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Analysis of *gemini pollen 3* mutant suggests a broad function of AUGMIN in microtubule organization during sexual reproduction in *Arabidopsis*

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Running head: GEM3/AUG6 is essential during sexual reproduction.

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SUMMARY

In flowering plants, male gametes arise via meiosis of diploid pollen mother cells followed by two rounds of mitotic division. Haploid microspores undergo polar nuclear migration and asymmetric division at pollen mitosis I to segregate the male germline, followed by division of the germ cell to generate a pair of sperm cells. We previously reported two *gemini pollen* (*gem*) mutants that produced twin-celled pollen arising from polarity and cytokinesis defects at pollen mitosis I in *Arabidopsis*. Here, we report an independent mutant, *gem3*, with a similar division phenotype and severe genetic transmission defects through pollen. Cytological analyses revealed that *gem3* disrupts cell division during male meiosis, at pollen mitosis I and during female gametophyte development. We show that *gem3* is a hypomorphic allele (*aug6-1*) of *AUGMIN subunit 6*, encoding a conserved component in the augmin complex, which mediates microtubule (MT)-dependent MT nucleation in acentrosomal cells. We show that MT arrays are disturbed in *gem3/aug6-1* during male meiosis and pollen mitosis I using fluorescent MT-markers. Our results demonstrate a broad role for the augmin complex in MT organization during sexual reproduction, and highlight *gem3/aug6-1* mutants as a valuable tool for the investigation of augmin-dependent MT nucleation and dynamics in plant cells.

INTRODUCTION

The development of the haploid gametophytes in flowering plants requires a highly coordinated and genetically regulated program leading to gamete delivery and fusion during double fertilization (McCormick, 2004; Berger and Twell, 2011). The male (pollen) and female (embryo sac) gametophytes are three- and seven-celled structures, which result from two or three rounds of mitoses following meiosis respectively. Gametophyte development involves several key processes, including nuclear migration, asymmetric division and cell fate specification. To achieve a better understanding of these processes requires further elucidation of events at the molecular level. This will require studies, which aim to extend current knowledge of mutants and genes involved in gametophyte development and gametogenesis (Drews and Koltonow, 2011; Ge *et al*., 2010; Twell *et al*., 2011).

A determinative process in male gametogenesis is the asymmetric microspore division (pollen mitosis I), which segregates the vegetative and generative (or germline) cells. In previous work we have characterized mutants which have helped to dissect the mechanisms underlying asymmetric division at pollen mitosis I (Park *et al*., 1998; Twell *et al*., 2002; Oh *et al*., 2005, Oh *et al*., 2011). These can be grouped into classes affecting three consecutive steps including microspore polarization involving nuclear migration towards the future germ cell pole, oriented nuclear division at the germ cell pole and asymmetric cytokinesis (Oh *et al*., 2011). *gemini pollen 1* (*gem1*) mutants are affected in the first of these steps, giving rise to similar daughter cells and failed germ cell differentiation (Park *et al*., 1998). The identification of *gem1* as an allele of MOR1, a member of the microtubule-associated protein (MAP) 215 family (Whittington *et al*., 2001), showed that nuclear migration is microtubule (MT)-dependent in *Arabidopsis* and tobacco (Twell *et al*., 2002; Oh *et al*., 2008). In microspores of *sidecar pollen* (*scp*) mutants, nuclear migration occurs normally, but a proportion of four-celled pollen containing an extra vegetative cell are produced owing to defects at the second step, which delay nuclear division and alter division orientation (Chen *et al*., 1997; Oh *et al*., 2010). The fact that SCP encodes LBD27, a male-specific member of the plant-specific LOB Domain transcription factor family, suggests that a specialized regulatory network operates to coordinate the timing of division and spindle orientation at pollen mitosis I. In *two-in-one* (*tio*) mutants microspore nuclear migration and orientated asymmetric division proceed normally, but fail during cytokinesis, leaving two daughter nuclei in a common cytoplasm (Oh *et al*., 2005). TIO is a plant ortholog of the Ser/Thr FUSED kinase, which is thought to operate in a signaling module through its interaction with phragmoplast associated KINESIN-12A and KINESIN-12B (Lee *et al*., 2007; Oh *et al*., 2012). Collectively, these results highlight the importance of MTs for asymmetric division at pollen mitosis I.

