutant.

(D) Semi-quantitative RT–PCR analysis of *nad5* transcript accumulation in *ppr78-2*. *nad5*, full-length *nad5*; *nad5*-i1+2 and *nad5*-i3+4, *nad5* transcripts with introns 1–2 and introns 3–4 spliced, respectively.

(E) Quantitative RT–PCR analysis of the steady-state levels of *nad5* transcripts in the *ppr78-2* mutant. Values represent the mean and SD of three biological replicates.

WT, wild-type; *ppr78-2, ppr78-2* homozygote. Scale bars: 1 cm; total RNAs were isolated from seedling roots, and cDNAs were normalized by amplification of the maize *actin* gene.

RT–PCR analyses reveal that *ppr78-2* is a null allele, and loss of PPR78 function causes a dramatic reduction in the accumulation of *nad5* mature mRNA.

nad5 Mature mRNA Stability Is Compromised in *ppr78* Mutant

To test the hypothesis that the reduction in *nad5* mature mRNA is caused by the instability itself, we used cRT–PCR to study whether degradation *nad5* transcripts were enriched in the *ppr78-1* mutant.

In W22, there were two major forms of mature *nad5* detected by cRT–PCR (Figures 5B and 7A): 5′ ends of the primary and secondary ones were 374 and 43–332 nt upstream of AUG, respectively, and 3′ ends of both types were about 92 nt downstream of UAA. The calculated transcript sizes of mature *nad5* based on the mapped termini were about 2477 and 2151–2440 nt, respectively, which matched the sizes of the two major PPR78-dependent transcripts detected by RNA gel blots (Figure 5B). In the *ppr78-1* mutant, both primary and processed *nad5* mature mRNAs were detected, but the truncated forms losing both AUG and UAA were highly enriched (Figure 7A and 7C): among the 27 processed *nad5* molecules analyzed, only three were mapped to the mature transcript ends, three had an intact 3′ end but lost AUG, while the remaining 21 lost both AUG and UAA. Sequencing of 35 *nad5* molecules from WT showed only three of them were truncated at the 5′end, but the other 32 were intact in both ends. These results reveal that loss of PPR78 leads to the degradation of *nad5* mature mRNA, thus confirms the requirement of PPR78 for *nad5* mature RNA stability.

To study how PPR78 protects *nad5* mature mRNA, we also used cRT–PCR to inspect the transcript termini of *nad1:nad5*, which had the same 3′ part as *nad5* mature mRNA. If PPR78 stabilizes *nad5* mature mRNA at the 3′ end by blocking 3′-to-5′ exonucleolytic cleavage, truncated *nad1:nad5* losing the 3′ part should be detected in the *ppr78* mutant. In WT, two major forms of *nad1:nad5* were amplified (Figure 7B and 7D). They were identical at the 3′ termini but different at the 5′ termini: the 3′ ends were about 92 nt downstream of *nad5* UAA, the same as the 3′ termini of mature *nad5*; their 5′ ends were mapped 592 and 193–354 nt upstream of *nad1* AUG, respectively. The calculated sizes of *nad1:nad5* were about 2438 and 2040–2200 nt, which were close to the sizes of two major forms of mature *nad5*. Besides the intact transcripts, we did not detect any degradation of *nad1:nad5* in the *ppr78-1* mutant. The cRT–PCR analyses exclude the possibility that PPR78 stabilizes *nad5* mature mRNA by serving as a blockade to 3′-to-5′ exonucleolytic decay at the 3′ end. In support of the cRT–PCR results, a PPR78 binding site was predicted at *nad5* 5′ UTR 29–41 nt upstream of AUG (Supplemental Figure 7). By contrast, no potential PPR78 binding site was found on *nad5* 3′ UTR, as well as intron 2a of *nad5* and the interspacer of *nad1:nad5*.

