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AtPPR2, an Arabidopsis pentatricopeptide repeat protein, binds to plastid 23S rRNA and plays an important role in the first mitotic division during gametogenesis and in cell proliferation during embryogenesis

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SUMMARY

Pentatricopeptide repeat (PPR) proteins are mainly involved in regulating post-transcriptional processes in mitochondria and plastids, including chloroplasts. Mutations in the Arabidopsis *PPR2* gene have previously been found to cause defects in seed development and reduced transmission through male and female gametophytes. However, the exact function of AtPPR2 has not been defined. We found that a loss-of-function mutation of *AtPPR2* leads to arrest of the first mitotic division during both male and female gametogenesis. In addition, the *Atppr2* mutation causes delayed embryogenesis, leading to embryonic lethality. Mutation in *emb2750*, which appears to be a weak mutant allele of the *AtPPR2* locus, also results in defective seeds. However, a majority of *emb2750* seeds were able to germinate, but their cotyledons were albino and often deformed, and growth of the *emb2750* seedlings were arrested after germination. *AtPPR2* is mainly expressed in plant parts that undergo cell division, and AtPPR2 protein was localized to chloroplasts. RNA immunoprecipitation and protein gel mobility shift assays showed that AtPPR2 binds to plastid 23S rRNA. Our study adds to a growing body of evidence that plastids and/or chloroplasts play a key role in cell division. AtPPR2 may modulate the translational process to fine-tune plastid function, thereby regulating cell division.

Keywords

AtPPR2; pentatricopeptide repeat proteins; gametogenesis; embryogenesis; cell division; plastid

INTRODUCTION

Pentatricopeptide repeat (PPR) proteins are characterized by tandem repeats of a degenerate 35 amino acid motif (Small and Peeters, 2000). The motif is predicted to fold into a helix-turn-helix structure, and is present in the ‘ α -solenoid’ superfamily, which also includes ankyrin repeat proteins and Puf domain RNA-binding proteins (Schmitz-Linneweber and Small, 2008). Although other eukaryotic organisms generally have relatively few PPR proteins, the PPR family has been massively expanded in plants. The Arabidopsis genome encodes at least 450 PPR proteins (Lurin *et al.*, 2004; Rivals *et al.*, 2006; O’Toole *et al.*,

2008), making it one of the largest protein families. Most plant PPR proteins characterized so far reside in either the mitochondria or plastids. They are thought to be sequence-specific RNA-binding proteins that are generally involved in various RNA post-transcriptional processes, including RNA editing, splicing, stabilization and translation (Saha *et al.*, 2007; O'Toole *et al.*, 2008). A few PPR proteins are involved in transcriptional regulation (Ikeda and Gray, 1999; Ding *et al.*, 2006; Pfalz *et al.*, 2006).

Gametogenesis and embryogenesis involve important cellular processes, including cell division, differentiation and morphogenesis, that are coordinated by various signals, such as hormones, developmental cues and environmental signals (Yang and Sundaresan, 2000; Berleth and Chatfield, 2002; Yadegari and Drews, 2004; Ma, 2005). Prior to gametogenesis, the diploid megasporocyte and microsporocyte undergo meiosis to give rise to haploid megaspores and microspores, respectively. During gametogenesis, microspores and megaspores undergo multiple cycles of mitotic division to produce male gametophytes (pollen) and female gametophytes (embryo sacs). Embryogenesis involves a regular sequence of cell division, differentiation and morphogenesis. Identification of genes involved in gametogenesis and embryogenesis continues to serve as an essential route towards understanding of fundamental cellular processes. A large number of nuclear genes encoding plastid or chloroplast proteins, including many PPR proteins, have been found to be essential for embryogenesis (Lurin *et al.*, 2004; Tzafrir *et al.*, 2004; Cushing *et al.*, 2005; Ajjawi *et al.*, 2010; Myouga *et al.*, 2010; Bryant *et al.*, 2011). Of at least 400 *EMB* genes in *Arabidopsis* that function in embryo development, approximately 30% encode chloroplast-localized proteins (Bryant *et al.*, 2011).

In this study, we performed functional characterization of an *Arabidopsis* PPR gene (*AtPPR2*; *Arabidopsis* Genome Initiative gene identification number *At3g06430*). Phenotypes associated with mutations at this locus have previously been reported by several groups in genome-wide mutational analyses. The Meinke group identified a T-DNA insertion line at this locus (*emb2750*) from a collection of at least 250 *emb* mutants defective in seed development (Tzafrir *et al.*, 2004). In addition, a large-scale phenotypic analysis of insertional mutations for nucleus-encoded, chloroplast-localized proteins identified three insertional lines at this locus that did not produce homozygous progeny (Myouga *et al.*, 2010). Another mutant allele at this locus was identified as one of 67 Ds element insertion lines that showed reduced transmission through male and female gametophytes (Boavida *et al.*, 2009). These reports indicate that *AtPPR2* may play an important role in embryogenesis and gametogenesis. *AtPPR2* shares a high sequence similarity to the maize PPR protein *ZmPPR2*. The null mutation of the maize *PPR2* gene leads to albino seedlings, but does not affect seed development or gametogenesis (Williams and Barkan, 2003), suggesting that the biological functions of *ZmPPR2* and *AtPPR2* may have diversified. However, the exact role of *AtPPR2* remains unknown.

We found that a loss-of-function mutation of *AtPPR2* leads to arrest of the first mitotic cell division during both microgametogenesis and megagametogenesis. The mutation also causes slow progression of embryogenesis and eventually leads to embryonic lethality. *AtPPR2* is localized in chloroplasts and binds to the plastid 23S ribosomal RNA. Our findings indicate that *AtPPR2* may control the translational process through binding to 23S rRNA to fine-tune plastid function for gametogenesis and embryogenesis.

