

## MAA3 (MAGATAMA3) Helicase Gene is Required for Female Gametophyte Development and Pollen Tube Guidance in *Arabidopsis thaliana*

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**The female gametophyte plays a central role in the sexual reproduction of angiosperms. We previously isolated the *maa3* (*magatama3*) mutant of *Arabidopsis thaliana*, defective in development of the female gametophyte, micropylar pollen tube guidance, and preventing the attraction of multiple pollen tubes. We here observed that the nucleolus of polar nuclei is small, and that the fusion of polar nuclei often did not occur at the time of pollination. The *MAA3* gene encodes a homolog of yeast *Sen1* helicase, required for RNA metabolism. It is suggested that *MAA3* may regulate RNA molecules responsible for nucleolar organization and pollen tube guidance.**

**Keywords:** *Arabidopsis thaliana* • Female gametophyte • *magatama3* (*maa3*) • Nucleolus • Pollen tube guidance • *Sen1* RNA helicase.

**Abbreviations:** DIC, differential interference contrast; FG, female gametophyte; RT-PCR, reverse transcription-PCR; snoRNA, small nucleolar RNA; TAIL PCR, thermal asymmetric interlaced PCR.

**Note:** The nucleotide sequence of *MAA3* cDNA has been submitted to GenBank with the accession number EU915246.

Angiosperms alternate between a diploid sporophytic generation and a haploid gametophytic generation. Female gametophytes (FGs) generate female gametes, attract male

pollen tubes and then function as the location for double fertilization in the sexual reproduction of angiosperms. The development and diversity of FGs had been studied in detail by the middle of the 20th century (Maheshwari 1950). However, few studies focused on the genetics and biochemistry of the FG, which was, therefore, called the 'forgotten generation' in 1979 by J. Heslop-Harrison (Brukhin et al. 2005).

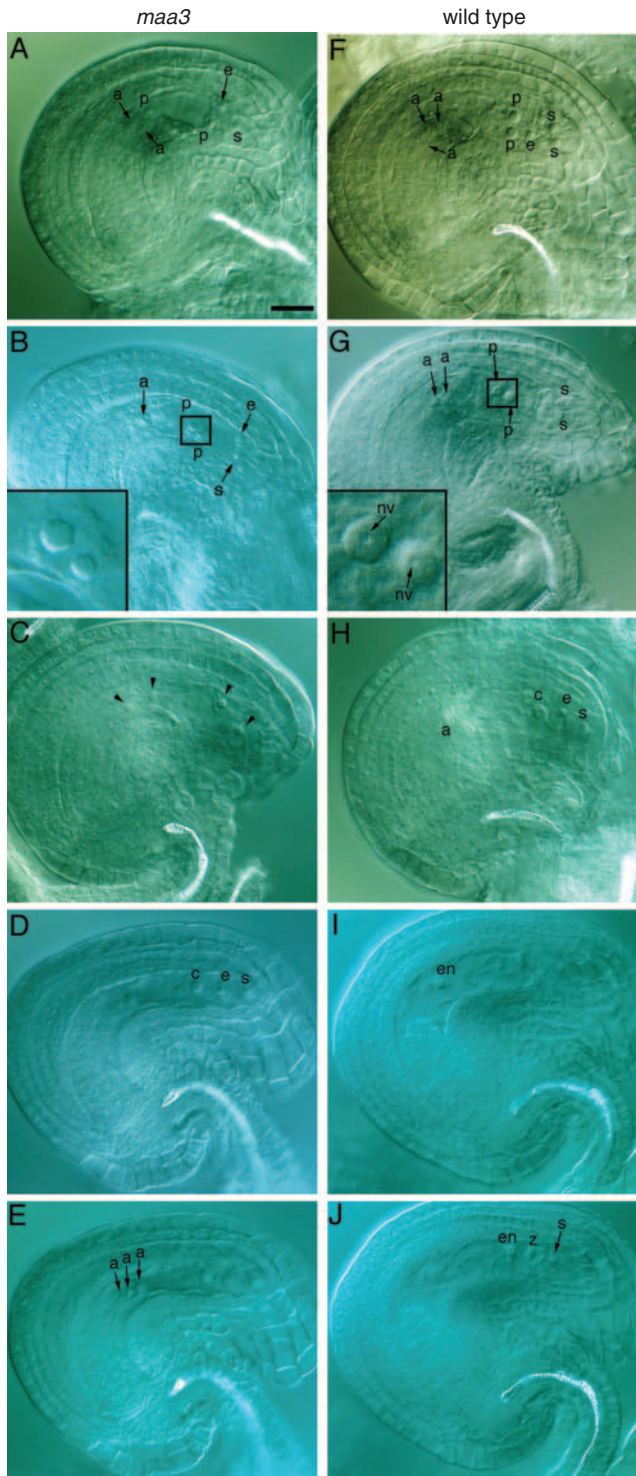
Molecular genetic analysis of *Arabidopsis thaliana* is now shedding light on the genetic function of the FG. A number of FG mutants have been isolated and analyzed (reviewed by Drews and Yadegari 2002, Brukhin et al. 2005, Kagi and Gross-Hardt 2007). We developed a method to isolate FG mutants using the production of half the normal number of seeds as a criterion (Shimizu and Okada 2000), which is typical for FG mutants (Moore et al. 1997). Using this method, we reported *maa1* (*magatama1*) and *maa3* mutants, in which FG development was delayed, and we focused on the defect in pollen tube guidance. Mutant FGs did attract pollen tubes from transmitting tissue onto the funiculus. However, the pollen tubes could not reach the FG, and they started to wander around the micropyle as if they had lost their way. This indicates that pollen tube guidance by the FG is composed of at least two steps, and the latter step, micropylar guidance, was defective in *maa1* and *maa3* mutants. In addition, the frequency of two pollen tubes on a funiculus is higher in these mutants. This suggests that the FG normally prevents the attraction of multiple pollen tubes, and could contribute to the prevention of polyspermy (Shimizu and Okada 2000).