Complex I Formation Is Impaired in the *ppr78-1* Mutant

In *Arabidopsis*, NAD5 is a key subunit of complex I, and the defect in *nad5* gene expression impairs complex I assembly and causes an arrest in plant development (Colas des Francs-Small et al., 2014). To study the effect of defective *nad5* gene expression in maize, crude mitochondria were isolated from both WT and *ppr78-1* mutant, and the abundance of complex I was compared by blue native polyacrylamide gel electrophoresis (BN–PAGE) (Figure 8A). The results showed that the mutant mitochondria had little assembled complex I and super-complex I + III_2, but there was no marked difference in the quantity of complex V (F_0F_1–ATP synthase). Moreover, in-gel NADH-dehydrogenase activity staining detected trace amounts

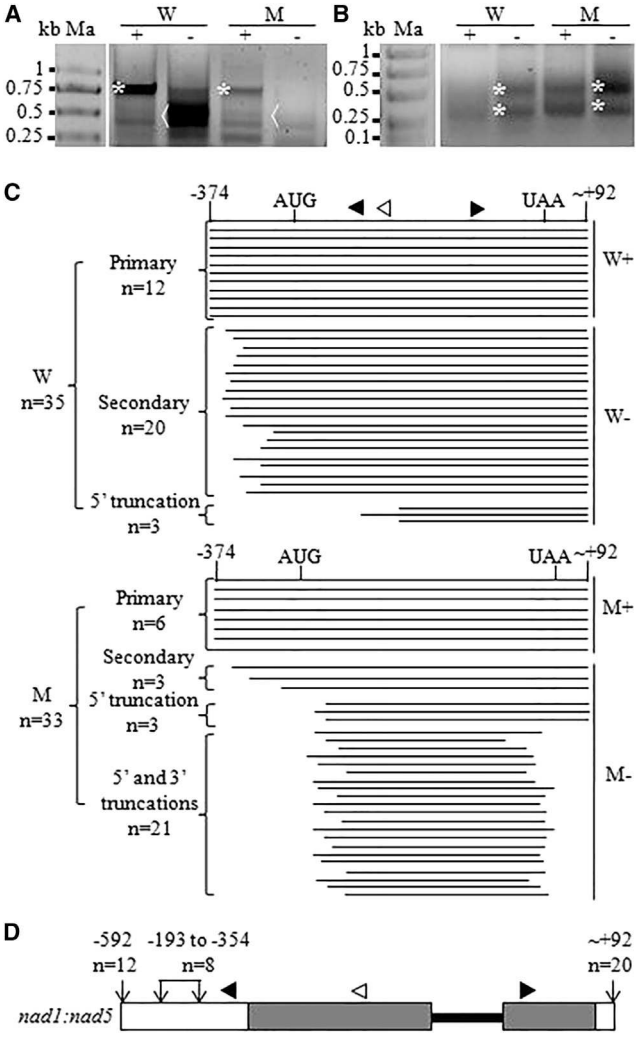

Figure 7. Transcript End Mapping of Mature *nad5* and *nad1:nad5* in the *ppr78-1* Mutant.

(A and B) Circular RT–PCR amplification of mature *nad5* and *nad1:nad5*, respectively. The bands as indicated by asterisks and curved lines were gel purified and sequenced by ligation into vectors.

(C) Transcript termini of mature *nad5* in the *ppr78-1* mutant. Each black bar represents a single sequenced clone and shows the position of the 5′ and 3′ ends relative to the translation start and stop codons. Gel fractions of the clones are indicated on the right.

(D) Transcript termini of *nad1:nad5*. The *nad1* and *nad5* coding regions are shown as gray boxes, the 5′ UTR of *nad1* and 3′ UTR of *nad5* as white boxes, the intergenic spacer linking *nad1* exon 5 and *nad5* exon 3 as a bold black line.

Positions of the 5′ and 3′ ends relative to the coding regions and the number of clones obtained at that position are indicated. The primers used for reverse transcription and PCR amplification are indicated by open and closed arrows, respectively. W, wild-type; M, *ppr78-1*; + and −, mitochondrial RNAs treated or not by RNA 5′ polyphosphatase, respectively; Ma, DNA marker.

of complex I and super-complex I + III_2 (Figure 8B), suggesting a residual NADH-dehydrogenase activity of complex I in the *ppr78-1* mutant. Together, these results show that the *nad5* transcript defects in *ppr78-1* have a significant impact on complex I accumulation and activity.

In mitochondria, the block in the electron transfer chain increases the activity of the alternative respiratory pathway (Karpova et al., 2002). This pathway is characterized by the expression of alternative oxidase (AOX). In maize, three homologous genes encode AOXs: *Aox1* (AY059646.1), *Aox2* (AY059647.1), and *Aox3* (AY059648.1). A complex I deficiency could activate the alternative respiratory pathway by increasing *Aox2* gene expression level (Li et al., 2014; Xiu et al., 2016). To analyze whether the impairment in the formation of complex I blocks the electron transfer chain in *ppr78-1* mutant, qRT–PCR was performed to investigate the expression level of the *Aox* genes (Figure 8C). As expected, the *Aox2* expression in *ppr78-1* mutant was dramatically upregulated to 358-fold over the WT level, indicating activation of the alternative respiratory pathway as the electron transport chain was blocked.