RESULTS

Loss-of-function mutation of *Atppr2* leads to embryonic lethality

The T-DNA-mutagenized line SALK_108269 from the Salk collection (Alonso *et al.*, 2003) was obtained from the *Arabidopsis* Biological Resource Center (<http://abrc.osu.edu/>). This

line was found to contain two T-DNA insertions: one in the *At2g23140* gene that encodes an E3 ubiquitin ligase (data not shown) and the other in the *At3g06430* gene that encodes a PPR protein (this study). PCR analysis was used to identify progeny plants in which the two insertions segregated away from each other. The progeny that carried a T-DNA insertion in *At3g06430* only are described here. Sequence analysis of the T-DNA flanking fragments by PCR revealed that this allele contains a T-DNA insertion in the first exon (Figure 1a).

At3g06430 consists of two exons, with an open reading frame that encodes a polypeptide with 486 amino acids. The protein is predicted by the pSORT (Nakai and Kanehisa, 1991) and ChloroP (Emanuelsson *et al.*, 1999) algorithms to contain a transit peptide for targeting the protein to chloroplasts. Searching the Prosite database (<http://www.expasy.ch/prosite/>) revealed that the protein includes 10 PPR repeats between amino acids 123 and 470. Of all Arabidopsis PPR proteins, this protein has the highest sequence similarity to maize PPR2 (Williams and Barkan, 2003). This T-DNA insertion allele is referred to here as *Atppr2-1* (abbreviated to *Atppr2*).

The *Atppr2* line did not display kanamycin resistance, probably because of silencing of the *nrp1* gene in the T-DNA region. PCR analysis was used to estimate the segregation ratio by detecting the presence of the insertion allele. Among 377 progeny from self-pollinated *Atppr2/+* plants, 205 individuals carried the insertion allele, and the remaining 172 individuals were homozygous for the wild-type (WT) allele. All progeny that carried the insertion allele were found to be heterozygous at this locus. Failure to identify any progeny homozygous for the *Atppr2* mutant allele raised the possibility that the mutation may cause embryonic lethality. The heterozygous *Atppr2/+* plants do indeed display partial sterility, and produce shorter siliques than WT plants when self-pollinated (Figure 1b,c). Microscopic observation revealed abnormal phenotypes of some developing seeds in the siliques of the heterozygous plants. These seeds appeared whitish approximately 5–8 days post-pollination (Figure 1d), later turned brown and shrunken (Figure 1e), and were not viable (see below).

The *Atppr2* mutation impairs development of both male and female gametophytes

If the *Atppr2* mutation only caused embryonic lethality, the progeny plants of self-pollinated *Atppr2/+* heterozygous plants would be expected to segregate in a 2:1 ratio (heterozygous:WT). The aberrant segregation ratio (205: 172 = 1.2:1) from the PCR analysis suggests that the *Atppr2* mutation also causes reduced transmission through male and/or female gametophytes. Microscopic observation of young siliques of the heterozygous plants revealed collapsed residues that were probably aborted ovules (Figure 1d,f). As shown in Table S1, approximately 27% (604/2225) of the ovules from young siliques of the self-pollinated *Atppr2/+* plants were aborted or unfertilized, compared to 3.6% in WT plants. Assuming that 50% of the ovules produced from the heterozygous plants carried the mutant allele, the result indicates that approximately half of the *Atppr2* ovules $[(604 - 40)/1113 = 50.7\%]$ from these plants were not functional. PCR analysis of the progeny from the reciprocal crosses showed that the transmission rate of the insertion allele through female gametophytes was approximately 53.9% (Table S2).

To determine whether the *Atppr2* mutation also affects pollen development, pollen viability staining (Alexander, 1969) was performed. Almost all pollen grains (442/445) from WT plants stained positive (Figure 2a); however, approximately a quarter of the pollen grains (132/522 = 25.3%) from the *Atppr2/+* plants were not viable (Figure 2a). The mutant pollen grains contained little cell content and were shrunken at the mature pollen stage (Figure 2b). Assuming that 50% of pollen grains produced in the heterozygous plants carry the insertion alleles, the result indicates that approximately half of the *Atppr2* pollen grains were viable whereas the other half were aborted. The result from reciprocal crosses showed that the transmission rate of the mutant allele through male gametophytes was approximately 55.8%.

To further determine the fate of *Atppr2* pollen, we crossed *Atppr2/+* plants with the *quartet 1 (qrt1)* mutant and obtained *Atppr2/+ qrt1/qrt1* plants from the F₂ progeny. The *qrt1* mutation causes the four haploid products of male meiosis (tetrad) from the same pollen mother cell to remain attached, even when the pollen is mature (Preuss *et al.*, 1994). Each tetrad from the *Atppr2/+ qrt1/qrt1* plants should contain two pollen grains with the *AtPPR2* allele and two with the *Atppr2* allele. Pollen viability staining of 280 tetrads from the *Atppr2/+ qrt1* plants (Figure 2c) revealed that 142 tetrads had two viable pollen grains and two aborted pollen grains, and 130 tetrads had four viable pollen grains. Only eight ‘tetrads’ displayed three viable pollen grains (Figure 2c, inset). Of 117 tetrads examined from *AtPPR2/AtPPR2 qrt1* plants, 114 tetrads had four viable pollen grains and three ‘tetrads’ had three viable pollen grains. It is likely that one pollen grain may have become detached during the staining process or was blocked from view under the microscope in the ‘tetrads’ with three visible pollen grains. The result also indicated that approximately half of the *Atppr2* pollen grains (142/280) were not viable. We did not find any tetrad in which one *Atppr2* pollen grain was viable and the other was defective, suggesting that two *Atppr2* pollen grains from the same tetrad generally shared the same fate: they either were both aborted or both developed into viable pollen.

