



RPS9M, a Mitochondrial Ribosomal Protein, Is Essential for Central Cell Maturation and Endosperm Development in *Arabidopsis*

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During double fertilization of angiosperms, the central cell of the female gametophyte fuses with a sperm cell to produce the endosperm, a storage tissue that nourishes the developing embryo within the seed. Although many genetic mutants defective in female gametophytic functions have been characterized, the molecular mechanisms controlling the specification and differentiation of the central cell are still not fully understood. Here, we report a mitochondrial ribosomal protein, RPS9M, is required for central cell maturation. RPS9M was highly expressed in the male and female gametophytes before and after double fertilization. The female gametophytes were defective in the *rps9m* mutant specifically concerning maturation of central cells. The morphological defects include unfused polar nuclei and smaller central vacuole in central cells. In addition, embryo initiation and early endosperm development were also severely affected in *rps9m* female gametophytes even after fertilized with wild type pollens. The RPS9M can interact with ANK6, an ankyrin-repeat protein in mitochondria previously reported to be required for fertilization. The expression pattern and mutant phenotype of RPS9M are similar to those of ANK6 as well, suggesting that RPS9M may work together with ANK6 in controlling female gametophyte development, possibly by regulating the expression of some mitochondrial proteins.

Keywords: *Arabidopsis thaliana*, central cell, embryo, endosperm, female gametophyte, mitochondrion, ribosomal protein

INTRODUCTION

In angiosperms, the egg and sperm cells form within the female and male gametophytes through a series of meiosis, mitosis, and cell fate determination, respectively (Ma and Sundaresan, 2010). During female gametophyte development in most flowering plants, the megaspore mother cell (MMC) undergoes meiosis to produce four haploid megaspores. Three of the megaspores undergo programmed cell death, leaving one survived megaspore to become functional megaspore (FM). Subsequently, the FM cell undergoes three rounds of mitosis without cellularization to produce an eight-nucleate structure. Then migration of nuclei and cellularizations result in a seven-celled female gametophyte consisting of one egg cell, two synergid cells, three antipodal cells, and one central cell containing two polar nuclei (Yang et al., 2010). In *Arabidopsis thaliana* and many other

species, two polar nuclei fuse to form a diploid nucleus in the central cell and the central cell vacuole enlarged before fertilization. (Yadegari and Drews, 2004).

By genetic and molecular approaches, a number of genes have been identified to be involved in female gametophyte development (Yadegari and Drews, 2004; Kaegi and Gross-Hardt, 2007; Liu and Qu, 2008; Sundaresan and Alandete-Saez, 2010; Yang et al., 2010; Martin et al., 2014), and some of them display maternal effects on seed formation (Huh et al., 2008; Berger and Chaudhury, 2009; North et al., 2010). In the past decades or so, mutants defective in central cell development have also been described, including *FEM111/AGL80* (Portereiko et al., 2006a), *AGL61* (Bemer et al., 2008; Steffen et al., 2008), and the *BiP* genes (*BiP1* and *BiP2*) (Maruyama et al., 2010). *AGL61* and *AGL80* encode the type I MADS-domain transcription factors and mutation of either of them results in smaller central cell with reduced vacuole. Consequently the central cell in either *agl61* or *agl80* mutant fail to give rise to endosperms after fertilization. Yeast two-hybrid (Y2H) assay show that *AGL61* interact with *AGL80*, suggesting that these two transcription factors may function as a heterodimer in controlling the expression of downstream genes during central cell development. The *BiP* genes encode molecular chaperones of the Hsp70 family in the endoplasmic reticulum. The central cell in *bip1bip2* double mutant show defects in the fusion of polar nuclei, which may result from altered nuclear membrane. After fertilization, the mutant shows aberration in endosperm nuclear division, indicating that central cell maturation plays an important role in endosperm development.

Mitochondria also play a critical role in female gametophyte maturation. Mutations in some genes encoding mitochondrial proteins cause defects in female gametophyte. These include *RPL21M/NFD1* (Portereiko et al., 2006b), *FIONA/SYCO* (Kaegi et al., 2010), *GCD1* (Wu et al., 2012) and *MSD1/OIWA* (Martin et al., 2013). *RPL21M* encodes the mitochondrial 50S ribosomal subunit L21 and is essential for the fusion of polar nuclei during female gametophyte maturation and the male-female gametophyte recognition during double fertilization. *FIONA* encodes the cysteinyl t-RNA synthetase and requires for the central cell development and mitochondrial cristae integrity. *GCD1* encodes the ribosomal protein L20 which is enriched in gametophytes and plays important roles in fusion of polar nuclei, maturation of female gametes, embryogenesis initiation, and endosperm development. *MSD1* encodes a mitochondrial Mn-superoxide dismutase, which is critical for distribution of reactive oxygen species (ROS) during embryo sac patterning. All these genes described above are important for central cell maturation and endosperm development, indicating the importance of mitochondria in female gametophyte maturation.

Our previous work identified *ANK6* gene as required for male-female gamete recognition. *ANK6* is an ankyrin protein located in the mitochondria and likely functions as a scaffold to mediate protein-protein interactions through its ankyrin repeat domains. Indeed, we found a member of σ -transcription initiation factor, *SIG5*, as a partner for *ANK6*.

Like in *ank6* mutant, a proportion of *sig5* embryo sacs are also arrested at late stages of female gametophyte development (Yu et al., 2010). In the same yeast two hybrid screen, a mitochondrial ribosomal small subunit protein, designated *RPS9M*, showed interaction with *ANK6*. *RPS9M* is highly expressed in both male and female gametophytes just like *ANK6*. The *rps9m* mutants have female gametophyte defects and, in particular, the central cells showed smaller vacuoles and defect in the fusion of polar nuclei. Although *rps9m* embryo sacs are capable of double fertilization, embryogenesis initiation and endosperm proliferation are both defective after fertilization whether mutant or wild type pollens are used for pollination. Together, these results suggest that *RPS9M* functions with *ANK6* and possibly other proteins (e.g., *SIG5*) in the control of mitochondrial gene expression during female gametophyte.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana (ecotype Columbia-0) plants were used in this study unless otherwise indicated. Seeds were surface sterilized in 70% ethanol for 5 min, stratified at 4°C for 2 days, and grown on half-strength Murashige and Skoog (Sigma¹) medium at 22°C under 16:8 h light: dark cycles for a week. Then plants were transferred to soil and incubated in a constant-temperature room at 22°C under 16 h light/8 h dark cycles. *Agrobacterium*-mediated transformation were as described previously (Clough and Bent, 1998). The transgenic lines were selected on 0.5 × Murashige and Skoog medium (Sigma) containing 50 µg/mL kanamycin (for pBI101-based vectors) or 30 µg/mL hygromycin B (for pCAMBIA 1300-based vectors).

Plasmid Constructs

The *RPS9M* cDNA is amplified by RT-PCR using the primer pair *RPS9M-F* and *RPS9M-R* and ligated into the pMD18-T vector (TaKaRa). To make the *ProRPS9M-GUS* construct for histochemical analysis, a 2.2-kb fragment upstream of the ATG starting codon of *RPS9M* was PCR amplified using the primers *RPS9M-PF* and *RPS9M-PR* and cloned into the pMD18-T vector, then subcloned into the pBI101.2 vector using the same primers. For subcellular localization analysis, the *RPS9M* CDS was amplified without its stop codon using the primer pair *RPS9M-GF* and *RPS9M-GR*, subsequently digested with *Xba* I and *Bam*H I, respectively, and then inserted into the plant binary vector pMD1-GFP to make the 35S-*RPS9M-GFP* fusion construct.

For CRISPR/Cas9 plant expression vectors construction. The chimeric sgRNA for *RPS9M* was constructed by cloning annealed oligos *RPS9M-sgF* and *RPS9M-sgR* into pBlueScript SK-sgRNA, then both the sgRNA and hSpCas9 were subcloned into the expression vector pCAMBIA1300 (Supplementary Figure S2A) as previously described (Feng et al., 2013; Mao et al., 2013).