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**Fig. 1** *maa3* and wild-type female gametophytes in *maa3* heterozygote pistils. (A–E) FGs with the *maa3* phenotype. (F–J) FGs with the wild-type phenotype. (A) *maa3* FG in the early FG5 stage. (B) *maa3* FG in the late FG5 stage. The nucleoli of polar nuclei are magnified four times in the inset. The two nucleoli were small, without internal structure, and their sizes were different. (C) FG in stage FG4. This was in the same pistil as

Here we report further analysis of the *maa3* mutant. *maa3* mutation was almost fully penetrant in FG lethality, and also showed a partial male gametophytic lethality (Shimizu and Okada 2000). Here, we molecularly identified the *MAA3* gene, and analyzed the development of *maa3* FGs.

To describe FG development, we use a staging system of FG development proposed by Christensen et al. (1997), which is based on the number of nuclei in an FG (Supplementary Table S1). Since homozygous *maa3* plants were never found, we observed FGs in heterozygous *maa3* plants, in which half of the FGs should inherit the *maa3* allele and the other half should have the wild-type allele.

It was reported that nuclei of FGs have a single nucleolus, which is much bigger than that of surrounding sporophytic cells, and that the nucleoli of polar nuclei are the largest within an FG (Willemsse and van Went 1984, Mansfield et al. 1990, Christensen et al. 1997). By differential interference contrast (DIC) microscopy, the nucleoli of FGs can be observed as a round structure (Fig. 1). In contrast, the nucleus cannot be observed clearly by DIC, since the density of nucleoplasm and cytoplasm is similar (Christensen et al. 1997, Moore et al. 1997).

In newly opened flowers of *maa3* heterozygotes, half of the FGs appeared as wild type in FG6, FG7 or fertilized stages. The other half of the FGs showed the following abnormalities (Fig. 1B). First, the fusion of polar nuclei did not occur, which corresponds to FG5 in terms of the numbers of nuclei (Shimizu and Okada 2000). This suggests that the development of *maa3* FG was delayed or arrested. Secondly, the nucleoli were small. This was conspicuous in the nucleoli of polar nuclei, both before and after nuclear fusion. In the wild type, nucleoli of polar nuclei are much bigger than other nucleoli of the FG, and have round internal structures (Fig. 1G). The internal structure of nucleoli was termed the nucleolar vacuole, but its function is not clear (Newcomb 1973;