Genetic complementation confirmed that *AtPPR2* functions in both gametogenesis and embryogenesis

Genetic complementation was performed to determine whether the embryonic lethality phenotype and the defect in male and female gametophyte development are indeed caused by the *Atppr2* mutation. A 3.2 kb genomic fragment of the *AtPPR2* gene was cloned from WT plants (Col-0), including approximately 1 kb upstream of the start codon. The construct containing the *AtPPR2* genomic clone (*PPR2Comp*) was transformed into *Atppr2/+* heterozygous plants. In the T₂ progeny of at least eight independent transformants (of 26 independent transgenic lines), plants homozygous for the *Atppr2* allele were identified and shown to carry the *PPR2Comp* transgene. These plants (*Atppr2Comp*) were indistinguishable from WT plants, and showed full fertility and normal ovule and pollen development (Figures 1c and 2a,b).

The *Atppr2* mutation blocks the first mitotic division during male and female gametogenesis

To reveal at what stage the *Atppr2* mutant pollen fails to proceed, we stained nuclei in developing pollen with DAPI. The *Atppr2/+* heterozygous plants in the *qrt1* background served as ideal material for the observation, as the two WT microgametophytes from the same tetrad can be used as a reference to reveal any defect in the two *Atppr2* microgametophytes. During normal microgametogenesis, a haploid microspore undergoes asymmetric cell division to produce a large vegetative cell that is diffusely stained and a small intensely stained generative cell. This is termed the bicellular stage. In many plant species such as *Arabidopsis*, the generative cell undergoes another cell division to generate two sperm cells (the tricellular stage) before pollination occurs.

We did not find any discernible difference between WT and *Atppr2* microspores at the unicellular stage (Figure 3a). At the bicellular stage, no difference was found between all four pollen grains in some tetrads (data not shown); however, in other tetrads, two mutant pollen grains remained at the unicellular stage (Figure 3b). Similarly, at the tricellular stage, all four pollen grains in some tetrads were normal, but in the other tetrads, the two *Atppr2* pollen grains degenerated (Figure 3c). We did not find any tetrads in which the mutant pollen grains were arrested at the bicellular stage, indicating that once the mutant pollen grains reached the bicellular stage, they were able to proceed to the tricellular stage to

become mature pollen. Abnormal pollen grains in the *Atppr2/+* heterozygous plants with the WT *QRT1* background were also found to be arrested at the unicellular stage.

To determine the stage at which female gametophyte development becomes abnormal due to the *Atppr2* mutation, cleared whole-mount ovules from *Atppr2/+* plants at various developmental stages were observed using differential interference contrast optics. During normal megagametogenesis, the nucleus of the functional megaspore undergoes three rounds of mitosis without cytokinesis to form two-, four- and eight-nucleate embryo sacs (Figure 3d–g). Subsequent cellularization gives rise to a seven-celled embryo sac. In *Atppr2/+* heterozygous plants, a majority of ovules from the same gynoecium exhibited the structure of normal megagametophytes at various developmental stages. All abnormal ovules were found to contain an ‘embryo sac’ that did not undergo nuclear division, and were arrested at the one-nucleate stage (Figure 3h–j). No mutant megagametophytes that were arrested at the two-nucleate or four-nucleate stages were found. The results indicate that the *Atppr2* mutation also blocks the first nuclear division during development of female gametophytes.

The *Atppr2* mutation causes delayed embryogenesis and abnormal embryo morphogenesis

Due to partial transmission of the mutant allele through both male and female gametophytes, approximately 10–15% of developing seeds from a silique of self-fertilized *Atppr2/+* plants were homozygous for the *Atppr2* mutation. To reveal the defect in *Atppr2* embryos, developing seeds at various developmental stages from self-pollinated heterozygous plants were prepared by the whole-mount method and observed using differential interference contrast optics. Normal embryo development progresses from the zygote through the globular, heart, torpedo and cotyledon stages, and finally to the curled-cotyledon stage and maturity (Figure 4).

No obvious difference before or at the globular stage was found between mutant and WT embryos. From the late globular stage to the early heart stage, developmental deviation of mutant embryos became apparent. Development of mutant embryos was not uniformly arrested at a particular stage, but became significantly slower than that of normal embryos (Figure 4). When normal embryos were at the heart stage (Figure 4c), 18% (27/148) of abnormal embryos were at the globular stage (Figure 4d). When normal embryos were at the torpedo stage (Figure 4e), 20% of abnormal embryos were at the globular to heart stage and often had abnormal morphology (Figure 4f–h). Of these abnormal embryos, 11% (19/179) were at the globular stage (Figure 4f) and 9% (17/179) were at the heart stage (Figure 4g,h). Similarly, when normal embryos had progressed to the cotyledon and curled-cotyledon stages (Figure 4i,m), the mutant embryos lagged behind (Figure 4j–l,n–p). Some mutant embryos developed beyond the torpedo stage, but they displayed abnormal morphology (Figure 4p).

The *emb2750* mutation affects embryogenesis but not gametogenesis

The *emb2750* mutant was identified as one of at least 250 mutants that showed defects in embryogenesis (Tzafrir *et al.*, 2004). Detailed characterization of this mutant has not been reported. The *emb2750* allele was obtained from the Arabidopsis Biological Resource Center (stock number CS16224). This allele contains a T-DNA insertion near the middle of the second exon in *AtPPR2* (Figure 1a). However, apart from the defect in seed development (Figure 5a,b), we did not find any defect in male or female gametophyte development in the *emb2750/+* heterozygous plants (Figure 5a,c), as no aborted ovules or pollen were observed from the heterozygous plants. A quarter ($447/1892 = 23.6\%$) of the seeds in the siliques from *emb2750/+* plants were abnormal. In addition, the progeny from self-pollinated *emb2750/+* plants grown in soil segregated at the ratio 2:1 (309 heterozygous, 152 WT).

Together, these results indicate that the *emb2750* mutation causes a defect in embryogenesis but does not affect male or female gametophyte development, indicating that *emb2750* is a weak mutant allele and that its partial function is sufficient to support normal gametogenesis. The defective embryogenesis phenotype of the *emb2750* was genetically complemented by the *AtPPR2* complementation transgene (data not shown).