¹www.sigmaldrich.com

Histochemical GUS Analysis

Transformed *Arabidopsis* lines carrying the *pRPS9M-GUS* fusion were selected based on Kanamycin resistance. The promoter activity was reflected by GUS activity visualized in the plant tissues using 5-bromo-4-chloro-3-indolylb-D-glucuronide (X-Gluc) as a substrate according to published protocols (Jefferson et al., 1987). In details, inflorescences and 2 week old seedlings were incubated in GUS staining solution [10 mg/mL X-Gluc, 0.1 M $K_3(Fe(CN)_6)$, 0.1 M $K_4Fe(CN)_6 \cdot 3H_2O$, 1 M Na_2EDTA , 0.2 M Na_3PO_4 (pH7.0)] at 37°C overnight. Then the plant tissues were decolorized for 30 min in each of the gradient ethanol solution (30, 40, 50, 60, 70%) and incubated in 75% ethanol at 37°C overnight. The samples were examined and photographed with an Olympus SZX12 microscope equipped with a camera.

Yeast Two-Hybrid (Y2H) Screen and Analysis

We used the CLONTECH Matchmaker GAL4 Two-Hybrid System 3 for the yeast (*Saccharomyces cerevisiae*) two-hybrid screen and analysis. For performing Y2H screen of *Arabidopsis* cDNA library, according to the method described as previous (Yu et al., 2010). The ANK6 CDS were fused to GAL4 DNA-binding domain in pGBKT7. AH109 cells were first transformed with pBD-ANK6 plasmid, then the transformants were transformed with the *Arabidopsis* cDNA library cloned in the prey vector pACT. The transformed cells were plated on synthetic dropout (SD) selection medium that lacked Trp, Leu, and His supplemented with 20 mM 3-AT to reduce the growth of false-positive colonies. The plates were incubated at 30°C for 3–12 days. The prey plasmid DNAs were isolated from yeast, transformed and isolated from *Escherichia coli*, and retransformed into yeast cells containing either the empty vector or the vector with bait to verify growth further. The clones that continued to grow in the –His and +3-AT medium after retransformation were selected for DNA sequencing. For Y2H analysis the interactions between RPS9M and ANK6, the *RPS9M* CDS and truncated *RPS9M* variants were fused to the GAL4 activation domain in pGADT7. Yeast strain AH109 was cotransformed with combinations of pGADT7 and pGBKT7 constructs and selected on SD medium lacking Leu and Trp (SD-LW). Cotransformants were then assayed for interaction and activation of the His and adenine reporter genes on SD medium lacking Leu, Trp, His, and adenine (SD-LWHA). For this, fresh colonies were grown in SD-LW at 30°C overnight to an OD_{600} of 1–2, the cells were pelleted and resuspended in 0.5 M sorbitol to an OD_{600} of 0.5, and 5 μ L of each cell suspension was spotted on SD-LWHA plates using a multichannel pipette and grown at 30°C for 2–3 days.

Bimolecular Fluorescence Complementation (BiFC) Assays

The ORF sequence of ANK6 and *RPS9M* was cloned into the plasmid pE3308 and pE3449 using the primer pairs list in Supplementary Table S2. Protoplasts were isolated from 4 week old *Arabidopsis* rosette leaves. Transient protoplast expression

was performed using the polyethylene glycol transformation method as described previously (Sheen, 2001).

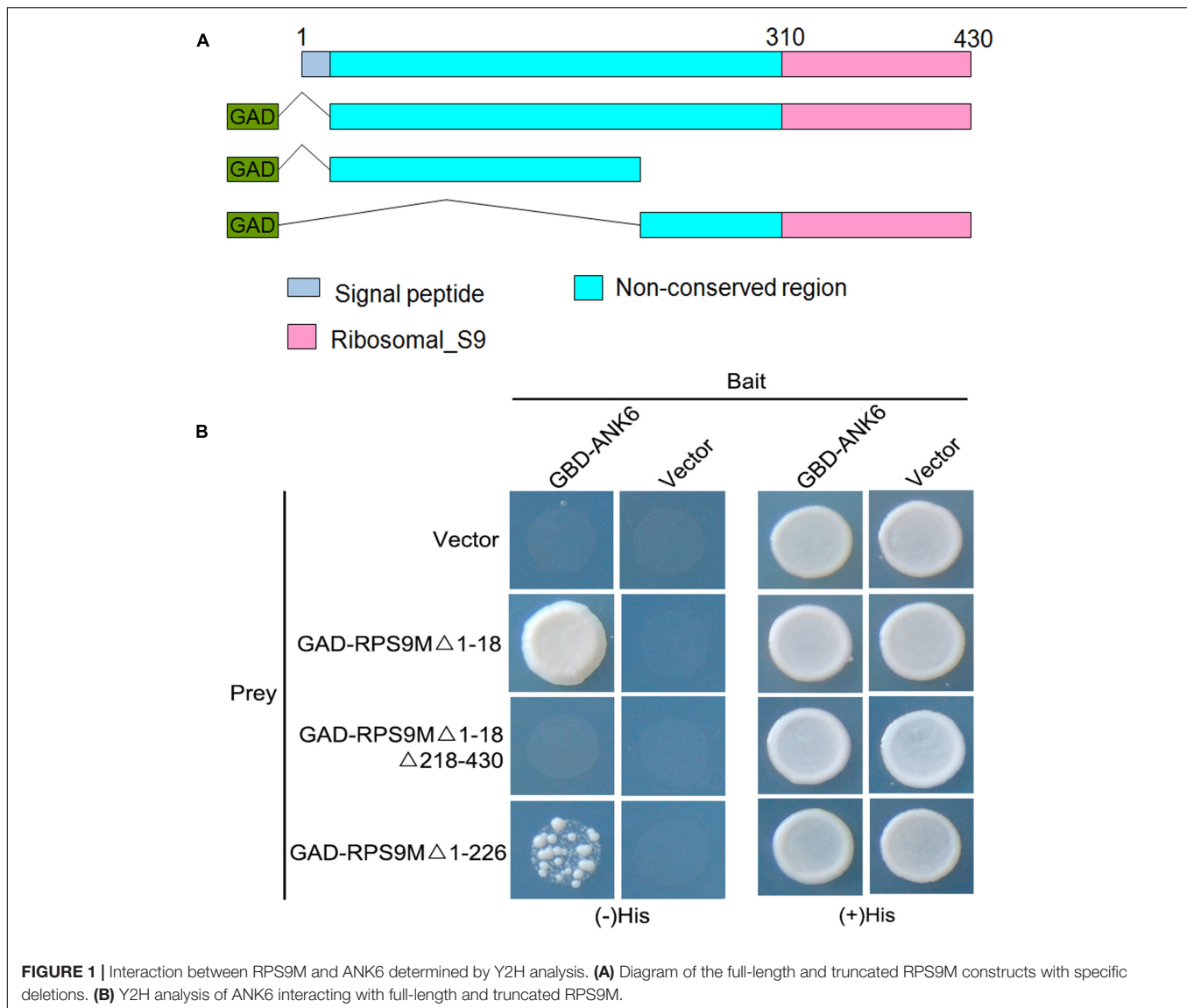
CLSM and Central Cell Vacuole Size Calculation

The confocal observation of ovules was performed according to the method described previously (Christensen et al., 1997) with slight modifications. Inflorescences were harvested and fixed in 4% glutaraldehyde (in 12.5 mM cacodylate, pH 6.9), and a vacuum was applied for the initial 20 min, after which they were in fixative overnight at room temperature. After fixation, the tissues were dehydrated through a conventional ethanol series with 20 min per step. After the dehydration, the tissue was cleared in 2:1 (v/v) benzyl benzoate: benzyl alcohol for a minimum of 1 h. Pistils or siliques were dissected, mounted with immersion oil, and observed with a Zeiss LSM510 META laser scanning microscope (Zeiss, Jena, Germany) with a 488-nm argon laser and an LP 530 filter. Images were edited with Zeiss LSM Image Browser software and Photoshop software. The central cell vacuole size was calculated according to the method as described previously (Wu et al., 2012). Single section image was captured when scanning to the largest sectional view of the central cell vacuole, and the size of central cell vacuoles were calculated using Image J software.