DISCUSSION

PPR78 Is Involved in *nad5* Mature mRNA Stability in Maize

PPR is a large protein family implicated in organellar gene expression, including RNA editing, intron splicing, RNA processing, RNA maturation, RNA stability, and translation initiation (Barkan and Small, 2014). In chloroplasts, many PPR proteins have been documented to confer RNA stability (Barkan and Small, 2014); in mitochondria, AtMTSF1 and AtMTSF2 are the few PPR proteins reported to stabilize RNA (Haïli et al., 2013; Wang et al., 2017). Although we could not rule out the possibility that PPR78 may take part in other aspects of mitochondrial gene expression, the evidence provided in this study demonstrates an essential role of PPR78 in *nad5* mature mRNA stability.

In *Arabidopsis*, generation of the mature *nad1* requires *trans*-splicing among three distinct precursors, including exon 1, exons 2–3, and exons 4–5 (Wang et al., 2017). The AtMTSF2 protein binds at the 3′ extremity of exons 2–3 and protects it from degradation by 3′-to-5′ exonucleolytic cleavage. In *Atmtsf2* mutant, the precursor exon 2–3 is degraded, which blocks the formation of mature *nad1* and leads to overaccumulation of the precursors exon 1 and exons 4–5. One major difference between AtMTSF2 and PPR78 is that the former protects precursor RNA, whereas the latter stabilizes the spliced mRNA. In maize, the mature *nad5* is assembled by three independent precursors: *nad5*T1, T2, and T3. In *ppr78* mutants, the abundance of mature *nad5* is severely decreased (Figures 4–6). Although the steady-state level of *nad5*T1 is also reduced, it seems unlikely the cause of the loss of *nad5* mature mRNA because (1) we could not detect increased accumulation of *nad5*T2+3, and (2) the transcript termini of *nad5*T1 are intact in the mutant. *nad1:nad5* contains the *nad5* intron 2b region, and its steady-state level is dramatically increased in the *ppr78* mutant. It is still possible that the *nad5*T1 intron 2a could interact with *nad1:nad5* directly to facilitate intron 2 *trans*-splicing, and the reduction of *nad5*T1 in *ppr78* mutant blocks the assembly of mature *nad5*. Another possibility is that PPR78 may be essential for *nad1:nad5* processing, and loss of function in PPR78 blocks the generation of *nad5*T2+3, thus mature *nad5*. However, our data argue against both of the two possibilities (see analysis in Results). Supporting an essential role of PPR78 in *nad5* mature RNA stability, we detected enrichment of truncation *nad5* mRNA in the *ppr78* null mutant.

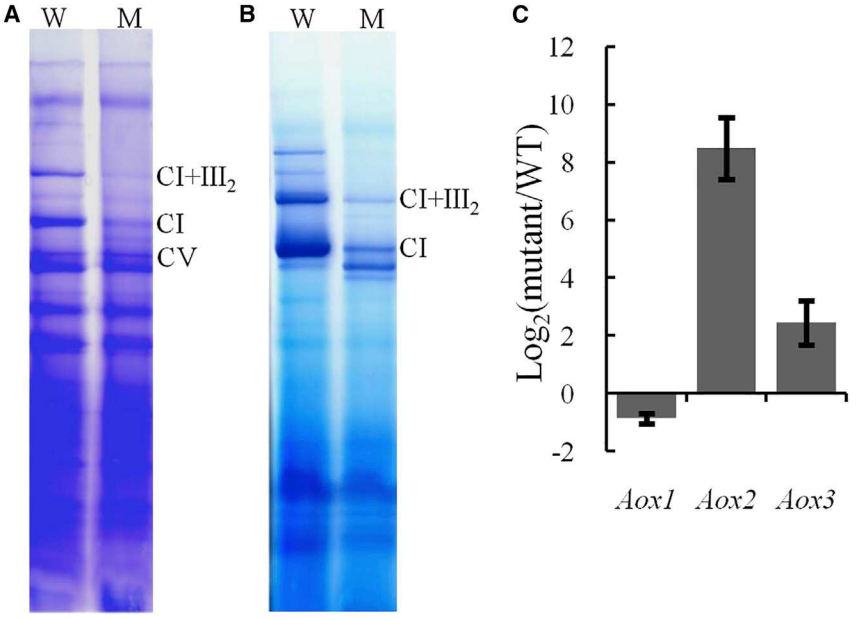

Figure 8. Reduced Abundance and NADH-Dehydrogenase Activity of Complex I in *ppr78-1* Mutant.