The *emb2750* embryos displayed similar defects to those of the *Atppr2* embryos, including retardation in development (Figure 5). The *emb2750* embryos started to show a defect when normal embryos from the same silique were at the heart stages (Figure 5f,g). However, a majority (approximately 70%) of *emb2750* embryos progressed to the cotyledon or later stages but with abnormal morphology (Figure 5r,s). In addition, mature *emb2750* seeds were not as shrunken as *Atppr2* seeds (Figure 6a). This prompted us to examine whether any mutant seeds from the *emb2750/+* plants or the *Atppr2/+* plants germinate. We germinated normal and mutant seeds on agar plates that contained half-strength MS salts and 3% sucrose. None of the mutant seeds from the *Atppr2/+* plants showed any signs of germination. However, approximately 70% of the *emb2750* mutant seeds germinated, but they produced albino cotyledons and did not undergo further development after germination (Figure 6b,c). In addition, the cotyledons of the *emb2750* seedlings were often deformed, and some of the mutant seedlings contained only one cotyledon (Figure 6c). PCR analysis using genomic DNA prepared from the albino seedlings confirmed that they were homozygous *emb2750* seedlings. Quantitative real-time RT-PCR analysis was used to detect *AtPPR2* transcripts from the albino *emb2750* seedlings. When the primer pair located upstream of the insertion site (P1 and P2 in Figure 1a) was used in the assay, the transcript level was approximately 60% of that in the WT plants (Figure 6d). However, little transcript was detected when primers downstream of the insertion site were used. The result suggests that the *emb2750* allele produces a truncated transcript that is partially functional.

Unlike the chloroplasts in the WT seedlings, there was no typical chloroplast structure or thylakoid membrane system in chloroplasts of the albino *emb2750* cotyledons (Figure S1). Instead, these plastids were vacuolated.

Expression patterns of *AtPPR2*

Quantitative real-time RT-PCR analysis detected *AtPPR2* transcripts in all organs examined (Figure 7a). The highest transcript levels were detected in developing siliques in which embryos were at globular to later stages. To further examine the expression patterns of *AtPPR2*, a GUS (β -glucuronidase) reporter construct was produced in which the GUS reporter gene was under the control of the 1.2 kb promoter region of *AtPPR2*. Nineteen of 21 independent transgenic lines exhibited similar GUS expression patterns. Consistent with the quantitative real-time RT-PCR results, the reporter gene was expressed in all major organs (Figure 7b–k). The highest GUS activity was detected in the parts that undergo cell division, such as newly emerging leaves (Figure 7b), the root apical meristem (Figure 7c), embryo sacs (Figure 7j), and developing seeds and embryos (Figure 7k,l). In expanded leaves, epidermal and mesophyll cells displayed very weak GUS activity, but vascular systems showed stronger GUS activity (Figure 7d). Strong GUS activity was also detected in anthers and pollen (Figure 7f–i). Consistent with the role of *AtPPR2* in early male gametogenesis, GUS activity was detected at the tetrad stage (Figure 7h).

AtPPR2 protein is localized in chloroplasts

To experimentally determine the subcellular localization of *AtPPR2*, it was fused at its C-terminus to eYFP, and fusion constructs, in which expression of the eYFP fusion is under the control of either the CaMV 35S promoter or the *AtPPR2* promoter, were introduced into Arabidopsis to generate stable transgenic lines. However, we were unable to detect any

eYFP signal. Neither did we detect the fusion protein in Western blot analysis using anti-YFP antibodies. These results suggest that the fusion protein may not be stable in the transgenic lines. As the AtPPR2 protein is predicted to have a chloroplast target signal peptide by the pSORT (Nakai and Kanehisa, 1991) and ChloroP (Emanuelsson *et al.*, 1999) algorithms, we fused the first 30 amino acids of AtPPR2 to eYFP and transiently expressed the fusion protein under the control of the CaMV 35S promoter in tobacco (*Nicotiana benthamiana*) leaves through *Agrobacterium*-mediated transformation. The fusion protein (AtPPR2N-eYFP) was found to be localized in the chloroplasts (Figure 8a). Similarly, we transiently expressed the fusion protein in protoplasts derived from Arabidopsis leaves, and found that the eYFP signal was localized in chloroplasts (Figure 8b).

AtPPR2 binds to 23S rRNA

To determine whether AtPPR2 is an RNA-binding protein and to which RNA it binds, we performed an RNA immunoprecipitation experiment. We generated transgenic Arabidopsis lines that express AtPPR2 fused to the FAST tag, an epitope tag consisting of the FLAG tag and the StrepII tag in tandem (Ge *et al.*, 2005). Expression of the fusion protein was under the control of the CaMV 35S promoter. The fusion construct was capable of complementing the *Atppr2* phenotype (data not shown). The AtPPR2-FAST protein was immunoprecipitated from crude protein extracts of the transgenic plants using an anti-FLAG M2 affinity gel. Transgenic plants expressing 35S::FAST were used as a negative control. We then extracted RNA associated with the pulled-down protein, and amplified the RNA molecules using RT-PCR. The sample derived from the PPR2-FAST precipitate showed a band with a size of approximately 350 bp and smearing around the band, but no visible DNA was amplified from the FAST precipitate (Figure 9a). The lane from the PPR2-FAST sample was divided into five zones (Figure 9a). DNA was extracted from the gel slices, cloned into a plasmid vector and transformed into *Escherichia coli*. A total of 12 transformants were found to contain an insert in the plasmid. Sequencing results revealed that these inserts (with sizes of 305, 311 and 381 bp) were derived from three different regions of the plastid genes *Atcg00950* or *Atcg01180*, both of which encode plastid 23S rRNA.