The study of mutants affecting haploid pollen mitosis I presents an alternative strategy to uncover the functions of genes with essential roles in cell division, as these cannot be isolated or studied routinely in the diploid phase due to homozygous lethality. In addition to the aforementioned genes, it is not

surprising that genes required for MT nucleation and organization have been identified based on binucleate mutant pollen phenotypes using reverse genetics. So far, multiple components of the γ-tubulin ring complex (γ-TuRC), the augmin complex and GCP-WD/NEDD1, which together control MT-dependent MT nucleation in acentrosomal plants, have been shown to be essential for cell division based on defects in pollen mitosis I (Ho *et al*., 2011; Hotta *et al*., 2012; Pastuglia *et al*., 2006; Zeng *et al*., 2009). Conversely, such mutations are usually homozygous lethal, which hampers the study of their functions in meiotic and somatic divisions.

In this study, we used a forward genetics approach to isolate an independent pollen mutant locus, *gem3*, which exhibits abnormalities typical of *gem1* and *gem2* (Park *et al*., 1998; Park *et al*., 2004). Detailed cytological analyses and the isolation of homozygous *gem3* mutants uncovered a range of cell division defects including those affecting both male and female development. We demonstrate that reduced expression of *GEM3*/*AUG6* causes gross abnormalities in meiotic and mitotic MT organization during pollen development in *Arabidopsis*. Our data strengthen the evidence for the broad function of the augmin complex during sexual reproduction in *Arabidopsis*.

RESULTS

Isolation and genetic analysis of *gemini pollen3*

Pollen grains from an EMS-mutagenized seed pool harboring multiple visible genetic markers in the Ler-0 background (Grini *et al*., 1999) were morphologically screened and a mutant with aberrant pollen grains and cell division defects was identified. Mature pollen harvested from open flowers of backcrossed mutants revealed a range of aberrant phenotypes, including twin-celled pollen, the signature phenotypes of *gem1 and gem2* mutants (Park *et al*., 1998; 2004), therefore, we named this mutant *gem3*.

The phenotypes of the majority (95.5 %) of 1,515 self-progeny derived from *gem3* heterozygous plants, was normal during vegetative and reproductive development. These segregated 46.7 % wild-type and 48.8 % heterozygous mutant, which were only distinguishable based on their pollen phenotypes (Figure 1; Figure S1). Conversely, the remaining 4.5 % of progeny were distinguishable at seedling stage and showed abnormal phenotypes that were more pronounced later in development. These plants were homozygous for the *gem3* allele and exhibited narrow, twisted and curled leaves, larger flowers with altered petal morphology and very short siliques compared to wild-type plants (Figure S1b-e).

We examined the transmission efficiency of the *gem3* allele through male and female gametophytes by carrying out reciprocal test-crosses to wild-type and scoring the numbers of F1 progenies based on their pollen phenotypes (Table S1). Transmission efficiency reflects how successfully the mutant gametes transmit their genomes to progeny compared to the wild-type gametes. The results showed that 20.8 % of the male and 84.1 % of the female gametes carrying the *gem3* allele were transmitted to the next generation, indicating that *GEM3* has a role in both sexes, with a greater requirement in the male (Table S1). Based on the observed frequencies of $gem3^{+/-}$ plants in F1 individuals from reciprocal test-crosses (Table S1), 45 % of progeny from self-fertilized $gem3^{+/-}$ plants were predicted to be wild-type, 47.1 % $gem3^{+/-}$ and 7.9 % $gem3^{-/-}$, in accordance with the observed 46.7 %, 48.8 % and 4.5%, respectively.

Mature pollen phenotypes of *gem3* mutants

The phenotypes of DAPI-stained pollen from open flowers of wild-type and heterozygous *gem3* plants are shown in Figure 1. Wild-type plants had 99 % (n = 2,833) normal tricellular pollen (Figure 1a) and 1.0 % abnormal pollen (Figure 1g), while $gem3^{+/-}$ plants had 26.8 % (n = 1,146) abnormal pollen (Figure 1b–l), comprising 18.8 % dead pollen (Figure 1g) and 8 %, which showed a mixture of phenotypes (Figure 1b–f, 1h–l). These included binucleate (Figure 1b–f) and uninucleate pollen grains (Figure 1h–l), with or without a dividing wall, which was observed to be complete (Figure 1c–d, 1i–j)

or incomplete (Figure 1e, 1k). Furthermore, the frequency of each type of mutant pollen class varied between mutant plants, although the overall frequency remained similar.