RESULTS

RPS9M Interacts with Ankyrin Protein ANK6

The ankyrin repeats are generally recognized as protein–protein interaction modules. We previously showed that mitochondrial ANK6 protein functions in double fertilization, suggesting that a protein complex mediated by this ankyrin repeat protein might participate in ovule development. We performed a Y2H screening by using ANK6 (amino acid residues 21–174) as a bait to screen a prey cDNA library. Several positive clones were obtained, including *RPS9M*, a bacterial-origin mitochondrial-type ribosomal protein S9 gene (AT3G49080) (Bonen and Calixte, 2006). It encodes a 430-aa protein, with a Ribosomal_S9 domain at the C terminus (amino acid residues 310–430) and a non-conserved region at the N-terminus, according to the SMART protein domain prediction program (Figure 1A). The sequence of the Ribosomal_S9 domain shows high similarity to the RPS9 protein of *Escherichia coli* and chloroplast ribosomal protein S9 of *Arabidopsis*. To identify the ANK6-interacting domain, three truncated *RPS9M* variants were fused with GAL4 activation domain (Figure 1A). The interaction between these variants and ANK6 were assayed by using the Y2H system (Figure 1B). ANK6 interacts with the C-terminus Ribosomal_S9 domain of *RPS9M*. *RPS9M* lacking the non-conserved N-terminus showed a reduced interaction activity with ANK6, suggesting a role for the N-terminus in regulating the interaction of *RPS9M* with ANK6 (Figure 1).



RPS9M Is a Mitochondrial Localized Protein and Interacts with ANK6 in Mitochondria

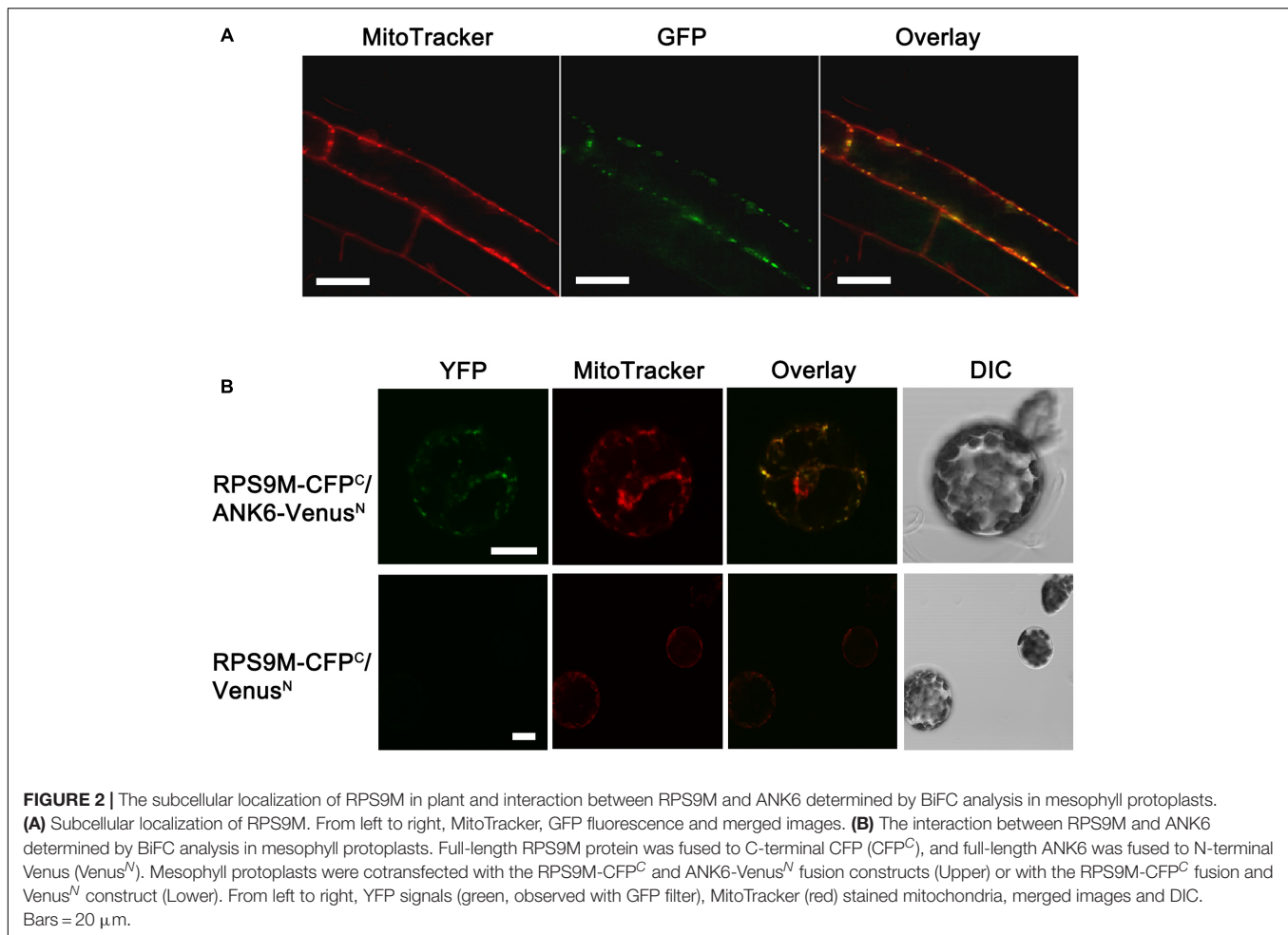
The RPS9M protein contains a mitochondrial pre-sequence that targets the protein to mitochondria according to SUBAcon. To investigate the subcellular location, an *RPS9M-GFP* gene cassette driven by the cauliflower mosaic virus 35S promoter was constructed and introduced into wild type plants (Supplementary Figure S4). The GFP fluorescent signals were detected in the roots of these transgenic plants under confocal microscopy. MitoTracker dye was used as a control to stain mitochondria of the roots. In the root epidermal cells of a *35S-RPS9M-GFP* line, the green fluorescent signals were colocalized with the MitoTracker fluorescent signals (**Figure 2A**), indicating that RPS9M protein was targeted to mitochondria.

To confirm the interaction between RPS9M and ANK6, we performed bimolecular fluorescence complementation assays in

plant cells. We co-expressed RPS9M tagged with C-terminal CFP and ANK6 tagged with N-terminal Venus in mesophyll protoplasts. The yellow/green fluorescence signals indicated interaction between RPS9M and ANK6. Mitochondrial dye was used to localize mitochondria. As shown in **Figure 2B**, the yellow/green fluorescence and the red dye signals were overlapped, supporting the notion that RPS9M and ANK6 interact with each other in mitochondria.