wild-type late FG5 (G) and wild-type FG6 (H). (D, E) *maa3* FG in the FG6 stage in two microscopic foci. (F) Wild-type late FG5. (G) Wild-type late FG5. Another synergid and an egg cell are out of focus. The nucleoli of polar nuclei were magnified four times in the inset to show the internal, round structure. (H) Wild-type FG6. (I, J) Two-nucleate endosperm stage of the wild type in two microscopic foci. (A) and (F) were in pistil 3 in Supplementary Table S1, (B) in pistil 4, (C), (G) and (H) in pistil 2, and (D), (E), (I) and (J) in pistil 5. Pistils 2 and 3 were fixed and observed before anthesis. Pistils 4 and 5 were after anthesis and autopollination. Scale bar = 20 μm. Arrowhead, nucleolus of the four-nucleate stage; a, nucleolus of the antipodal cell; c, nucleolus of fused polar nuclei in the central cell; e, nucleolus of the egg cell; en, nucleolus of the endosperm; nv, nucleolar vacuole; p, nucleolus of polar nuclei; s, nucleoli of the synergid cell; z, nucleolus of the zygote.

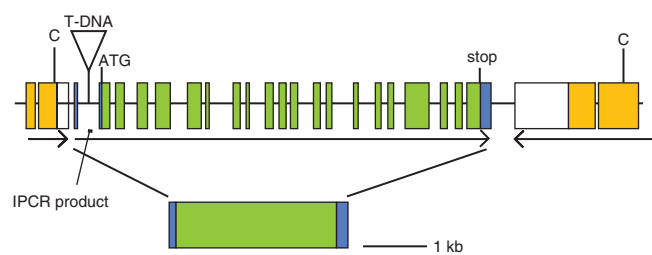
reviewed by Willemse and van Went 1984). In contrast, the nucleoli of polar nuclei of *maa3* FG were small, and no internal structures were observed (Fig. 1A, B). We also noted that the size of the two polar nuclei was often different in *maa3* (Fig. 1B).

To examine whether the development of FGs was delayed, we examined all FGs in seven pistils of *maa3* heterozygotes (Supplementary Table S1). In wild-type pistils, Christensen et al. (1997) reported that the development of FGs is well synchronized. In pistils of *maa3* heterozygotes, we found that the development of *maa3* FGs was delayed, and that synchronization of FG development was disturbed (Supplementary Table S1). Moore et al. (1997) reported that synchronization after fertilization is low in wild-type pistils, since the arrival times of pollen tubes at FGs are not synchronized. To determine the developmental stages of older pistils more clearly, we also observed two pistils that were emasculated, hand-pollinated with wild-type pollen and then harvested 12 h later (as described by Shimizu and Okada 2000). About half of the FGs completed fertilization and were at the zygote or two-nucleate endosperm stage. In contrast, about half of the FGs showed the *maa3* phenotype with small nucleoli in late FG5 or FG6 stages. These results indicate that the development of *maa3* FGs was slow, and the fusion of polar nuclei did not occur in many *maa3* FGs. It is suggested that some FGs could continue development even after the time of pollination, and could accomplish fusion of polar nuclei and fertilization, since reciprocal crossing showed that *maa3* mutations could be transmitted through the female at a low frequency (3%; Shimizu and Okada 2000). It is also suggested that *maa3* homozygosity is lethal in embryogenesis or the early stage of seedling development, since homozygous plants were never recovered.

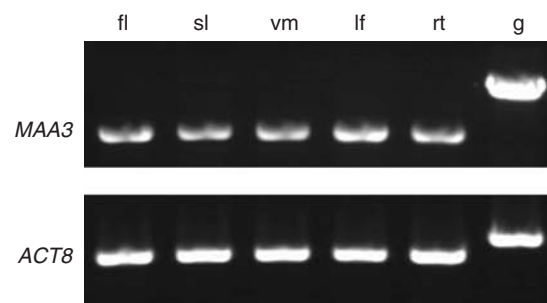
The *maa3* mutant was isolated from T-DNA insertional lines. Inverse PCR, DNA gel blotting, genetic mapping and complementation experiments strongly suggested that disruption of the At4g15570 gene resulted in the *maa3* phenotype (Supplementary Text 1).

To study the genomic structure of the MAA3 gene, the cDNA sequence of MAA3 including the full-length coding region was determined. The longest cDNA recovered from the shoot apex tissue was 2,706 bp long, and the exon–intron structure is consistent with the latest release of TAIR (www.arabidopsis.org, GenBank NC\_003075). MAA3 spans >6 kb, and is composed of 21 exons. The ATG start codon is located in the second exon, and the first exon does not contain any coding sequence. The T-DNA was inserted in the first intron (Fig. 2), and presumably disturbed the expression of MAA3.