Homozygous *gem3* plants showed a much more severe abnormal pollen phenotype compared to heterozygous plants (Figure 2). Tricellular pollen grains were observed at a frequency of 34.4 % (n = 1,321) and their sizes were highly variable. In fact, only 1.2 % were similar in size to normal tricellular pollen, whereas the remaining 33.2 % were larger with brighter DAPI-stained nuclei indicating higher DNA content (Figure 2a–c). As observed in *gem3* heterozygotes, dead pollen was the most frequent aberrant class (Figure 2j–l, 52.7 %), together with binucleate (Figure 2d–f) and uninucleate (Figure 2g–i) pollen grains, with or without a dividing wall (13.2 %). These common types of aberrant pollen were also found to have variable sizes (Figure 2d–l).

Defects appear at pollen mitosis I and meiosis in heterozygous and homozygous *gem3* plants, respectively

In order to determine when the first abnormalities appear, we examined DAPI-stained spores dissected from staged anthers of wild-type and *gem3* mutant plants. Typical progression of pollen development was observed in wild-type plants (Figure 3). Meiosis results in a tetrad of microspores enclosed within a thick callose wall (Figure 3a). Free microspores released from the tetrad (Figure 3b) gradually increase in size and develop exine (Figure 3c). Microspores become polarized with the nucleus displaced to a future germ cell pole (Figure 3d) and a curved cell plate separates two unequal daughter cells (Figure 3e). The smaller generative cell, which has a nucleus stained intensely with DAPI, undergoes pollen mitosis II to generate two sperm cells (Figure 3f–h). In heterozygous *gem3* plants, pollen development from the tetrad to the polarized microspore stages did not differ from that in the wild-type. Aberrant phenotypes first became obvious at early bicellular stage (Figure 3m–p, 3r), with cell plates that were straighter (Figure 3m) or divisions that were symmetric (Figure 3n), in contrast to those in normal pollen (Figure 3e), or with incomplete cell plates (Figure 3o–p). On the other hand, in homozygous *gem3* plants, aberrant phenotypes were evident at the tetrad stage (Figure 3i–l, 3q). Both wild-type (n = 228) and *gem3* heterozygotes (n = 322) had 100 % normal tetrads

composed of four equally sized microspores (Figure 3a, 3q). However in *gem3* homozygotes (n = 384), the majority (68.6%) were severely aberrant with highly variable numbers of spores from monad to polyads with over 10 nuclei/micronuclei (Figure 3i–l, 3q). Even relatively normal-looking tetrads in *gem3* homozygotes gave rise to mutant microspores undergoing defective pollen mitosis I, which accounts for the high proportion of abnormal pollen grains observed at the mature pollen stage (98.8%).

To further investigate male meiotic defects in *gem3* homozygous plants, we examined chromosomal behavior in dividing pollen mother cells in prefixed inflorescences. At late prophase I, condensation of chromosomes to form bivalents seemed similar in both wild-type and *gem3* mutants (Figure 4a–b). At metaphase I, however, the bivalents were present at different locations. In wild-type and heterozygous *gem3* plants, chromosomes always aligned in the center of dividing meiocytes, whereas those in *gem3* homozygous plants were frequently found at the periphery (Figure 4c–d). This suggests that spindle position and/or its bipolar structure, which captures five bivalents in the center, is disturbed in *gem3*. At telophase I, normally two clusters of five chromosomes translocate to the opposite poles along the long axis of the cell (Figure 4e), whereas in *gem3*$^{-/-}$ plants, chromosomes are either scattered as multiple clusters or separated at the cell periphery (Figure 4f). At the dyad stage of prophase II, wild-type and *gem3*$^{+/-}$ plants produce two equal nuclei that are separated by an organelle band in the center (Figure 4g), whereas in *gem3*$^{-/-}$ plants, lagging chromosome(s) or highly unequal nuclei are observed (Figure 4h). At later stages in wild-type and *gem3*$^{+/-}$ plants, two sets of chromosomes aligned at metaphase II plates separate into four groups of five chromatids, yielding four nuclei of equal size (Figure 4i, 4k, 4m). However, in *gem3*$^{-/-}$ plants, chromatid separation occurred on one side of the cell or resulted in lagging chromatids, giving rise to various numbers of chromatid groups or nuclei/micronuclei (Figure 4j, 4l, 4n). Together, these results strongly suggest that there are gross abnormalities in position, structure, and function of the meiotic spindles in *gem3*$^{-/-}$ plants.