RPS9M Is Expressed in Both Male and Female Gametophyte

In order to reveal the function of *RPS9M* gene, we first examined the expression pattern of *RPS9M* using transgenic plants harboring the reporter construct with *RPS9M* promoter fused to the β -glucuronidase (GUS) gene. Seven GUS transgenic lines were selected and their GUS activities were identified with similar expression patterns. Strong GUS activities were

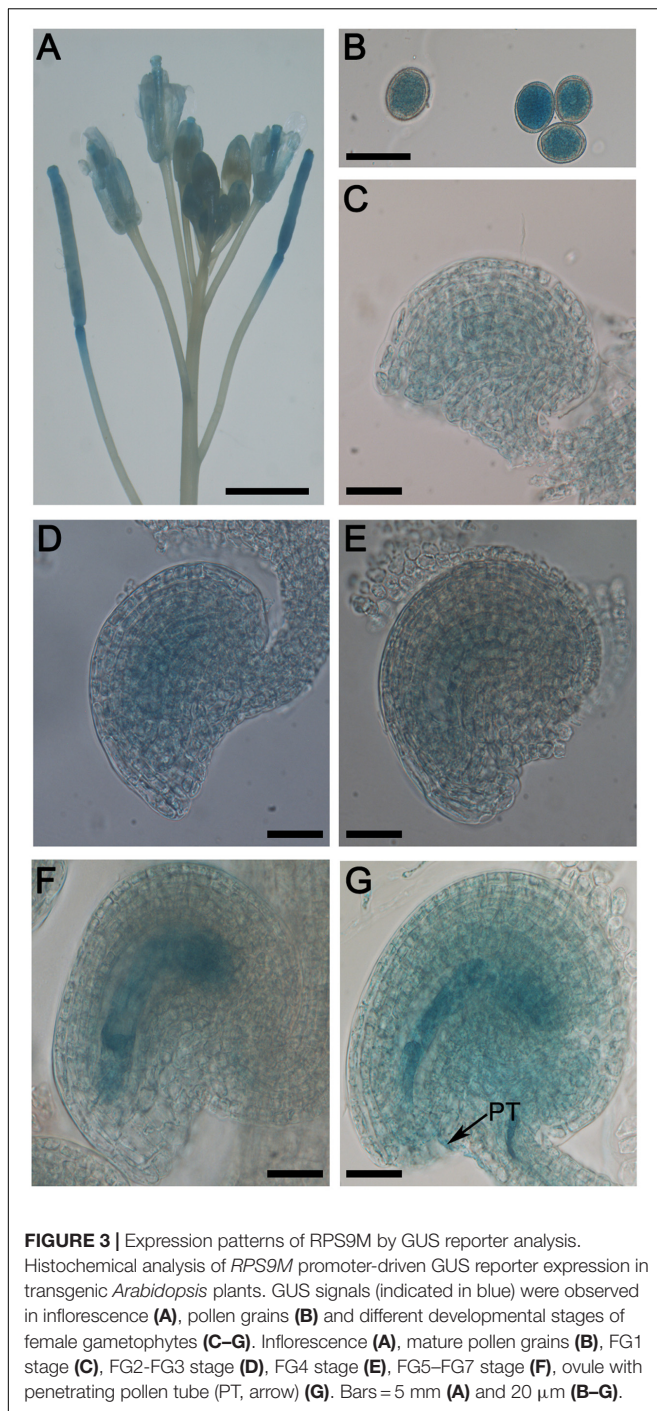


detected in inflorescence particularly in the anthers and pistils of T₂ transgenic lines (Figure 3A and Supplementary Figures S1A,B). In anthers, GUS activities were detected throughout the developmental stages, especially in the mature pollen grains (Figure 3B and Supplementary Figures S1A,B). In pistils, GUS activities were detected in female gametophytes at different developmental stages (Figures 3C–G), particularly in the mature female gametophyte stage (Figure 3F). The *RPS9M* promoter was also highly active during double fertilization (Figure 3G). After fertilization, GUS signals were detected in young siliques, especially in embryo and endosperms (Supplementary Figures S1B,D,E), but no GUS signal was detected in mature seeds (Supplementary Figure S1C). GUS signals were also observed in young leaves and root tips (Supplementary Figure S1F). These expression patterns suggest that *RPS9M* may participate in the development of male and female gametophytes as well as in embryogenesis.

***RPS9M* Mutant Displayed Empty Seed Sets in Siliques**

To determine the function of *RPS9M* gene in plant development, CRISPR/Cas9 gene editing approach was adopted to produce mutations in the *RPS9M* gene (Jiang et al., 2013; Mao et al.,

2013; Zhang et al., 2014). We designed two sgRNA that targeted the coding sequence of *RPS9M* gene and transformed into wild type plants. A series of heterozygous mutants were obtained in the T₁ generation. Most of the mutants involved insertions or deletions of a few nucleotides, with rare cases of sizable deletions of 30 or more nucleotides (Supplementary Figures S2B–D). In this study we chose two mutant lines for further research, one contain a deletion of 32 nucleotides, equivalent to the sequence of +271–+302 (named as *rps9m-1*) and the other contain a deletion of 5 nucleotides equivalent to the sequence of +375–+379 (named as *rps9m-2*) (Figure 4A). Genetic segregation in T₂ generation produced mutant plants that did not contain T-DNA insertion sequences or sgRNA/Cas9 fragments. Genotyping of +/*rps9m-1* and +/*rps9m-2* in T₃ and T₄ generation led to identification of several heterozygous mutant plants, but no homozygous mutant plants. No homozygous mutant was obtained even after +/*rps9m* plants were selfed, implying that the *rps9m* mutant allele may cause gametophyte defect or homozygous embryo lethality. The heterozygous plants grew normally in the vegetative stage. However, after fertilization, about 50% ovules in both +/*rps9m-1* and +/*rps9m-2* siliques were aborted at early stage (Figures 4B,C). Analysis of the progeny of +/*rps9m-1* and +/*rps9m-2* plants and other mutant



lines in T₄ generation revealed a segregation ratio of wild type to heterozygous plants close to 1:0.2 (Supplementary Table S1). These data suggested that not only the female gametophyte, but also the male gametophyte were defective when RPS9M function was disrupted.

To further examine the function of RPS9M in reproduction, reciprocal cross-tests between the heterozygous *+/-rps9m-1* mutant and wild type plants were performed. When *+/-rps9m-1* pistils were pollinated with wild type pollens, only a

few heterozygote plants were identified in the progeny, the transmission of *rps9m-1* allele through female gametophyte was reduced to 3% ($n = 151$; $P < 0.001$) (Table 1). In addition, siliques resulting from this cross exhibited about 50% aborted seed sets. These observations indicate that RPS9M lose-of-function mutation almost completely disrupt the female gametophyte function. When wild type ovules were pollinated with *+/-rps9m-1* pollens, we found the transmission efficiency of the *rps9m-1* male gametophyte was significantly reduced (TE = 21%; $n = 167$; $P < 0.001$), compared with that of the wild type allele (Table 1), indicating that the male gametophyte function is also affected, albeit to a lesser extent compared to the defect of the female gametophyte. Similar results were obtained when reciprocal cross-tests were done between *+/-rps9m-2* and wild type plants that the transmission of female gametophyte was almost completely blocked and the transmission of male gametophyte was reduced to 24% (Table 1). These results demonstrate that loss of RPS9M function indeed cause defects in both two gametophytes, especially the female gametophyte.

To verify that the observed phenotypes were caused by loss-of-function of the *AtRPS9M* gene, we performed genetic complementation experiment by introducing 35S-RPS9M-GFP construct into *+/-rps9m-1* and *+/-rps9m-2* mutant plant. In the T₂ populations, we obtained 5 independent lines that were homozygous for both the *rps9m-1* allele and the RPS9M transgene, and 3 independent homozygous lines for *rps9m-2* and RPS9M transgene. In the progenies of these transgenic lines, silique seed sets were fully restored to the wild type level (Figures 4B,C), demonstrating that the RPS9M transgene fully complemented the *rps9m* phenotype.

Female Gametophyte Development Is Affected in *rps9m* Mutants

Genetic analysis of the *rps9m* mutant suggested a defect in female gametophyte function. To further examine this defect, we monitored ovule development in wild type and *+/-rps9m* plants by using whole-mount clearing and confocal laser scanning microscopy (CLSM). We first analyzed female gametophytes at the terminal development stage (stage FG7). We emasculated the wild type, *+/-rps9m-1* and *+/-rps9m-2* flowers (stage 12c) and kept them growing for 24 h, then fixed the ovule tissues for confocal analysis. Wild type female gametophytes at this stage have one egg cell, one central cell, and two synergid cells, whereas the three antipodal cells were undetected. The two polar nuclei have fused to form a diploid nucleus in most of the central cells (Figure 5A).