The MAA3 gene encodes a protein with 818 amino acid residues. The protein domain identification tool, SMART (Letunic et al. 2006), identified a DEAD-like helicase superfamily domain (DEXDc domain) at position 254–550.



**Fig. 2** Schematic drawing of the genomic structure of the MAA3 locus. The upper line shows position 60,001–70,000 bp of the ATCA4 contig, and the cDNA structure is shown below. Green and blue boxes indicate coding and non-coding exon regions of the MAA3 gene, respectively. An arrow indicates the direction of transcription. Orange and white boxes indicate the neighboring genes (coding regions in orange) predicted by GENSCAN software. The genomic fragment between two points labeled C was used for complementation. The position of the T-DNA insertion and the inverse PCR product is shown.



**Fig. 3** Expression analysis of the MAA3 gene. RT-PCR of the MAA3 gene with the ACT8 gene as control. Fl, flower; sl, silique; vm, shoot apex including vegetative meristem; lf, leaf; rt, root; g, genomic DNA.

Protein BLAST searches identified a number of homologs with a DEAD-like helicase domain. Among them, Sen1 proteins showed the highest homology throughout the entire length of MAA3 (28% identity and 48% conserved amino acids with GenBank accession No. AAB63976). Three other genes encoding Sen1 homologs (At4g30100, At1g16800 and At2g19120) were identified in the genome of *A. thaliana*.

To assay the expression pattern of MAA3, reverse transcription–PCR (RT–PCR) was conducted. The MAA3 gene was expressed at a similar level in flower, silique, shoot apex, leaf and root tissues (Fig. 3).

We report here the observation of FG development of *maa3* mutants. The growth of the *maa3* FGs was delayed, and the nucleoli of polar nuclei were small. As we reported before, the pollen tube guidance was defective (Shimizu and

Okada 2000). We identified the *MAA3* gene as encoding a homolog of yeast *Sen1* helicase protein.

Recent reports have suggested that RNA metabolism is critical for the development of FGs. Three genes are shown to be necessary for the specification of the egg cell: *LACHESIS* encoding a homolog of yeast splicing factor PRP4, *GAMETOPHYTIC FACTOR1/CLOTHO* encoding a protein with high similarity to Snu114 proteins of yeast and animals, and *ATROPOS* encoding a homolog of SF3a60 responsible for pre-spliceosome formation (Gross-Hardt et al. 2007, Moll et al. 2008).

*Sen1* (*splicing endonuclease1*) is essential in budding yeast *Saccharomyces cerevisiae*, and is necessary for various aspects of RNA metabolism. *sen1-1* was first described as a mutant with reduced *in vitro* endonuclease activity and *in vivo* accumulation of unspliced pre-tRNAs (Winey and Culbertson 1988). *sen1* mutants also show defects in the processing and amount of rRNA, small nuclear and nucleolar RNAs, the localization of nucleolar proteins, pre-mRNA metabolism, transcriptional termination, nuclear fusion, chromosomal maintenance, transcription-coupled DNA repair and the distribution of RNA polymerase II across the yeast genome (Ursic et al. 1995, Steinmetz et al. 2001, Steinmetz et al. 2006, Kawauchi et al. 2008, and references therein). Mutations in the human *Sen1* ortholog, Senataxin, cause human neurological diseases (Moreira et al. 2004).

*Arabidopsis thaliana* has four homologs of *Sen1*, one of which is *MAA3*. In yeast, it was shown that the C-terminal 1,214 amino acids are essential for growth, whereas the N-terminal 898 amino acids are dispensable (DeMarini et al. 1992). Although *MAA3* is shorter than yeast *Sen1*, the entire length of *MAA3* shows homology with the essential C-terminal region of *Sen1*, suggesting that the protein function is conserved.