Cell division defects during female gametophytic development in *gem3* mutants

To investigate reduced fertility in *gem3* homozygotes we opened mature green siliques in wild-type and *gem3* mutant plants (Figure 5). Most of the seeds produced by wild-type plants were normal (95.4 %) with limited evidence of aborted seeds or ovules (Figure 5a, 5d, 5g), and *gem3*$^{+/-}$ siliques contained only slightly lower levels of normal seeds (85.0 %), with an equivalent increase in aborted ovules (Figure 5b, 5e, 5g). Conversely, in *gem3* homozygous plants, almost all ovules (98.2%) were aborted, leading to a residual level of seed set (Figure 5c, 5f, 5g). The seeds produced by *gem3* homozygous plants were irregular or larger than normal and were often shriveled at the mature seed stage (red asterisks, Figure 5f).

This raised the question of whether the high frequency of aborted ovules observed in *gem3* homozygotes arose from fertilization failure due to *gem3* mutant pollen or from abnormal female gametogenesis. Examination of cleared ovules from mature pistils of unopened buds revealed that *gem3*$^{-/-}$ ovules contain very small embryo sacs compared to those in wild-type ovules. Further observations using confocal laser scanning microscopy were consistent with these results (Figure 5h–m). Wild-type ovules contained fully expanded embryo sacs, which reached the 8-nucleate stage (female gametophytic stage 5, FG5) after completing three rounds of mitosis (Figure 5h), or the 4-cell stage (FG7) following the subsequent nuclear fusion of two polar nuclei and the degeneration of three antipodal cells (Figure 5i–j). Conversely, *gem3* mutant ovules contained much smaller embryo sacs arrested at either the FG1 stage with a single surviving functional megaspore overlying three degenerating megaspores (Figure 5k–l), or at the FG2-3 stages with two nuclei after the first round of mitosis (Figure 5m). This result indicated an important role for *GEM3* function in mitotic progression during embryo sac development.

Map-based cloning of *GEM3*

We used map-based cloning to identify the *GEM3* gene (Figure S2). We first generated an F2 population from a cross between *gem3*$^{+/-}$ (L*er*) and wild-type Columbia (Col-0) plants. To obtain recombinants in the vicinity of the *gem3* locus, we analyzed 873 F2 plants that showed the wild-type

pollen phenotype using existing and additionally designed markers (Table S2). As a result, 20 recombinants delimited the *gem3* locus to a ~233-kb region between the markers, MPO12b and M1324, and further down to a ~71-kb region on the BAC clone MNF13, which consists of 17 predicted genes (Figure S2). We sequenced several candidate genes and found some changes within the At5g40730, At5g40740, At5g40750, At5g40770, and At5g40840 sequences in *gem3* mutant plants (Table S3). We also initiated complementation analysis by transforming heterozygous *gem3* plants using wild-type sequences for some of the candidate genes within the identified region. The At5g40740 gene successfully rescued *gem3* pollen phenotypes, whereas two other candidate genes did not (Table S4). In *gem3* mutant plants, At5g40740, which encodes AUGMIN subunit 6 (AUG6), contained two single nucleotide polymorphisms (SNPs). The first SNP resides in the splice acceptor site (AG to AA) between the intron 5 and the exon 6, which induces two alternative splice acceptor sites (Figure 6a and 6c). The second SNP, G2107A, is located in the exon 12 and results in a single amino acid change, D703N (Figure 6a and Figure S3). Both SNPs are G-A substitutions typical for guanine alkylation mutations resulting from EMS mutagenesis (Grini *et al*., 1999). Semi-quantitative RT-PCR analysis suggested that both wild-type and mutant transcripts are expressed at similar levels (Figure 6b). In addition, random sequencing of 10 full-length cDNA clones from *gem3* homozygotes gave four and six clones of the respective mutant transcripts, showing that both mutant transcripts are expressed. The two mutant transcripts are predicted to be translated into a 221-amino acid polypeptide owing to a premature stop codon immediately after the first SNP, or a 736-amino acid polypeptide with an in-frame five amino acid deletion and an additional single amino acid change (Figure 6c, Figure S3). Moreover, transgenic plants containing the gene construct proAUG6-AUG6-dHA (Figure 6d) successfully complemented homozygous and heterozygous *gem3* pollen phenotypes. This construct also recovered sporophytic phenotypes at the seedling stage (Figure 6e–h) and at later mature plant stages, such as twisted organs and short siliques in *gem3*$^{-/-}$ plants. This result confirms that *gem3* mutant phenotypes arise from the identified mutations in *AUG6*.