On the other hand, ovules in *+/-rps9m-1* and *+/-rps9m-2* heterozygous flowers at the same stage showed differences from the wild type. Specifically, about 47% ($n = 720$) ovules in *+/-rps9m-1* plants and 45% ($n = 630$) embryo sacs in *+/-rps9m-2* pistils contained unfused polar nuclei (Figures 5B–D), indicating deflections of polar nuclei fusion in *rps9m* female gametophytes. In addition, we measured the central cell vacuole size in the embryo sacs, as shown in Figure 5E, the wild type central cell vacuole size at this stage usually developed to about 510 μ m², but in *rps9m* sacs, the size of central cell vacuole was only about 390 μ m² in *rps9m-1* and 365 μ m² in *rps9m-2* which were

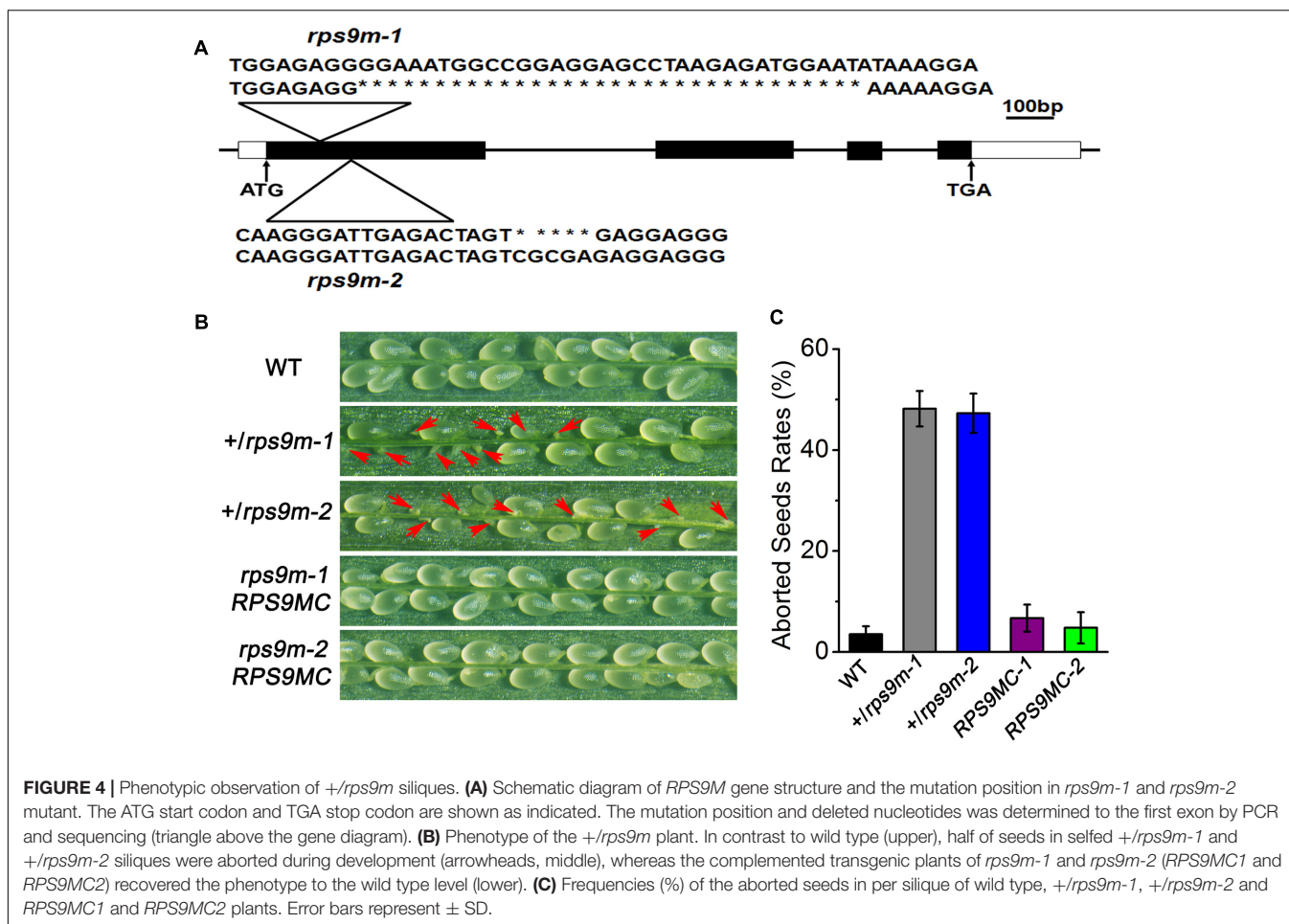


TABLE 1 | Transmission of *rps9m* alleles to F1 progenies after reciprocal crosses.

Parental genotypes		Progeny genotypes		Expected ratio	Observed ratio
Female	Male	WT	<i>+/rps9m</i>		
<i>+/rps9m-1</i>	WT	146	5	1:1	1:0.03 ^a
WT	<i>+/rps9m-1</i>	138	29	1:1	1:0.21 ^a
<i>+/rps9m-2</i>	WT	154	3	1:1	1:0.02 ^a
WT	<i>+/rps9m-2</i>	187	44	1:1	1:0.24 ^a

^aSignificantly different from the expected 1:1 segregation ratio based on χ^2 values ($P < 0.001$); +, *RPS9M* allele.

significantly smaller than those of wild type. We next observed the morphology of egg cells and synergid cells, no obvious difference was found among *rps9m-1*, *rps9m-2* and wild type (Figures 5A–C and Supplementary Table S4). These observations suggesting that disruption of *RPS9M* inhibited fusion of polar nuclei and affected central cell maturation. To further exclude the possibility that *RPS9M* affects the earlier developmental stages of female gametophyte, we examined female gametophytes from flowers at earlier development stages. No phenotypic variations appeared between wild type and *rps9m* ovules during nuclear mitosis stages (Supplementary Figure S3 and Supplementary Table S3), indicating that macrospore nuclear proliferations and migrations were not affected in *rps9m* female gametophytes.

Together, the data above indicate that the mutation of *RPS9M* specifically affects the final maturation process of the central cell during female gametophyte development.

Pollen Tube Guidance and Fertilization Is Not Impaired in *rps9m*

Several studies show that central cell defects sometimes affect other processes, such as pollen tube guidance and fertilization (Christensen et al., 2002; Portereiko et al., 2006b; Chen et al., 2007). In order to test whether *rps9m* embryo sacs affect pollen tube guidance, pollens from a transgenic line expressing *pLAT52-GUS* were used as donors to pollinate wild type, *+/rps9m-1*