Gel mobility shift assays confirmed that AtPPR2 binds to 23S rRNA (Figure 9b). In the assay, four rRNA species encoded by the plastid genome were incubated separately with purified AtPPR2-FAST protein, followed by UV irradiation. Samples were then separated by native polyacrylamide gel electrophoresis and detected by Western blotting using the anti-FLAG M2 antibody. As shown in Figure 9(b), only 23S rRNA, but not the other three rRNAs (16S, 5S and 4.5S), caused a mobility shift of AtPPR2.

Quantitative real-time RT-PCR was used to compare the rRNA transcript levels between the *emb2750* albino seedlings and WT seedlings. As shown in Figure 10(a), the levels of 23S rRNA in 7-day-old seedlings were similar between the mutant and WT, but the levels of the other three chloroplast rRNAs were approximately 20–50% lower in the mutant seedlings. Northern blotting analysis showed that the 23S rRNA transcript patterns between the mutant and WT were similar (Figure 10b). The transcript levels of the Rubisco large subunit (*RbcL*) gene and the *PsbA* gene, both of which are encoded in the plastid genome, were reduced by approximately one-third in the mutant seedlings. However, the level of RbcL protein in the 7-day-old mutant seedlings was <10% of that in the WT seedlings (Figure 10c,d). Similarly, the level of PsbA protein was 80% lower in the mutant (Figure 10d). These results suggest a general reduction in translation efficiency in plastids.

DISCUSSION

A large majority of the PPR proteins that have been characterized to date were found to function in post-transcriptional processes in mitochondria and plastids/chloroplasts (Saha *et al.*, 2007; Schmitz-Linneweber and Small, 2008). Mutations in many PPR genes cause lethality or give rise to discernible phenotypes (Lurin *et al.*, 2004; Tzafrir *et al.*, 2004; Cushing *et al.*, 2005; Kocabek *et al.*, 2006). We found that a loss-of-function mutation of *AtPPR2* reduces the transmission rates through both male and female gametophytes by approximately 50%, by causing arrest of the first mitotic division in gametogenesis. The mutation also impairs embryo development, leading to embryonic lethality. *AtPPR2* apparently does not function in a specific step in embryogenesis, but is instead involved in a more general cellular process, presumably in cell proliferation. The *AtPPR2* gene is mainly expressed in regions that undergo cell proliferation. In epidermal and mesophyll cells of expanded leaves, expression of *AtPPR2* was barely detectable, suggesting that *AtPPR2* may not play a significant role in plastid metabolism in these cells.

AtPPR2 and *ZmPPR2* of maize share over 60% sequence identity at the amino acid level, and are considered to be orthologous (Williams and Barkan, 2003). However, other than the albino seedling phenotype, the *Zmppr2* null mutation does not lead to any defect in embryogenesis or gametogenesis (Williams and Barkan, 2003). In addition, the *Zmppr2* seedlings were morphologically normal, and grew for 2 weeks in soil at a near normal rate, although the mutant seedlings lack plastid rRNA. In contrast, *AtPPR2* is essential for embryogenesis. The *emb2750* mutation, which appears to be a weak allele at the *AtPPR2* locus, also leads to defective embryos. *emb2750* seeds germinated but seedling development was arrested after germination. If *ZmPPR2* and *AtPPR2* are orthologous, *AtPPR2* may have gained an additional function. However, it cannot be ruled out that the function of *ZmPPR2* may not have been fully revealed by its null mutation, as it may share partial functional redundancy with another maize PPR gene. Maize mutations at the *ZmPPR4* and *ZmPPR5* loci cause similar phenotypes to that of *Zmppr2* (Schmitz-Linneweber *et al.*, 2006; Beick *et al.*, 2008). An alternative explanation is that plastid/chloroplast translation may not be essential for embryogenesis in some plant species (Zubko and Day, 1998; Asakura and Barkan, 2006; Bryant *et al.*, 2011).

Genes encoded in the genome of the present-day plastid are involved in chloroplast biogenesis and photosynthesis. As Arabidopsis embryos can develop well beyond germination without photosynthesis, a plastid function separate from photosynthesis is believed to account for the embryonic-lethal phenotype caused by mutations in plastid PPR proteins (Schmitz-Linneweber and Small, 2008; Bryant *et al.*, 2011). *AtPPR2* probably functions in translation by acting as a plastid 23S rRNA-binding protein. Lack *AtPPR2* probably leads to general dysfunction of plastid functions. Although photosynthesis may not be essential for embryogenesis or gametogenesis, other primary metabolites produced in plastids, such as amino acids, fatty acids, vitamins and phytohormones, may be required for these developmental processes. Therefore, disruption of general functions of plastids may contribute to the defects in gametogenesis and embryogenesis caused by the *Atppr2* mutation.

Sporogenesis produces haploid microspores and megaspores from diploid micro- and megasporocytes, respectively, through meiosis. A single *Atppr2*^{+/+} micro- or megasporocyte produces two spores (daughter cells) with the mutant allele and two with the wild-type allele. The cytoplasm (including plastids) is partitioned into the four daughter cells, each of which is expected to contain functional plastids from the mother cell. During subsequent gametogenesis, micro- and megaspores undergo multiple rounds of mitotic division to form micro- and megagametophytes. In the *Atppr2* daughter cells, the plastid function is expected

to gradually deteriorate because of the failure to produce new AtPPR2 protein. Our results showed that approximately 50% of *Atppr2* microspores or megaspores undergo the first mitotic division to start gametogenesis, presumably because of the presence of a threshold amount of functional AtPPR2 in plastids inherited from the mother cells. If the *Atppr2* mutation simply causes a nutritional defect due to plastidial metabolic malfunction, the *Atppr2* micro- and megagametophytes would be arrested during the subsequent developmental processes due to further deterioration of plastid-based metabolism. However, it appears that, once the *Atppr2* micro- and megaspores pass through the first mitotic division, they are capable of completing the normal gametogenesis process and develop into functional gametophytes. Thus, the gametogenesis phenotypes caused by the *Atppr2* mutation cannot be fully explained by insufficient plastid-produced metabolites. Alternatively, it is possible that another related gene with AtPPR2-like function may be activated after the first nuclear division, allowing the mutant gametophytes that passed through the first division to continue the developmental process.