In silico data show that *AUG6* is expressed in various tissues and developmental stages (Arabidopsis eFP browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Table S5) and our independent analysis of *AUG6* gene expression is consistent with these data. A promoter fragment extending ~2-kb upstream

of the start codon of *AUG6* directed GUS expression in anthers from young buds to open flowers (Figure 6i), and in young roots and emerging leaves of seedlings (Figure 6j). RT-PCR analysis showed that the *AUG6* gene is expressed throughout pollen development at a similar level (Figure 6k). Expression of a fusion protein from a proAUG6-H2B-mCherry construct showed that the *AUG6* promoter is active during pollen development (Figure 6l) and in young roots in seedlings (Figure 6m). These data support the hypothesis that augmin may function throughout the plant life cycle.

MT arrays are abnormally organized in *gem3* mutant plants

The observation of *gem3* mutant pollen phenotypes by DAPI staining and the identification of *GEM3* as *AUG6* strongly suggested that *gem3* mutants have defects in MT organization and function. To visualize MT arrays together with separating chromosomes/nuclei in dividing cells, we transformed heterozygous *gem3* plants with two *Agrobacterium* strains harboring the reporter constructs proUBQ14-GFP-TUA6 and proUBQ14-H2B-mCherry. We observed MT arrays and sister chromosomes/nascent nuclei at or after pollen mitosis I in wild-type and heterozygous *gem3* plants (Figure 7a–j). In normal microspores at pollen mitosis I, mitotic arrays, the spindle and phragmoplast, are organized near the future germ cell pole (Figure 7a–c), separating two daughter cells of unequal size (Figure 7d–e). However, in *gem3* mutant microspores, elongated spindles were observed towards the center of dividing cells (Figure 7f). Likewise, mutant phragmoplasts formed near the center (Figure 7g) and often failed to expand centrifugally (Figure 7h–j), or were irregularly curved (Figure 7i). In *gem3* mutant pollen, two nascent nuclei were observed to be more equal in size (Figure 7g–j) compared to those in wild-type pollen (Figure 7c–e), showing that division asymmetry was no longer tightly controlled. Further, the phenotypes of *gem3* cells with two interphase nuclei and short phragmoplasts (Figure 7g–j) are reminiscent of *drosophila* S2 cell cultures, which take longer to form metaphase and telophase spindles, when the augmin complex component Dgt6 is down-regulated by RNAi (Bucciarelli *et al*., 2009).

We further examined dividing pollen mother cells of wild-type and *gem3*$^{-/-}$ plants (Figure 7k-y). At late prophase I, meiocytes looked similar in wild-type and *gem3*$^{-/-}$ plants. Both had condensed chromosomes and some MTs appeared as intense dots around the nucleus and in the cytoplasm (Figure 7k, 7p, 7u). However, while the wild-type spindles maintain chromosomes tightly grouped at the metaphase plate (Figure 7l), mutant spindles leave chromosomes more loosely positioned at the center (Figure 7q) or displaced to one side (Figure 7v). Similarly, at or immediately after telophase I, when wild-type plants have relatively dense MTs positioned between two equal chromosomes/nuclei (Figure 7m), mutants show unequal chromosomes/nuclei with less bright and more randomly distributed MTs (Figure 7r) or elongated, curved spindles (Figure 7w). Similar spindle abnormalities were also observed during meiosis II (Figure 7n–o, 7s–t, 7x–y). Compared to the two normal spindles, which are distantly separated with tightly attached chromatids (Figure 7n–o), mutant spindles are disturbed. Examples include those with loosely attached chromatids (Figure 7s–t, 7y) and those that are close to each other and displaced (Figure 7x). In telophase II, mutant spindles often appear more curved than normal spindles (Figure 7t, 7y). Some abnormalities observed in MTs of *gem3*$^{-/-}$ meiocytes, including their lower density and curved and/or elongated spindles, are consistent with the defects reported in other *aug* mutant cells (Goshima *et al*., 2007 and 2008; Ho *et al*., 2011; Hotta *et al*., 2012). Although there may be additional aspects to be investigated further in the *gem3* allele, our data shows that AUG6 deficiency causes disorganization of MT structures which are essential for successful male gamete production in *Arabidopsis*.

DISCUSSION

In this study, we isolated heterozygous *gem3* mutants that produce abnormal binucleate or bicellular pollen grains arising from defects in cell division at pollen mitosis I. We showed that homozygous *gem3* progeny have severe defects in male meiosis and female gametophytic mitosis. Detailed cytological analyses and map-based cloning of *GEM3* revealed that phenotypes arise due to disruption

of AUG6 functions in the organization of MT structures during reproduction, most likely via augmin complex-mediated MT nucleation that enables the generation of new MTs from existing ones.