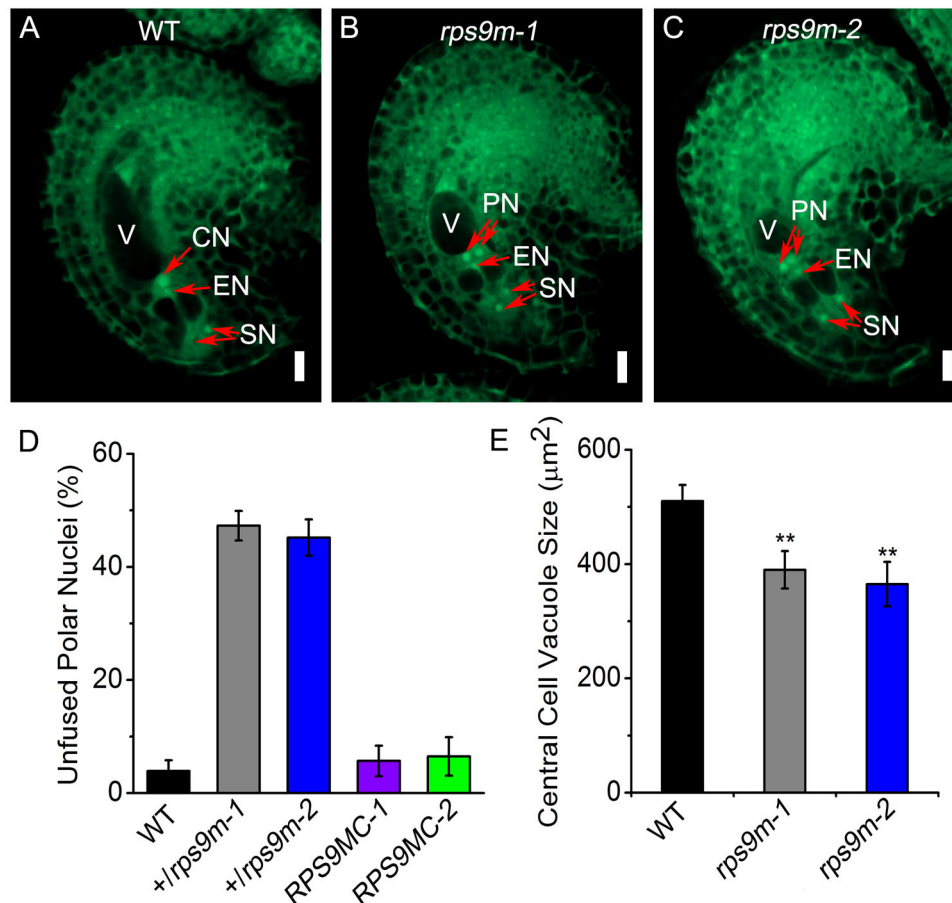
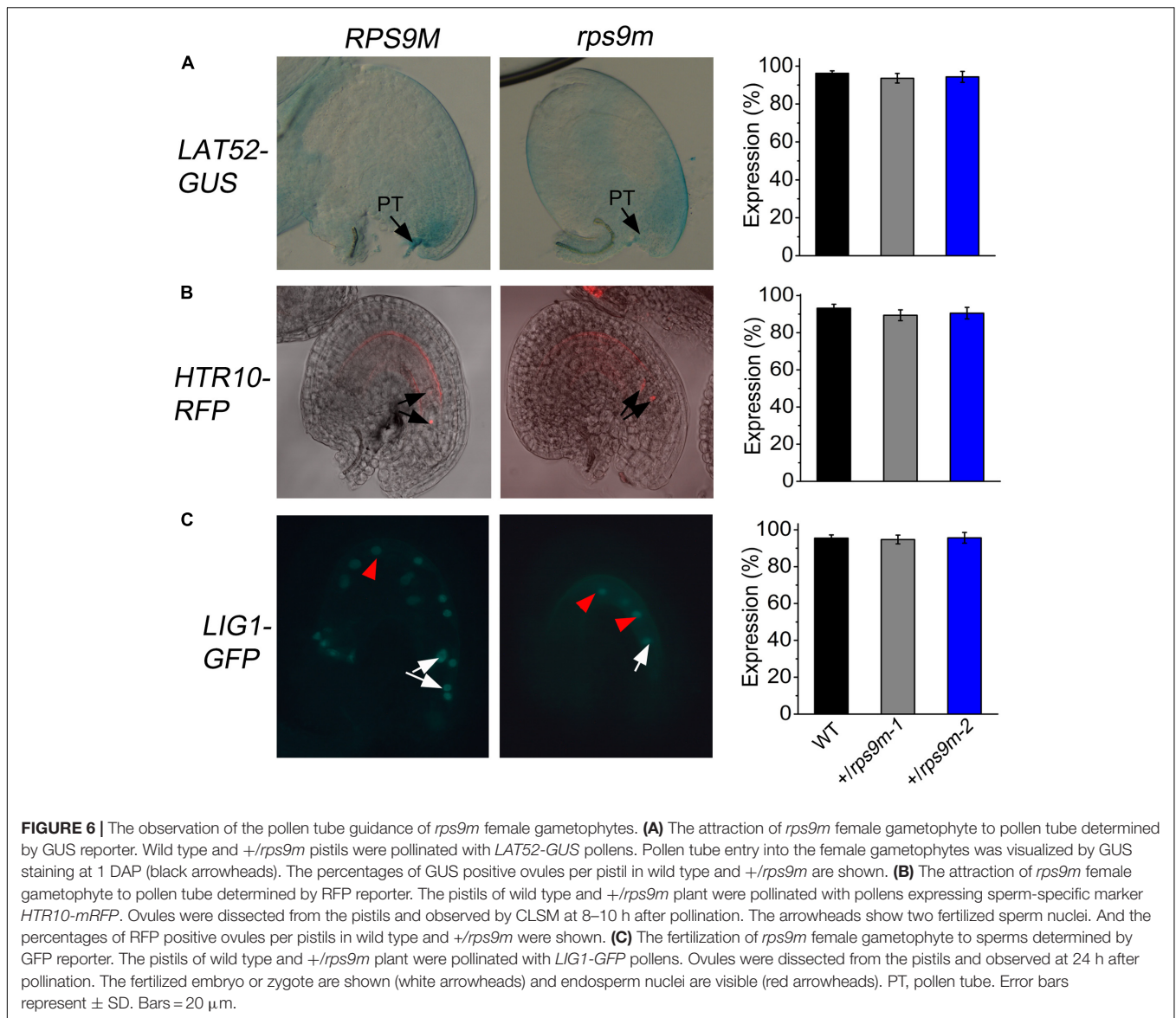


FIGURE 5 | The observation of central cell development in *rps9m* female gametophyte by CLSM. **(A)** Wild type female gametophyte at FG7 stage. The female gametophyte contains two synergid nucleus, one egg nucleus and a secondary central cell nucleus. **(B)** The *rps9m-1* female gametophyte at the same pistil. The two polar nucleus still unfused and the central cell vacuole is smaller compared to that in wild type. **(C)** The *rps9m-2* female gametophyte at the same stage. **(D)** Frequencies (%) of unfused polar nuclei in wild type, +/*rps9m-1*, +/*rps9m-2* and the complemented transgenic plants of +/*rps9m-1* and +/*rps9m-2* at this stage. **(E)** The central cell vacuole size of wild type, *rps9m-1* and *rps9m-2*. CN, central cell nucleus; EN, egg cell nucleus; PN, polar nucleus; SN, synergid nucleus; V, vacuole. Error bars represent \pm SD. Bars = 10 μm . ** $P < 0.05$, Student's *t*-test.

and +/*rps9m-2* pistils (Twell et al., 1989). The ovules were collected and GUS activities were analyzed at 24 h after pollination. Almost all ovules in wild type (96%, $n = 190$), +/*rps9m-1* (93%, $n = 240$) and +/*rps9m-2* (94%, $n = 220$) pistils had a pollen tube entered (Figure 6A), indicating that pollen tube guidance was not impaired in *rps9m* female gametophyte. To further investigate whether mutation of *RPS9M* in female gametophyte impact fertilization process, a transgenic line expressing *pHTR10-mRFP1*, a sperm-nuclear protein labeling monomeric red fluorescent protein, was used as pollen donors (Ingouff et al., 2007). We pollinated wild type, +/*rps9m-1* and +/*rps9m-2* pistils with *pHTR10-RFP1* pollens. By 12 h after pollination, the RFP signals can be detected in 93% ($n = 96$) ovules of wild type, 89% ($n = 117$) ovules of +/*rps9m-1* and 90% ($n = 120$) ovules of +/*rps9m-2* plants at positions of the egg cell and central cell (Figure 6B), indicating disruption of *RPS9M* in female gametophyte does not impact pollen reception and sperm cell migration. The possibility of fertilization

was evaluated by duration of RFP signals in *rps9m* female gametophyte after pollination, since the RFP signals elapsed quickly as soon as the fertilization was completed. About 1–2 days after pollination, no RFP signals can be detected in both wild type and *rps9m* female gametophytes, suggesting the double fertilization process is completed in not only wild type but *rps9m* female gametophytes as well. To ascertain that the double fertilization occurred in *rps9m* female gametophyte, a transgenic line expressing DNA LIGASE 1 nuclear protein and green fluorescent protein (GFP) fusion protein is used as paternal line (Ingouff et al., 2009). We therefore pollinated +/*rps9m-1*, +/*rps9m-2* and wild type pistils with pollens containing LIG1-GFP. Up to 1 days after pollination (DAP), the paternal expression of LIG1-GFP labeled both the embryo and endosperm in seeds produced from WT (95%, $n = 135$), +/*rps9m-1* (94%, $n = 170$) and +/*rps9m-2* (96%, $n = 174$) pistils (Figure 6C), indicating double fertilization is not affected in *rps9m* female gametophyte.