(A) Coomassie blue staining of mitochondrial extracts separated by blue native polyacrylamide gel electrophoresis.

(B) In-gel NADH-dehydrogenase activity staining for complex I.

(C) Quantitative RT–PCR analysis of *Aox* gene expression. Values represent the means and SD of three biological replicates. W, wild-type; M, *ppr78-1*; RNA normalization was performed against the maize *actin* gene; CI, complex I; CV, complex V; CI + III_2, super-complex I + III_2.

PPRs are RNA-binding proteins. Previous results show chloroplast PPR proteins could bind to the 5′ or 3′ termini of the target transcripts and serve as a barrier for RNA decay in either 5′ or 3′ direction (Pfalz et al., 2009; Prikryl et al., 2011; Zhelyazkova et al., 2012; Zoschke et al., 2016). The mitochondrial PPR proteins AtMTSF1 and AtMTSF2 could also protect their RNA targets by blocking 3′-to-5′ exonucleolytic cleavage (Haïli et al., 2013; Wang et al., 2017). In the absence of the protecting proteins, truncated transcripts losing either the 5′ or 3′ end are enriched. *nad1:nad5* has the same 3′ end as *nad5* mature mRNA. The intact 3′ terminus of *nad1:nad5* in the *ppr78* mutant argues against the possibility that PPR78 stabilizes mature *nad5* at its 3′ end by blocking 3′-to-5′ exonucleolytic cleavage. It is also unlikely that PPR78 serves as a barrier to 5′-to-3′ degradation because *nad5* transcripts losing not only translation start but also stop codons are enriched in the mutant. Moreover, increasing evidence suggests that exonuclease with 5′-to-3′ activity may be absent in plant mitochondria (Binder et al., 2013; Ruwe et al., 2016). The present data imply that the mitochondrial PPR78 may stabilize mature *nad5* in a different way, by which it simultaneously protects the 5′ and 3′ termini of the full-length mRNA. Further experiments (e.g., RNA-binding assay) are necessary to unravel the underlying mechanism whereby PPR78 stabilizes mature *nad5* in maize.

PPR78 Is Required for Maize Seed Development

The mitochondrion is the power house for cellular activity, and the energy of ATP is generated by production of a proton gradient via the electron transport chain, consisting of complex I, II (succinate dehydrogenase), III (cytochrome c reductase), mobile electron protein cytochrome C, and complex IV (cytochrome c oxidase) and V. Disturbance of the electron transport chain may compromise energy production and arrest seed development in maize (Sosso et al., 2012; Li et al., 2014; Sun et al., 2015; Xiu et al., 2016; Yang et al., 2017). In *empty pericarp16* mutant, the defect in *nad2* gene expression reduces complex I assembly, blocks the electron transport chain, and arrests embryo and endosperm development at a very early stage (Xiu et al., 2016). In the *ppr2263* mutant, defective editing at the *cob*-908 site causes a reduction in the formation of complex III and partially arrests maize seed development (Sosso et al., 2012). PPR78 protein is required for *nad5* mature mRNA stability. In the *ppr78* null mutants, the defect in *nad5* transcript accumulation severely compromises complex I assembly, and thus causes the *smk* phenotype with partial arrest in embryogenesis and endosperm development.

METHODS

Plant Materials

The maize *ppr78-1* and *ppr78-2* alleles were obtained from the Uniform*Mu* transposon-tagging population, which was created by introgressing *Mu* active lines into the inbred W22 background (McCarty et al., 2005). The WT plants used in this study were either siblings of the mutant or W22 inbred. The maize plants were grown in field conditions at South China Agricultural University. The kernel tissues used for total RNA extraction and crude mitochondria isolation were endosperm plus embryo.

Seed Germination Test

To test the seed germination rate, *ppr78-1* mutant kernels were surface sterilized by 30% (v/v) hypochloric acid for 20 min, rinsed three times in distilled water, then sown in sterile soil. The germination rate was recorded 2 weeks after the seeds were sown.