It was found that the two mutant pollen grains in each tetrad from an *AtPPR2*/- *qrt1/qrt1* plant were either both defective or both normal. One possible explanation is that pollen mother cells (meiocytes) in an anther may have variable amounts of plastids. The two mutant microspores from a mother cell with a higher amount of AtPPR2-containing functional plastids may be capable of undergoing normal gametogenesis. Similarly, asymmetric distribution of plastids in a megasporocyte may also result in megaspores with variable amount of inherited functional plastids. As a result, *Atppr2* megaspores with inherited plastids above a threshold level may undergo normal megagametogenesis, whereas the others are arrested at the one-nucleate stage.

We could not rule out the possibility that AtPPR2 may prevent production of a detrimental product encoded by an unidentified plastid DNA sequence. Many male-sterility restorer genes encode PPR proteins that prevent production of corresponding cytoplasmic male sterility factors encoded by the plastid or mitochondrial genomes (Bentolila *et al.*, 2002; Braundmeier *et al.*, 2002; Brown *et al.*, 2003; Desloire *et al.*, 2003; Koizuka *et al.*, 2003; Komori *et al.*, 2004; Wang *et al.*, 2006; Gillman *et al.*, 2007; Kazama *et al.*, 2008; Uyttewaal *et al.*, 2008). However, the fact that AtPPR2 binds to 23S rRNA makes it more likely that AtPPR2 is involved in the general translation process in plastids/chloroplasts. The AtPPR2-modulated translation process may fine-tune the plastid function (including metabolic pathways) to meet the needs of dividing cells. The first mitotic division of gametogenesis could be a more critical checkpoint to determine whether gametogenesis should proceed or not. The Arabidopsis GUN1 (GENOME UNCOUPLED1) protein is also a chloroplast-localized PPR protein that functions as a retrograde chloroplast-derived signal to modulate expression of nuclear genes involved in photosynthesis (Jarvis, 2007; Koussevitzy *et al.*, 2007). The disruption of normal plastid functioning caused by the *Atppr2* mutation may generate a retrograde signal which blocks cell division. Further elucidation of the molecular event mediated by AtPPR2 could provide novel insights into the role of plastid function in controlling cell division.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *Atppr2-1* T-DNA insertion line (SALK_108269) and *emb2750* (stock number CS16224) were obtained from the Arabidopsis Biological Resource Center (<http://abrc.osu.edu/>). Arabidopsis plants were grown in a growth room under a 15 h light/9 h dark cycle at a light intensity of 125 mol m⁻² sec⁻¹ provided by cool-white fluorescent bulbs, at 50% humidity and 21°C.

The procedures for genotype determination of the insertion lines, *Arabidopsis* transformation and genetic complementation are given in Appendix S1. Primer sequences and their uses are given in Table S3.

Light microscopy and electron microscopy

For pollen viability staining, pollen grains were collected, placed in droplets of Alexander staining solution (Alexander, 1969), and stained for 30 min before observation with a dissecting microscope. For DAPI (4',6-diamidino-2-phenylindole) staining, the pollen nuclei were stained with DAPI as previously described (Park *et al.*, 1998), and observed using a 340–380 nm excitation filter and a 450–480 nm emission filter and a Nikon Eclipse 800 widefield microscope (Nikon Instruments, <http://www.nikon.com/>). SEM observation of unfixed pollen was performed using a Hitachi TM-1000 table-top scanning electron microscope (Hitachi High-Technologies Corp., <http://www.hitachi-hitec.com>).

Whole-mount ovules and embryos

To examine defects in ovule and embryo development, flowers and siliques from *Atppr2* heterozygous plants at various developmental stages were cleared as previously described (Yadegari *et al.*, 1994), and observed using a Nikon Eclipse 800 wide-field epifluorescence microscope and differential interference contrast (DIC) optics.

GUS reporter assay

To produce the *PPR2Promoter::GUS* construct, the promoter of *AtPPR2* was amplified from Col-0 genomic DNA using primers PPRpro5' and PPRpro3', and inserted upstream of the GUS reporter gene in the binary vector pBAR-GUS which is derived from pCB302 (Xiang *et al.*, 1999). GUS activity staining was performed as previously described (Xia *et al.*, 1996). For thin sectioning after GUS staining, the stained samples were fixed in FAA at 4°C overnight, embedded in Paraplast+ (SPI Supplies, <http://www.2spi.com/>; CAT#8889503002), sectioned at 8 µm thickness, and observed under a Nikon Eclipse 800 wide-field microscope.

RNA isolation, quantitative real-time RT-PCR and RNA blotting

Total RNA was isolated from plants using Trizol reagent (Invitrogen, <http://www.invitrogen.com/>) according to the manufacturer's instructions. The isolated RNA was treated with RNase-free DNase (Promega, <http://www.promega.com/>; CAT#M6101) and purified using Qiagen RNeasy mini columns (<http://www.qiagen.com/>) according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). The *ACTIN2* transcript was used as an internal control to normalize RNA quantity. Primers qPPR2-1 and qPPR2-2 were used to determine the *AtPPR2* gene expression levels in WT plants. Primers P1–P6 were used to determine transcript levels of *AtPPR2* in the *emb2750* seedlings. Three biological replicates were included in the quantitative real-time RT-PCR analysis. For Northern blotting analysis of the plastid 23S rRNA transcript, 10 µg total RNA was loaded onto a 1.5% formaldehyde agarose gel. The RNA blot was autoradiographed onto a phosphor screen (Amersham, <http://www.5.amershambiosciences.com/>).

eYFP reporter assay for determining AtPPR2 subcellular localization

The *AtPPR2* sequence encoding the first 30 amino acid residues was PCR-amplified using the primer pair PPR2N-eYFP5' and the PPR2NeYFP3', cloned into pCR_BluntII-TOPO, and sub-cloned into the pCAM35S::eYFP binary vector to generate pCAM35S::AtPPR2N-eYFP. The construct was transiently transformed into *N. benthamiana* leaves by agroinfiltration as previously described (Wachter *et al.*, 2007). Transient expression of

pCAM35S::AtPPR2N-eYFP and pCAM35S:: eYFP in *Arabidopsis* mesophyll protoplasts was performed as previously described (Yoo *et al.*, 2007). The eYFP signal was observed using a Nikon C1 confocal microscope (488 nm argon laser line, 500–530 nm bandpass detection for YFP, 650–700 nm bandpass detection for chlorophyll).