Embryogenesis and Endosperm Proliferation Are Defective in *rps9m* Mutants

In wild type ovules, fertilized egg cell (zygote) undergoes mitosis to form the embryo and the fertilized central cell gives rise to endosperm following fertilization, ultimately developing into mature seeds. But in *+rps9m-1* siliques approximately 50% seeds aborted, the observed seed abortion phenotype was probably due to abnormal development of the embryo and/or the endosperm (Yadegari and Drews, 2004; Berger et al., 2006). We therefore analyzed embryo and endosperm development in *+rps9m-1* siliques at different time points after pollination. Briefly, the *+rps9m-1* flowers were self-pollinated. By 2 days after pollination (DAP), 91.2% of the young seeds ($n=437$) contained both an embryo and endosperms. However, 41.5% of the seeds were quite different from the other half in size and

endosperm cavity (Table 2). The wild type endosperm usually had 16 or more endosperm nuclei. In contrast, majority of the mutant endosperms only had two (19.5%) or four (14.6%) nuclei, and about 7.4% mutant endosperm arrested immediately. The mutant embryo development was also arrested, about 22% of the embryos arrested at the zygote stage, and 19.6% of them divided once to produce a 2-cell embryo before arrested in *+rps9m-1* silique (Figures 7A–D and Table 2). At 3 DAP, the aborted seeds collapsed. Similar results were observed from the analysis of *+rps9m-2* plants that half of seeds were aborted, and the endosperms arrested at two (17%) or four (21%) nuclei stage in the aborted seeds (Table 2). When we pollinated *+rps9m-1* and wild type ovules with *LIG1-GFP* pollens, at 2 DAP, the paternal expression of *LIG1-GFP* was detected in both the embryo and endosperms in seeds produced from wild type and *+rps9m-1* plants. But in *+rps9m-1* siliques,

TABLE 2 | Percentage of aborted endosperm and embryo in *rps9m* mutant.

Female × male	Endosperm (%)				Embryo (%)			Unfertilized (%)	n
	1nuc	2nuc	4nuc	>4nuc	Zygote	2 cells	>2 cells		
+/ <i>rps9m-1</i> × +/ <i>rps9m-1</i>	7.4	19.5	14.6	49.7	21.9	19.6	49.7	8.8	437
+/ <i>rps9m-2</i> × +/ <i>rps9m-2</i>	2.7	17.2	21.4	51.3	14.5	26.8	51.3	7.4	452
+/ <i>rps9m-1</i> × WT	4.8	21.7	18.4	48.6	21.2	23.7	48.6	6.5	463
+/ <i>rps9m-2</i> × WT	3.1	16.8	24.5	50.7	15.3	29.1	50.7	4.9	446
WT × +/ <i>rps9m-1</i>	0.0	0.0	1.6	92.7	1.6	0.0	92.7	5.7	375
WT × +/ <i>rps9m-2</i>	0.0	0.0	1.9	92.9	0.0	1.9	92.9	5.2	381
WT × WT	0.0	0.0	0.0	94.6	0.0	0.0	94.6	5.4	249

+, *RPS9M* allele; nuc, nucleus.

nearly half of seeds were aborted at the same stage as we observed in +/*rps9m-1* self-pollination siliques (Figures 7E–H and Table 2). On the other hands, when +/*rps9m-1* pollens were used to pollinate wild type stigmas, embryo and endosperm developed normally (Figures 7I,J and Table 2). These results suggest that defects in female gametophyte lead to seed abortion in +/*rps9m-1* plants. When we pollinated WT and +/*rps9m-1* pistils with *pRPS9M-GUS* pollens. The paternally derived *pRPS9M-GUS* was expressed in the zygote and endosperms soon after fertilization in both wild type and *rps9m-1* ovules (Figures 7K,L), indicating the expression of the *RPS9M* allele in +/*rps9m-1* ovule is not silenced epigenetically.