Genomic DNA Extraction and Allelism Test

Maize genomic DNA was extracted using the urea extraction method (Tan et al., 2011). The genotypes of *ppr78-1* and *ppr78-2* were determined using the *Mu* TIR primer paired with the *PPR78* gene-specific primer (Supplemental Table 1). To determine the genetic allelism between *ppr78-1* and *ppr78-2*, the *ppr78-1*/+ and *ppr78-2*/+ plants were reciprocally crossed, and the phenotypes of *ppr78-1*/*ppr78-1*, *ppr78-2*/*ppr78-2* and *ppr78-1*/*ppr78-2* were characterized.

Histology of *ppr78-1* Seed Development

Developing WT and *ppr78-1* mutant kernels were harvested from self-pollinated *ppr78-1*/+ ears at 9, 13, and 18 DAP. The kernels were cut longitudinally into three slices, and the central slice containing the embryo was fixed in 4% (w/v) paraformaldehyde overnight at 4°C. The kernel samples were dehydrated, infiltrated, and embedded as described (Zhang et al., 2013). Embedded sections were de-waxed in absolute xylene, rehydrated by passing through an ethanol series (100%–50%), then stained in 1% w/v safranin O (Sigma, USA) for 18–24 h. The stained slides were rinsed in tap

water, air-dried, then dehydrated in 1:1 xylene:ethanol and 100% absolute xylene. The resulting sections were mounted by Canada Blasam and observed using light microscopy (Eclipse E80i, Nikon, Japan).

Immunohistochemistry Analysis

WT and *ppr78-1* mutant kernels were harvested from the same ear of a self-pollinated *ppr78-1*/+ plant at 13 DAP. Immunohistochemistry analysis was performed as described previously (Liu et al., 2013).

Subcellular Localization

A DNA fragment of the *PPR78* gene containing the potential mitochondrial signal peptide (amino acids 1–320) was amplified from maize cDNA of W22 inbred. The PCR product was cloned into pENTR/D-TOPO (Invitrogen, USA), and concomitantly introduced into the binary vector pGWB5 by Gateway site-specific recombination. The resulting construct was transiently expressed in tobacco (*Nicotiana tabacum*) epidermal leaves and colocalization of GFP and MitoTracker signals was inspected using an Olympus FluoView FV1000 confocal microscope (Olympus, Japan).

Isolation of Crude Mitochondria, BN–PAGE, and NADH-Dehydrogenase Activity Assay

Isolation of crude mitochondria from maize kernels, BN–PAGE, and in-gel NADH-dehydrogenase activity assay were performed as described (Sun et al., 2015).

RNA Extraction, RT–PCR, and cRT–PCR

RNAs were extracted using the TRIzol reagent (Invitrogen, USA) and treated by DNase I (NEB, UK). Possible DNA contamination was controlled by PCR amplification using RNA as the template. One microgram of DNA-free RNA was reverse transcribed using random hexamer primers by SuperScript III (Invitrogen, USA).

To compare the steady-state transcript levels of the 35 protein coding mitochondrial genes, semi-quantitative PCR was performed at an annealing temperature of 57°C using the primers described previously (Xiu et al., 2016). The PCR cycles were adjusted between 28 and 40 depending on the transcript level of the corresponding gene. To analyze the transcript levels of *nad5* intron spliced or unspliced products and *nad1:nad5*, RT–PCR was performed at an annealing temperature of 57°C for 28–41 cycles. cDNA normalization was performed by amplification of the maize *actin* gene (NM_001155179.1) at an annealing temperature of 57°C for 27–30 cycles.

qRT–PCR was performed on cDNAs synthesized from three biological replicates using iQ SYBR Green Supermix (Bio-Rad, USA). Each determination included three repeats, and relative transcript levels were measured with respect to the transcript of the maize *actin* gene. Reactions were run on a Light-Cycler 480 Real-Time PCR System (Roche, USA), and data were evaluated with LIGHT-CYCLER 480 (v1.5). Quantitative analysis on the abundance of the 35 mitochondria-encoded transcripts and *nad5* intron spliced or unspliced products was performed using the primers listed in Supplemental Table 2.