RNA immunoprecipitation assay and gel mobility shift assay

To produce the 35S promoter::AtPPR2-FAST construct, the AtPPR2 gene was PCR-amplified using primers PPR2-FAST5' and PPR2-FAST3'. The PCR product was cloned into pCR-BluntII-TOPO, cut out using *KpnI*/*SpeI*, and ligated into *KpnI*/*SpeI*-digested pBAR35::FLAG vector. The construct was then transformed into Col-0 plants.

The RNA immunoprecipitation experiment was performed as described previously (Peritz *et al.*, 2006; Jensen and Darnell, 2008) with some modifications. Up to 2 g of leaves from the 35S::AtPPR2-FAST transgenic plants and 35S::FAST transgenic plants (control) were ground into powder in liquid N₂. The powder was suspended in 8 ml ice-cold lysis buffer containing 100 mM KCl, 5 mM MgCl₂, 10 mM HEPES (pH 7.0), 100 U ml⁻¹ RNasin RNase inhibitor (Promega, <http://www.promega.com/>), 2 mM vanadyl ribonucleoside complex solution (Sigma, <http://www.sigmaaldrich.com/>) and 10 µl ml⁻¹ protease inhibitor cocktail (Sigma). The extracts were centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was incubated with anti-FLAG M2 affinity gel (Sigma) for 2 h, and then washed five times (4 min each) with ice-cold lysis buffer. RNA was extracted from the gel samples using Trizol (Invitrogen) according to the manufacturer's instructions, except that, before precipitation of RNA, 5 µg GlycoBlue™ (Ambion, <http://www.ambion.com>) was added to the aqueous phase to facilitate the precipitation of RNA. The RNA pellet was dissolved in diethylpyrocarbonate-treated water. Extracted RNA samples were ligated with 5' RNA linker RL5 (/5Biosg/rArGrG rGrArG rGrArC rGrArU rGrCrC rC/3Phos/) and 3' RNA linker RL3 (rGrGrG rGrArU rGrGrC rGrGrC rUrUrC rCrUrG rC/3Bio/), and then reverse-transcribed using primer DP3. The resulting cDNA molecules were then PCR-amplified using primers DP5 and DP3. The resulting DNA samples were separated through agarose gel electrophoresis, extracted from the gel slides, cloned into the pCR-BluntII-TOPO vector, and transformed into *E. coli*. Plasmid DNA was isolated from transformants, sequenced, and BLAST-searched against the *Arabidopsis* transcript database (the TAIR9 coding sequence database).

The gel mobility shift assay was performed as described previously (Nakamura *et al.*, 2003; Ryder *et al.*, 2008) with some modifications. AtPPR2-FAST protein was affinity-purified from protein extracts of the *Arabidopsis* transgenic line carrying 35S::AtPPR2-FAST using anti-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions. Coding sequences of 23S rRNA and control rRNA (16S rRNA, 5S rRNA and 4.5S rRNA) were cloned into pCR®-Blunt II-TOPO vector (Invitrogen), and transcribed *in vitro* using SP6 RNA polymerase (NEB, <http://www.neb.com>). Protein concentration was determined by CB-X™ protein assay (G-Biosciences, <http://www.gbiosciences.com>), and RNA concentration was measured using a NanoDrop™ spectrophotometer (Thermo Scientific, <http://www.thermofisher.com/>). One microgram of purified AtPPR2-FAST protein was incubated with various amounts of 23S rRNA and control rRNAs at room temperature for 20 min. The samples were UV cross-linked with 1.5 Joule of irradiation by placing the samples 4.5 cm away from a UV source (Stratagene, <http://www.stratagene.com>). Samples were then separated by native PAGE, and protein was detected by Western blotting using anti-FLAG M2 antibody (Sigma).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The *Atppr2* mutation impairs seed and ovule development

(a) Schematic representation of the *AtPPR2* gene and the two *Atppr2* T-DNA insertion alleles. Open boxes, filled boxes and lines represent untranslated regions (UTR), exons and introns respectively. The numbers 1, 269, 982 and 1580 indicate the positions of the ATG start codon, the T-DNA insertion sites in the *Atppr2* allele and the *emb2750* allele, and the TGA stop codon, respectively, in the *AtPPR2* coding sequence. P1–P6 indicate the positions of the primers used in PCR and RT-PCR analysis.

(b, c) The *Atppr2* mutation leads to partial sterility. Shown are developing (b) and mature (c) siliques from WT (Col-0) and *Atppr2*/+ plants.

(d) Opened siliques 8 days post-pollination showing whitish abnormal seeds (black arrows) and aborted ovules (red arrows) from an *Atppr2*/+ plant. *Atppr2Comp*: homozygous *Atppr2* mutant that carried the *AtPPR2* complementation construct.

(e) Seeds from mature siliques of self-pollinated *Atppr2*/+ plants. The arrows indicate abnormal seeds.

(f) Portion of a silique from an *Atppr2*/+ plant. The arrow indicates an aborted ovule.

Scale bars = 1 cm [upper panel of (b)], 1 mm [lower panel of (b), and (c)] and 300 μm (d, e).

Figure 2. The *Atppr2* mutation leads to pollen abortion

(a) Viability staining of mature pollen grains from WT, *Atppr2/+* and *Atppr2Comp* plants. The arrows indicate aborted pollen grains.