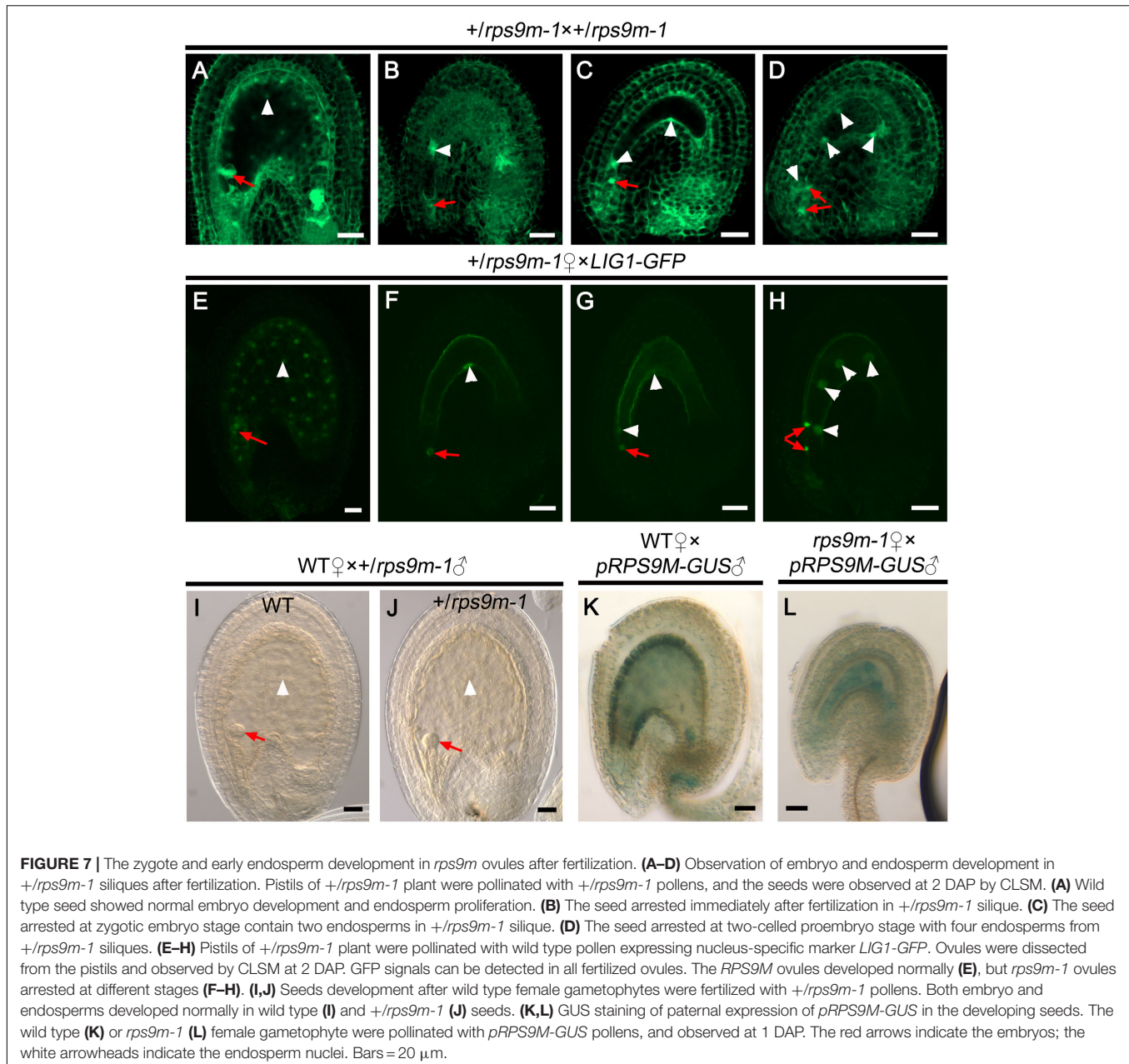
DISCUSSION

In plants and animals, mutants of ribosomal protein genes display various specific developmental defects (Byrne, 2009; Gilbert, 2011; Horiguchi et al., 2011, 2012; Terzian and Box, 2013). Phenotypic diversity of ribosomal protein mutants may result from different patterns of gene expression or extraribosomal function of some ribosomal proteins (Ferreyra et al., 2010). Mitochondrion as a semi-autonomous organelle also contains ribosome to translate proteins encoded by mitochondrial genome. In *Arabidopsis*, several mutants in mitochondrial ribosomal protein genes have been reported (Portereiko et al., 2006b; Zhang et al., 2015). However, most of the mitochondrial ribosomal protein genes are still not functionally characterized. Here, we identified a mitochondrial ribosomal protein *RPS9M* that plays an important role in polar nuclei fusion, central cell development and endosperm proliferation. *RPS9M* physically interacts with ANK6, an ankyrin-repeat protein in mitochondria previously reported to be required for female gametophyte development and fertilization (Yu et al., 2010), suggesting that *RPS9M* may work together with ANK6 in controlling female gametophyte development.

Genetic characterization of heterozygous +/*rps9m* mutant plants indicates that *rps9m* mutation affects female gametophyte development. At the last development stage of *rps9m* female gametophyte, the two polar nuclei fail to fuse into one and the central cell vacuole is smaller than that in wild type (Figure 5). Lacking *RPS9M* probably leads to general dysfunction of mitochondria. Besides *RPS9M*, mutations in several other

mitochondrial ribosomal proteins also caused gametophytic or seed development defects. These include *HUELLENLOS* (Skinner et al., 2001), *RPS11* (*NFD3*) (Portereiko et al., 2006b), *RPL21M* (*NFD1*) (Portereiko et al., 2006b) and *RPL18* (*HEART STOPPER*, *HES*) (Zhang et al., 2015). *HUELLENLOS* encode a mitochondrial ribosomal protein L14 that is homologous to eubacterial ribosome protein L14 and containing high similarity to cytosolic L23 and chloroplast ribosome L14 protein in *Arabidopsis*; mutation of *HUELLENLOS* lead to integuments reduced or absent, and ovule primordia collapse. Disruption of *RPS11* and *RPL21M* both cause polar nuclei fusion defection; moreover, *rpl21m* female gametophyte have a defect in fusion of sperm nuclei in both the egg cell and the central cell during fertilization. *HEART STOPPER* (*HES*) encode a mitochondrial ribosomal L18 protein, *hes* mutant showed no defections during gametophyte development but seed development was affected, embryos were arrested at the late globular stage and endosperms uncellularized in homozygous *hes* mutant seeds. Silencing of the nuclear *RPS10* gene disturbs the ratio between the small and large subunits of mitochondrial ribosome and changes the efficiency of translation of mRNAs for OXPHOS and ribosomal proteins (Kwasniak et al., 2013). Considering that *RPS9M* is a component of the mitoribosome, disruption of *RPS9M* may impact the integrity or stability of mitoribosome and thereby reducing the translation efficiency of some mitochondrial genes involved in mitochondrial function such as ATP synthesis, and ultimately affecting gametophyte development.

Although *RPS9M* is expressed in female gametophyte tissues throughout the developmental stages (Figure 3), disruption of *RPS9M* only impacts central cell development. No obvious morphological defects are observed in other cells such as egg cell and synergid cells of the *rps9m* female gametophyte (Figure 5). One plausible explanation for this phenomenon is that mutation of *RPS9M* impacts energy supply from mitochondria. The early development of *rps9m* female gametophyte is normal as the wild type mitochondria from the diploid MMC provide sufficient nutrients (ATPs, carbon skeletons) to the mutant female gametophyte. However, along with the further development of the *rps9m* female gametophyte, the new-born mitochondria have defect in energy supply. Inside the wild type embryo sac, the distribution of mitochondria is not uniform but largely concentrated in the central cell, whereas the egg cell and synergid cells contain only a few of mitochondria (Martin et al., 2014),



suggesting that the central cell may need more energy for its development. The mutant central cell suffers more severely than other cells in the *rps9m* female gametophyte possibly due to defects in mitochondria failing to produce enough energy for polar nuclei fusion and central cell development.

Our data show that *rps9m* female gametophytes can be successfully fertilized (Figure 6), but the endosperm proliferation and embryo initiation were arrested after fertilization even when *rps9m* ovules were fertilized with wild type pollens (Figure 7), indicating that central cell development is critical for early endosperm development and embryo initiation. Recent studies provide evidence for the maternal control over embryogenesis and endosperm development in plant (Xin et al., 2012; Baroux

and Grossniklaus, 2015). During central cell maturation, high levels of metabolic activities, large number of mitochondria, and enriched starch and lipid reserves are detected (Liu et al., 2010), suggesting that the central cell is primed for the immediate endosperm proliferation upon sperm cell delivery. But in *rps9m* ovules, the central cell maturation processes are defective and these may directly affect this “priming” process for endosperm development. Our data showed that the paternal-derived *de novo* *RPS9M* transcripts were detected in the zygote and endosperm cells immediately after fertilization in both *rps9m* ovules and wild type ovules, indicating that embryo initiation and endosperm development are independent of paternal source of *RPS9M* protein. Thus, the seed development defect in *rps9m*

mutant may suffer from early defects in *rps9m* female gametes, which cannot be compensated by later supply of the RPS9M protein(s).

RPS9M physically interacts with ANK6 both *in vitro* and *in vivo* (Figures 1, 2B), suggesting that they are members of a protein complex in mitochondria during female gametophyte development. As an adaptor protein, ANK6 is presumed to mediate protein–protein interactions through its ANK repeat motifs, suggesting that more mitoribosomal proteins or other proteins may be recruited by ANK6 to work together with RPS9M during gametophyte developmental processes. In our previous work, an RNA polymerase transcription initiation factor, SIG5, was proven to physically interact with ANK6 in mitochondria, and the *sig5* ovules also show female gametophyte defections as *ank6*, indicating they are essential proteins in female gametophyte development (Yu et al., 2010). In eubacteria, the transcription of mRNAs and translation of protein processes are coupled, and some proteins and complexes including RNA polymerase subunits and cofactors may be involved in both transcription and protein synthesis (Kaczanowska and Ryden-Aulin, 2007). Future work will test the hypothesis that coupled transcription/translation processes in plant mitochondria may be operated by a complex, including SIG5 and RPS9M working together, to control the transcription and translation of some mitochondrial gene(s) during gametophyte development and fertilization.

ACCESSION NUMBERS

The accession numbers for the genes used for this article are as follows: *At3g49080* (*AtRPS9M*), *At5g61230* (*AtANK6*), and *At5g24120* (*AtSIG5*).

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AUTHOR CONTRIBUTIONS

CL, FY, LC, SL, and DL designed the research. CL, FY, LT, XyH, HT, ZX, and XhH performed the research. CL, FY, LT, DL, SL, and LC analyzed data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2017.02171/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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