gem3, a weak mutant allele of *AUG6*

A typical phenotype reported in the *Arabidopsis aug* mutants, *aug1*, *aug3*, *aug4*, and *aug5* (Ho *et al*., 2011; Hotta *et al*., 2012), is the generation of binucleate pollen, which is also found in *gem3*/*aug6-1* plants. Likewise, depletion of any of the HAUS, human augmin, subunits caused indistinguishable phenotypes in HeLa cells, showing their co-dependence for this function (Lawo *et al*., 2009). However, none of the reported *Arabidopsis aug* mutants were found to produce the signature 'twin-cell' pollen phenotypes with a persistent dividing wall seen in *gem3*/*aug6-1* (Figure 1). In addition, only the *gem3*/*aug6-1* mutants produced homozygotes, which display male meiotic defects (Figure 3 and Figure 4). This implied that *gem3* might represent a weak mutant allele in an *AUG* subunit gene. One of the two SNPs found in the *gem3* allele causes alternative splicing, which in turn produces two mutant proteins, containing, 221 aa (augmt1) and 736 aa (augmt2) (Figure 6 and Figure S3). The longer mutant protein lacks five in-frame amino acids compared to the wild-type 741-aa AUG6. We asked whether this longer aug6 mutant protein would be functional. When we transformed $gem3^{+/-}$ with the proAUG6-aug6mt2-dHA construct encoding the 736-aa mutant polypeptide, 17 $gem3^{+/-}$ and 8 $gem3^{-/-}$ plants containing the construct showed a significant increase in the frequency of wild-type pollen grains, compared to those without the construct (Table S6). This shows that *gem3* is a weak *aug6* allele, which can still provide sufficient AUG6 function to allow some augmin complexes to be functional, which enables the survival of homozygous mutants.

Mutant *gem3* microspores exhibit normal nuclear migration prior to pollen mitosis I, with wild-type and $gem3^{+/-}$ plants showing 98.6 % (n = 433) and 97.9 % (n = 332) polarized microspores, respectively. In addition, two nuclei were almost always detected in dividing microspores of heterozygous *gem3* plants after pollen mitosis I (99.5%, n = 722). This shows that nuclear migration and division at pollen mitosis I are not affected in *gem3*. Therefore, more centrally located cell plates (Figure 3m–n) do not result from nuclear division in unpolarised microspores, but are more likely

attributable to abnormal spindle elongation. Unlike *gem3* mutants, *gem1* microspores display incomplete polarization, suggesting that the MAP215 family protein, GEM1/MOR1, has a role in MT organization and function during nuclear migration in *Arabidopsis* (Park *et al*., 1998; Twell *et al*., 2002). Similarly, when TMBP200, the tobacco MOR1/GEM1 orthologue, was depleted by RNAi, the typical two-step nuclear migration in tobacco microspores was severely disturbed (Oh *et al*., 2010). Since other *aug* mutants display defects at pollen mitosis I (Ho *et al*., 2011; Hotta *et al*., 2012), it would be interesting to examine whether these could provide further role of augmin for nuclear migration towards the germ cell pole.

Augmin function and the *gem3* mutant

The identification of several *Dgt* genes, which encode members of the eight-subunit augmin complex, has started to reveal their role in a centrosome-independent mechanism to rapidly increase new MTs by binding to preformed MTs and recruiting the γ-TuRC within the mitotic spindles (Goshima *et al*., 2007; 2008). The loss of one subunit causes destabilization of the augmin complex and reduces MT density within the spindle, resulting in perturbed chromosome alignment and segregation (Goshima *et al*., 2008; Lawo *et al*., 2009). The augmin complex is a conserved mediator of centrosome-independent, but γ-TuRC-dependent, MT nucleation in multiple species, including *Drosophila*, *Xenopus*, human, and moss (Nakaoka *et al*., 2012). In addition, its function is not restricted to mitotic spindles but extends to cytokinetic anaphase spindle MTs (Uehara *et al*., 2009; Uehara and Goshima, 2010) and meiotic spindle assembly in male (Savoian and Glover, 2014) and female (Petry *et al*., 2011; Colombié *et al*., 2013) *Drosophila*. Moreover, reconstituted human augmin is a Y-shaped complex that can adopt multiple conformations (Hsia *et al*., 2014), providing structural information that can be used to understand how the augmin complex functions.