A conspicuous phenotype of the *maa3* mutant was the small size of nucleoli of polar nuclei, which was observable both before and after their nuclear fusion. The nucleolus is a subnuclear structure that is not bound by a membrane, in which rRNA is transcribed and modified by small nucleolar RNA (snoRNA), and the ribosomal subunits are assembled. Considering that the yeast *sen1* mutant is defective in the processing of snoRNA and rRNA, and in the localization of nucleolar proteins, it is suggested that misprocessing of RNA resulted in the small size of the nucleoli in *maa3* mutants. In addition, *maa3* FGs often did not accomplish polar nuclear fusion. Consistent with this, an allele of the yeast *sen1* mutant (isolated as *cik3-1*) showed a defect in nuclear fusion (Ursic et al. 1995). Partial male gametophytic lethality (Shimizu and Okada 2000) also suggests a role for *MAA3* helicase in the development of male gametophytes. In studying yeast *sen1* mutants, Kim et al. (1999) stated that it is difficult to present a single hypothesis that accommodates all of the complicated and apparently unrelated phenotypes of *sen1*. Similarly, it may not be feasible to determine which of the *maa3* pleiotropic phenotypes are primary or secondary.

Another conspicuous phenotype of *maa3* is the defect in micropylar pollen tube guidance and in preventing the attraction of multiple pollen tubes (Shimizu and Okada 2000). Here we showed that the *MAA3* gene, encoding a yeast *Sen1* helicase homolog, is necessary for pollen tube guidance by FGs. To connect a helicase gene and pollen tube guidance, two possibilities can be discussed, which are not necessarily mutually exclusive. First, the defect in pollen tube guidance may be a consequence of the delayed growth of *maa3* FGs. Another possibility is that *MAA3* helicase could be involved more directly in the regulation of RNA molecules responsible for pollen tube guidance, e.g. an mRNA encoding a guidance molecule. Recently, the cellular and molecular mechanism of pollen tube guidance has been extensively studied (Higashiyama et al. 2001, Marton et al. 2005, reviewed by Higashiyama and Hamamura 2008). *In vitro* experiments using *Torenia fournieri* demonstrated that synergid cells emit a diffusible signal(s) of pollen tube guidance (Higashiyama et al. 2001). The role of synergid cells is also supported by a study of the *MYB98* gene, which is expressed in synergid cells of *A. thaliana* and is necessary for pollen tube guidance (Kasahara et al. 2005). The central cell is also involved in pollen tube guidance, since the *CENTRAL CELL GUIDANCE (CCG)* gene connected to a central cell-specific promoter rescued the micropylar pollen tube guidance defect caused by the *ccg* mutation (Chen et al. 2007). *MAA3* may regulate RNA molecules responsible for pollen tube guidance in the synergid cells and/or in the central cell. Cell-specific rescue of *MAA3* could reveal which cells are responsible for pollen tube guidance.

## Materials and Methods

The *maa3* mutant was identified as described by Shimizu and Okada (2000) in screens of Wassilewskija (*Ws-2*) plants transformed by T-DNA of the pGV3850:HPT vector, which confers hygromycin resistance. The *A. thaliana* ecotype *Ws-2* was used as the wild type in microscopic observations, and *Col-0* in molecular experiments. Seeds were sown on vermiculite and grown under continuous white fluorescent light at 22°C. To assay hygromycin resistance, a rosette leaf was kept for 3 d on an agar plate (1× Gamborg's B5, 2% sucrose, 0.8% Bactoagar, 50 µg ml<sup>-1</sup> hygromycin B, pH 5.7–5.8). The leaves may stay green or become white, and are judged to be resistant or susceptible, respectively.

For microscopic observations, pistils were fixed in a 9:1 mixture of ethanol and acetic acid overnight, having been kept under a gentle vacuum for the first 30 min. They were then treated with 90 and 70% ethanol for 20 min each, and cleared in Hoyer's solution (7.5 g of gum arabic, 100 g of chloral hydrate, 5 ml of glycerol in 30 ml of water), and observed by DIC (Nomarski) optics after dissection.

Primers and PCRs in the study are described in Supplementary Table S2, and the identification of *MAA3* in

Supplementary Text 1. Thermal asymmetric interlaced (TAIL) PCR and inverse PCR were conducted as described by Oyama et al. (1997) and Ishiguro et al. (2001), respectively. *ACT8* was amplified as a control in RT-PCR (An et al. 1996). We digested genomic DNAs isolated individually from four plants having the *maa3* phenotype with *HindIII* and analyzed the fragments by DNA gel blotting (Southern hybridization) as described by Oyama et al. (1997).

Protein domain prediction was conducted using SMART (Letunic et al. 2006). Gene structure was estimated by GENSCAN (<http://genes.mit.edu/GENSCAN.html>). Homology search was performed using NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and TAIR BLAST (<http://www.arabidopsis.org/Blast/>).

### Supplementary Material

Supplementary Material are available at PCP Online.

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