For cRT–PCR, RNAs obtained from crude mitochondria were treated by RNA 5′ polyphosphatase (Epicentre, USA) to remove two phosphates from the primary 5′ ends. After this treatment, the primary transcripts could be circularized as the processed transcripts (Forner et al., 2007). The RNAs treated by RNA 5′ polyphosphatase were purified using the PureLink RNA Mini kit (Invitrogen, USA). 0.5 μg of RNAs treated or not by 5′ polyphosphatase were self-ligated using T4 RNA ligase 1 (NEB, UK) according to the recommended protocol. Equal amounts of circular RNAs were reverse transcribed using gene-specific primers (Supplemental Table 1), followed by one round of PCR amplification. The cRT–PCR products were separated by 1% agarose gel, and the bands amplified were recovered and cloned into pEASY-T1 Simple vector (TransGen Biotech, China).

RNA Gel Blot Hybridization

RNA gel blot hybridization was performed using the DIG Northern Starter Kit (Roche, USA) according to the manufacturer's protocol. The DNA fragments used to prepare the hybridization probes were amplified by the primers given in Supplemental Table 1. The PCR products were separated in 1% agarose gel, recovered, and cloned into pEASY-Blunt Simple vector, which contained a T7 promoter upstream of the insertion site. Before *in vitro* transcription, the construct was linearized by proper restricted endonuclease to make sure that there were few vector sequences in the resulting probes.

ACCESSION NUMBERS

Sequence data for maize *PPR78* can be found in the GenBank data library under accession number GenBank: KM596506.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

B.C.T. and Y.F.Z. designed the experiments, Y.F.Z., M.S., and F.S. performed the experiments, Y.F.Z. and B.C.T. analyzed the data, and Y.F.Z. wrote the paper.

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REFERENCES

Ban, T., Ke, J., Chen, R., Gu, X., Tan, M.H., Zhou, X.E., Kang, Y., Melcher, K., Zhu, J.K., and Xu, H.E. (2013). Structure of a PLS-class pentatricopeptide repeat protein provides insights into mechanism of RNA recognition. J. Biol. Chem. **288**:31540–31548.

Barkan, A., Rojas, M., Fujii, S., Yap, A., Chong, Y.S., Bond, C.S., and Small, I. (2012). A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. PLoS Genet. **8**:e1002910.

Barkan, A., and Small, I. (2014). Pentatricopeptide repeat proteins in plants. Annu. Rev. Plant Biol. **65**:415–442.

Binder, S., Stoll, K., and Stoll, B. (2013). P-class pentatricopeptide repeat proteins are required for efficient 5' end formation of plant mitochondrial transcripts. RNA Biol. **10**:1511–1519.

Cheng, S., Gutmann, B., Zhong, X., Ye, Y., Fisher, M.F., Bai, F., Castleden, I., Song, Y., Song, B., Huang, J., et al. (2016). Redefining the structural motifs that determine RNA binding and RNA editing by pentatricopeptide repeat proteins in land plants. Plant J. **85**:532–547.

Clifton, S.W., Minx, P., Fauron, C.M., Gibson, M., Allen, J.O., Sun, H., Thompson, M., Barbazuk, W.B., Kanuganti, S., Tayloe, C., et al. (2004). Sequence and comparative analysis of the maize NB mitochondrial genome. Plant Physiol. **136**:3486–3503.

Colas des Francs-Small, C., Falcon de Longevialle, A., Li, Y., Lowe, E., Tanz, S.K., Smith, C., Bevan, M.W., and Small, I. (2014). The

pentatricopeptide repeat proteins TANG2 and ORGANELLE TRANSCRIPT PROCESSING439 are involved in the splicing of the multipartite *nad5* transcript encoding a subunit of mitochondrial complex I. Plant Physiol. **165**:1409–1416.

Delannoy, E., Stanley, W.A., Bond, C.S., and Small, I.D. (2007). Pentatricopeptide repeat (PPR) proteins as sequence-specificity factors in post-transcriptional processes in organelles. Biochem. Soc. Trans. **35**:1643–1647.

Forner, J., Weber, B., Thuss, S., Wildum, S., and Binder, S. (2007). Mapping of mitochondrial mRNA termini in *Arabidopsis thaliana*: t-elements contribute to 5' and 3' end formation. Nucleic Acids Res. **35**:3676–3692.

Gully, B.S., Cowieson, N., Stanley, W.A., Shearston, K., Small, I.D., Barkan, A., and Bond, C.S. (2015). The solution structure of the pentatricopeptide repeat protein PPR10 upon binding atpH RNA. Nucleic Acids Res. **43**:1918–1926.