In higher plants, the augmin complex was shown to control acentrosomal MT nucleation and organization. Ho *et al*. (2011) first identified *Arabidopsis* AUG3 based on its sequence homology to an animal orthologue, and demonstrated cell division defects that originate from disorganized spindle and phragmoplast MT arrays in dividing *aug3-1* microspores. They further proposed the presence of

the augmin complex in plants by showing the interaction of AtAUG3 with AtAUG1 *in vivo*. Hotta *et al*. (2012) further showed that the augmin complex consists of eight subunits, including two that are plant-specific, and evidence that AUG1, AUG4, AUG5, and AUG7 are essential for proper organization of the spindle and phragmoplast in dividing microspores or root cells. These authors also showed that γ-tubulin is not localized towards the minus end of MTs in root cells of homozygous *aug7-1* plants. These results strongly suggested that the augmin complex recruits γ-TuRC, which then nucleates and organizes MTs for mitotic progression in plants. Furthermore, augmin subunits/complexes have been reported to function beyond mitotic MT organization in *Arabidopsis*. Cao *et al*. (2013) showed typical phenotypes that arose from altered cortical microtubule organization in *aug8* mutant hypocotyl cells and demonstrated that AUG8 is a MT plus-end binding protein that promotes MT reorientation in hypocotyls. More recently, Liu *et al*. (2014) demonstrated that the augmin complex recruits γ- TuRC to cortical MTs and initiates MT-dependent MT nucleation. Thus, augmin functions not only in mitotic MTs in dividing cells, but also in cortical MTs in interphase plant cells.

A limitation is that augmin function has only been demonstrated via mutant phenotypes or AUG-fluorescent protein expression in restricted types of plant cells. Due to the absence of homozygous mutants, the essential functions of AUG1, AUG3, AUG4, and AUG5 for spindle and phragmoplast MT organization could only be visualized in dividing microspores (Ho *et al*., 2011; Hotta *et al*., 2012). For AUG7, homozygous mutants with reduced *AUG7* expression revealed its function in mitotic MT arrays in root cells but not in reproductive cells due to aborted inflorescences (Hotta *et al*., 2012). For AUG8, phenotypic disturbance linked to disorganized cortical MTs was shown in elongating hypocotyls cells, and its function in MT branching was also visualized in hypocotyls (Cao *et al*., 2013). MT-dependent MT nucleation, visualized with AUG3-GFP and AUG7-GFP fusions, was found in cortical MTs in leaf epidermal cells (Liu *et al*., 2014). For AUG6, *aug6* mutants were not available, but knockdown lines expressing an artificial microRNA that targets *AUG6* showed that MT nucleation differed interphase cortical MTs in leaf pavement cells (Liu *et al*., 2014). Although all plant cells may utilize a common mechanism for MT nucleation and dynamics, unique features of MT

arrays occur in different cell types. For example, MT-dependent MT nucleation occurs at a ~40° angle on cortical MTs but at a ~20° angle on the spindle and phragmoplast MTs (Hashimoto, 2013). Furthermore, plant somatic cells generally build symmetric spindles with planar phragmoplasts, but microspores construct asymmetric spindles and curved phragmoplasts (Otegui and Staehelin, 2000). Thus, mechanisms involving unidentified cofactors may operate to assemble specific MT arrays in unique ways. Given that *gem3* heterozygotyes exhibit defects mainly at pollen mitosis I, and *gem* homozygous plants show a variety of phenotypes including male meiosis and female gametophytic defects, the *gem3/aug6-1* allele of *AUG6* may serve as a valuable tool for the comparative study of augmin-dependent MT nucleation and dynamics in a variety of cell types. In conclusion, we provide evidence that reduced expression of AUG6 disturbs mitotic and meiotic divisions due to malformed MT arrays in *Arabidopsis*, which strongly supports a broader role for the augmin complex in centrosome-independent MT organization during sexual reproduction in plants.

EXPERIMENTAL PROCEDURES

Plant growth and selection

Arabidopsis plants were grown in a temperature-controlled room at 22°C and a daily cycle of 16-h light/8-h dark. The *gem3* mutant plant originating from the Landsberg *erecta* (L*er*) ecotype was backcrossed three-times with L*er* wild-type plants. *Arabidopsis* plants were transformed by a floral-dipping method (Clough and Bent, 1998). Transgenic plants were grown on Murashige and Skoog (MS) media (Duchefa) containing 50 mg/L kanamycin, 20mg/L hygromycin, or 110mg/L gentamycin.

