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.
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 (Willemse 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;
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. 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 including vegetative meristem; leaf, leaf, root; genomic DNA.

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). 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 upper line shows position 60,001–70,000 bp of the ATFC4 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. 2 Schematic drawing of the genomic structure of the MAA3 locus. The upper line shows position 60,001–70,000 bp of the ATFC4 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; If, leaf, rt, root; g, genomic DNA.

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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).
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, GAMETO PHYTIC FACTOR1/CLOTHO encoding a protein with high similarity to Snu114 proteins of yeast and animals, and ATROPOS encoding a homolog of SF3a60 responsible for 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 neurodevelopmental 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 (Urset 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 cgg 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 Wassilewskijia (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, 20 µg ml⁻¹ hygromycin B, 0.8% Bactoagar, 50 µg ml⁻¹ hygromycin B, pH 5.7–5.8). The leaves may stay green or becomes 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 chlroral 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 Plant Cell Physiol. 49(10): 1478–1483 (2008) doi:10.1093/pcp/pcn130 © The Author 2008.
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.

Funding
The Japanese Ministry of Education, Science, Culture, and Sports Grants-in-Aid for Special Research on Priority Areas (Nos. 14036220 and 119043010); the University Research Priority Program of Systems Biology/Functional Genomics of the University of Zurich; the Japan Society for the Promotion of Science (Nos. 14036220 and 119043010); the University Research Program of the University of Tsukuba; the Japan Society for the Promotion of Science for critical discussion.

The authors would like to acknowledge Ueli Grossniklaus and the University of Zurich; the Japan Society for the Promotion of Science (to K.K.S.).

Acknowledgments
The authors would like to acknowledge Ueli Grossniklaus and Rie Shimizu-Inatsugi for critical reading of the manuscript, and Takashi Araki and members of the Okada laboratory for critical discussion.

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(Received July 31, 2008; Accepted August 29, 2008)