Haïli, N., Arnal, N., Quadrado, M., Amiar, S., Tcherkez, G., Dahan, J., Briozzo, P., Colas des Francs-Small, C., Vrielynck, N., and Mireau, H. (2013). The pentatricopeptide repeat MTSF1 protein stabilizes the *nad4* mRNA in *Arabidopsis* mitochondria. Nucleic Acids Res. **41**:6650–6663.

Hammani, K., Takenaka, M., Miranda, R., and Barkan, A. (2016). A PPR protein in the PLS subfamily stabilizes the 5'-end of processed rpl16 mRNAs in maize chloroplasts. Nucleic Acids Res. **44**:4278–4288.

Hauler, A., Jonietz, C., Stoll, B., Stoll, K., Braun, H.P., and Binder, S. (2013). RNA PROCESSING FACTOR 5 is required for efficient 5' cleavage at a processing site conserved in RNAs of three different mitochondrial genes in *Arabidopsis thaliana*. Plant J. **74**:593–604.

Holzle, A., Jonietz, C., Torjek, O., Altmann, T., Binder, S., and Forner, J. (2011). A RESTORER OF FERTILITY-like PPR gene is required for 5'-end processing of the *nad4* mRNA in mitochondria of *Arabidopsis thaliana*. Plant J. **65**:737–744.

Jonietz, C., Forner, J., Holzle, A., Thuss, S., and Binder, S. (2010). RNA PROCESSING FACTOR2 is required for 5' end processing of *nad9* and *cox3* mRNAs in mitochondria of *Arabidopsis thaliana*. Plant Cell **22**:443–453.

Karpova, O.V., Kuzmin, E.V., Elthon, T.E., and Newton, K.J. (2002). Differential expression of alternative oxidase genes in maize mitochondrial mutants. Plant Cell **14**:3271–3284.

Li, X.J., Zhang, Y.F., Hou, M., Sun, F., Shen, Y., Xiu, Z.H., Wang, X., Chen, Z.L., Sun, S.S., Small, I., et al. (2014). *Small kernel 1* encodes a pentatricopeptide repeat protein required for mitochondrial *nad7* transcript editing and seed development in maize (*Zea mays*) and rice (*Oryza sativa*). Plant J. **79**:797–809.

Liu, Y.J., Xiu, Z.H., Meeley, R., and Tan, B.C. (2013). *Empty pericarp5* encodes a pentatricopeptide repeat protein that is required for mitochondrial RNA editing and seed development in maize. Plant Cell **25**:868–883.

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., Caboche, M., Debast, C., Gualberto, J., Hoffmann, B., et al. (2004). Genome-wide analysis of *Arabidopsis* pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16**:2089–2103.

Luro, S., Germain, A., Sharwood, R.E., and Stern, D.B. (2013). RNase J participates in a pentatricopeptide repeat protein-mediated 5' end maturation of chloroplast mRNAs. Nucleic Acids Res. **41**:9141–9151.

Maier, R.M., Neckermann, K., Igloi, G.L., and Kossel, H. (1995). Complete sequence of the maize chloroplast genome - gene content, hotspots of divergence and fine-tuning of genetic information by transcript editing. J. Mol. Biol. **251**:614–628.

Manavski, N., Guyon, V., Meurer, J., Wienand, U., and Brettschneider, R. (2012). An essential pentatricopeptide repeat protein facilitates 5' maturation and translation initiation of *rps3* mRNA in maize mitochondria. Plant Cell **24**:3087–3105.

McCarty, D.R., Settles, A.M., Suzuki, M., Tan, B.C., Latshaw, S., Porch, T., Robin, K., Baier, J., Avigne, W., Lai, J.S., et al. (2005). Steady-state transposon mutagenesis in inbred maize. Plant J. **44**:52–61.

O'Toole, N., Hattori, M., Andres, C., Iida, K., Lurin, C., Schmitz-Linneweber, C., Sugita, M., and Small, I. (2008). On the expansion of the pentatricopeptide repeat gene family in plants. Mol. Biol. Evol. **25**:1120–1128.

Pfalz, J., Bayraktar, O.A., Prikryl, J., and Barkan, A. (2009). Site-specific binding of a PPR protein defines and stabilizes 5' and 3' mRNA termini in chloroplasts. EMBO J. **28**:2042–2052.

Prikryl, J., Rojas, M., Schuster, G., and Barkan, A. (2011). Mechanism of RNA stabilization and translational activation by a pentatricopeptide repeat protein. Proc. Natl. Acad. Sci. USA **108**:415–420.