(b) Scanning electron micrographs of mature pollen grains from WT, *Atppr2/+* and *Atppr2Comp* plants. The enlargement shows a close-up view of WT and *Atppr2* pollen grains. The arrows indicate aborted pollen grains.

(c) Viability staining of mature pollen grains from WT and *Atppr2/+* plants in the *qrt1* background. The arrow in the inset points to a 'tetrad' with three visible pollen grains. Scale bars = 50 μ m (a, c) and 20 μ m (b).

Figure 3. The *Atppr2* mutation leads to arrest of the first mitotic cell division in male and female gametogenesis

(a–c) DAPI staining of male gametophytes at the unicellular (a), bicellular (b) and tricellular (c) stages from *Atppr2*^{+/+} plants with corresponding bright-field images.

(d–j) Whole-mount preparations of ovules at various stages. The arrows indicate the nuclei.

(d–g) Normal female gametophytes at the one- (d), two- (e), four- (f) and eight-nucleate (g) stages. In (g), the two polar nuclei have fused to form a large central nucleus, and the antipodal nuclei have degenerated and become invisible in the mature embryo sac. (h–j)

Atppr2 mutant female gametophytes that remained at the onenucleate stage during the whole female gametogenesis process. The ovules in (e) and (h), (f) and (i), and (g) and (j) are from the same pistils. Ap, antipodal nuclei; Ccn, central cell nucleus; Ec, egg cell; Syn, synergid nuclei; Vac, vacuole.

Scale bars = 20 μ m (a–c) and 40 μ m (d–j).

Figure 4. The *Atppr2* mutation leads to delayed progression of embryogenesis

Whole-mount preparations of developing seeds at various stages from *Atppr2*/+ plants. With the exceptions of (a) and (b), embryos in the same row are from the same silique. Scale bar = 25 μ m.

(a, b, c, e, i, m) Normal embryos at the octant (a), globular (b), late globular to early heart (c), early torpedo (e), cotyledon (i) and curled-cotyledon (m) stages.

(d, f-h, j-l, n-p) Defective embryos.

Figure 5. The *emb2750* mutation impairs embryogenesis but does not affect gametogenesis

(a, b) Opened siliques (a) and seeds (b) from WT and *emb2750/+* plants.

(c) Viability staining of pollen grains from WT and *emb2750/+* plants in the *qrt1* background.

(d–s) The *emb2750* mutation causes defect in embryogenesis. With the exception of (d) and (e), embryos in the same row are from the same silique. (d–f, h, l, p) Normal embryos at the globular (d), late globular (e), heart (f), early cotyledon (h), curled-cotyledon (l) and mature (p) stages. (g, i–k, m–o, q–s) Defective embryos.

Scale bars = 300 μ m (a, b), 50 μ m (c) and 25 μ m (d–s).

Figure 6. Some *emb2750* mutant seeds were able to germinate and produce albino seedlings
 (a) WT, *Atppr2* and *emb2750* seeds.
 (b) Germination of WT, *Atppr2* and *emb2750* seeds.
 (c) Close-up view of WT and *emb2750* seedlings.
 (d) Transcript levels of *AtPPR2* in WT and *emb2750* seedlings determined by quantitative real-time RT-PCR. The locations of the primer pairs used in the analysis are indicated in Figure 1(a). Bars represent standard deviation. The expression level for WT was arbitrarily set to 1.
 Scale bars = 300 μ m (a, b) and 0.5 mm (c).

Figure 7. Expression patterns of *AtPPR2*

(a) *AtPPR2* transcript levels as determined by quantitative real-time RT-PCR in various organs of WT plants. Se, seedlings; Rt, roots; St, stems; RL, rosette leaves; Cl, cauline leaves; Flb, flower buds; Inf, Inflorescences; Ofi, open flowers; Silq1, siliques from the zygote to globular stages; Silq2, siliques from the globular to heart stages; Silq3, siliques from the heart to cotyledon stages.

(b–l) Expression patterns of *AtPPR2* revealed by the GUS reporter assay. GUS staining results for *AtPPR2Pro::GUS* transgenic Arabidopsis plants: seedling (b), root tip (c), plant at the 6–8 true leaf stage (d), inflorescence (e), flower (f), anther with near mature pollen grains (g), thin sections of two anthers at the tetrad stage (h) and stage 12 (i), ovule (j), and developing seeds at the heart stage (k) and curled-cotyledon stage (l).

Scale bars = 1 cm (b, d, e), 200 μ m (f, g) and 50 μ m (c, h–l).

Figure 8. AtPPR2 is localized in chloroplasts. The AtPPR2N-eYFP fusion protein co-localized with chlorophyll in tobacco leaf epidermal cells (a) and Arabidopsis leaf protoplasts (b)
Scale bars = 20 μ m (a) and 40 μ m (b).

Figure 9. AtPPR2 is a 23S rRNA-binding protein

(a) Agarose gel electrophoresis of cDNA from RNA precipitated with AtPPR2-FAST. No DNA was visible from the control sample (FAST).

(b) Gel mobility shift assay revealed that AtPPR2 binds to 23S rRNA but not to the other three plastid rRNA molecules.

Figure 10. The *emb2750* line showed altered expression of plastid-encoded rRNAs and *RbcL*
 (a) Levels of the four plastid-encoded rRNAs and the *RbcL* and *PsbA* transcript in 7-day-old seedlings revealed by quantitative real-time RT-PCR. The expression level in WT was set to 1.
 (b) RNA blot showing transcript patterns revealed by probing with the 23S rRNA gene fragment. Total RNA was isolated from 7-dayold seedlings.
 (c) Coomassie blue staining of total protein extracts from the WT and *emb2750* seedlings, indicating significant reduction of the *RbcL* level in the mutant. The arrow indicates the *RbcL* band.
 (d) Detection of *RbcL* and *PsbA* by immunoblotting using anti-*RbcL* and anti-*PsbA* antibodies, respectively.