Ruwe, H., Wang, G., Gusewski, S., and Schmitz-Linneweber, C. (2016). Systematic analysis of plant mitochondrial and chloroplast small RNAs suggests organelle-specific mRNA stabilization mechanisms. Nucleic Acids Res. **44**:7406–7417.

Sosso, D., Mbelo, S., Vernoud, V., Gendrot, G., Dedieu, A., Chambrier, P., Dauzat, M., Heurtevin, L., Guyon, V., Takenaka, M., et al. (2012). PPR2263, a DYW-subgroup pentatricopeptide repeat protein, is required for mitochondrial *nad5* and *cob* transcript editing, mitochondrion biogenesis, and maize growth. Plant Cell **24**:676–691.

Stern, D.B., Goldschmidt-Clermont, M., and Hanson, M.R. (2010). Chloroplast RNA metabolism. Annu. Rev. Plant Biol. **61**:125–155.

Stoll, B., Zendler, D., and Binder, S. (2014). RNA processing factor 7 and polynucleotide phosphorylase are necessary for processing and stability of *nad2* mRNA in *Arabidopsis* mitochondria. RNA Biol. **11**:968–976.

Sun, F., Wang, X., Bonnard, G., Shen, Y., Xiu, Z., Li, X., Gao, D., Zhang, Z., and Tan, B.C. (2015). *Empty pericarp7* encodes a mitochondrial E-subgroup pentatricopeptide repeat protein that is required for ccmFN editing, mitochondrial function and seed development in maize. Plant J. **84**:283–295.

Tan, B.C., Chen, Z., Shen, Y., Zhang, Y., Lai, J., and Sun, S.S. (2011). Identification of an active new mutator transposable element in maize. G3 (Bethesda) **1**:293–302.

Wang, C., Aube, F., Planchard, N., Quadrado, M., Dargel-Graffin, C., Nogue, F., and Mireau, H. (2017). The pentatricopeptide repeat protein MTSF2 stabilizes a nad1 precursor transcript and defines the 3 end of its 5-half intron. Nucleic Acids Res. **45**:6119–6134.

Wang, Z., Zou, Y., Li, X., Zhang, Q., Chen, L., Wu, H., Su, D., Chen, Y., Guo, J., Luo, D., et al. (2006). Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell **18**:676–687.

Wu, W., Liu, S., Ruwe, H., Zhang, D., Melonek, J., Zhu, Y., Hu, X., Gusewski, S., Yin, P., Small, I.D., et al. (2016). SOT1, a pentatricopeptide repeat protein with a small MutS-related domain, is required for correct processing of plastid 23S-4.5S rRNA precursors in *Arabidopsis thaliana*. Plant J. **85**:607–621.

Xiu, Z.H., Sun, F., Shen, Y., Zhang, X.Y., Jiang, R.C., Bonnard, G., Zhang, J.H., and Tan, B.C. (2016). EMPTY PERICARP16 is required for mitochondrial nad2 intron 4 cis-splicing, complex I assembly and seed development in maize. Plant J. **85**:507–519.

Yang, Y.Z., Ding, S., Wang, H.C., Sun, F., Huang, W.L., Song, S., Xu, C., and Tan, B.C. (2017). The pentatricopeptide repeat protein EMP9 is required for mitochondrial ccmB and rps4 transcript editing, mitochondrial complex biogenesis and seed development in maize. New Phytol. **214**:782–795.

Yin, P., Li, Q., Yan, C., Liu, Y., Liu, J., Yu, F., Wang, Z., Long, J., He, J., Wang, H.W., et al. (2013). Structural basis for the modular recognition of single-stranded RNA by PPR proteins. Nature **504**:168–171.

Zhang, Y.F., Hou, M.M., and Tan, B.C. (2013). The requirement of WHIRLY1 for embryogenesis is dependent on genetic background in maize. PLoS One **8**:e67369.

Zhelyazkova, P., Hammani, K., Rojas, M., Voelker, R., Vargas-Suarez, M., Borner, T., and Barkan, A. (2012). Protein-mediated protection as the predominant mechanism for defining processed mRNA termini in land plant chloroplasts. Nucleic Acids Res. **40**:3092–3105.

Zoschke, R., Watkins, K.P., Miranda, R.G., and Barkan, A. (2016). The PPR-SMR protein PPR53 enhances the stability and translation of specific chloroplast RNAs in maize. Plant J. **85**:594–606.