Genetic analysis and map-based cloning of *gem3/aug6-1*

Genetic transmission of *gem3* through the male and the female was determined as described previously (Park *et al*., 1998). For map-based cloning of the *GEM3* locus, mapping populations were generated using *gem3* mutants in the L*er* background (Grini *et al*., 1999). Fresh pollen grains from columbia (Col-0) wild-type plants were crossed to *gem3*$^{+/-}$. F1 plants were self-fertilized and F2 seeds

were used as mapping populations. Genomic DNA extracted from 873 F2 plants with normal pollen phenotype was analyzed using molecular markers identified through TAIR (http://www.arabidopsis.org) or designed for use in the present study (Table S2). Several candidate genes located within a 71-kb region of chromosome 5 were sequenced using DNA fragments, PCR-amplified using genomic DNAs and/or cDNAs from wild-type and *gem3* mutant plants.

Vector construction and generation of transgenic *Arabidopsis*

To examine gene expression, a 1,940-bp fragment immediately before the start codon of *AUG6* was amplified with specific primers (Table S2) and cloned upstream of the GUS and H2B-mCherry coding regions, generating proAUG6-GUS-dHA and proAUG6-H2B-mCherry, respectively. For complementation analysis, wild-type *AUG6* or mutant type 2 *aug6* full length cDNA sequence was inserted between proAUG6 and a double HA sequence by replacing the GUS fragment from proAUG6-GUS-dHA, which resulted in proAUG6-AUG6-dHA or proAUG6-aug6mt2-dHA, respectively. To visualize chromosomes/nuclei and MTs, two constructs of pPZP221-proUBQ14-SmRSGFP-TUA6 (Oh *et al*., 2010; 2012) and pER8-proUBQ14-H2B-mCherry were transformed into *gem3*$^{+/-}$ plants. proUBQ14-H2B-mCherry was constructed by replacing proAUG6 from proAUG6-H2B-mCherry with a proUBQ14 fragment. The verified constructs were transformed into the *Agrobacterium tumefaciens* GV3101 strain, which was used for floral dipping with wild-type or *gem3*$^{+/-}$ plants.

Gene expression analyses

AUG6 gene expression was investigated directly by RT-PCR analysis or indirectly using promoter-reporter lines. RT-PCR analysis was performed using developing spores and mature pollen, which were enriched following the procedure described by Honys *et al*. (2004). Promoter reporter lines, proAUG6-GUS-dHA and proAUG-H2B-mCherry, were analyzed as described by Oh *et al*. (2010).

Microscopy

Phenotypic analysis of DAPI-stained mature pollen/developing spores using UV epifluorescence microscopy was carried out as previously described (Oh *et al*., 2010). For callose staining, spore samples freshly dissected on a microscope slide were mixed with 1µl 0.1% aniline blue in 0.1 M K_2HPO_4 and mounted with a droplet of anti-fade medium (Vectorshield). Confocal laser scanning microscopic analysis (CLSM) of embryo sacs from wild-type and *gem3*$^{-/-}$ plants was carried out as described previously (Christensen *et al*., 1998), using an LSM700 microscope (Carl Zeiss). To detect GFP-tubulin and histone-mCherry, anthers containing meiocytes and microspores were dissected from fresh inflorescences in 0.3 M Mannitol solution and viewed immediately using an LSM700 microscope with a 100X Plan Apo 1.4 N.A. oil immersion objective. GFP and mCherry were excited at wavelengths of 488 and 561 nm, respectively.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.A.O., P.E.G., D.T. and S.K.P.; Investigation, S.A.O., J.J., H.J.P. and P.E.G.; Writing-Original Draft, S.A.O. and S.K.P.; Writing-Review & Editing, S.A.O., P.E.G., D.T. and S.K.P.; Funding Acquisition, S.A.O. and S.K.P.; Supervision, S.K.P.

CONFLICT OF INTEREST

There is no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Sprorophytic phenotypes of *gem3* mutant plants.

Figure S2. Map-based cloning of *GEM3/AUG6*.

Figure S3. Alignment of protein sequences deduced from the full length cDNA clones in a vector containing a double HA sequence.

Table S1. Genetic transmission of *gem3* mutant allele.

Table S2. Primer sequences used in this study.

Table S3. Summary of genetic lesions detected in some candidate genes within the *gem3* locus.

Table S4. Summary of complementation analyses.

Table S5. *In silico AUG6* gene expression profiles.

Table S6. Complementation analysis.

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Figure Legends

Figure 1. Pollen phenotypes in open flowers of wild-type and heterozygous *gem3* plants after DAPI-staining. (a) Wild-type tricellular pollen. (b–l) Abnormal pollen phenotypes in *gem3*. (b–f) Pollen grains with two nuclei. (g) Aborted pollen. (h–l) Pollen grains with a single nucleus. Complete or partial dividing walls are indicated by arrowheads. In some cases, the structured outer pollen wall (exine) appears disturbed, exposing the smooth (intine) layer beneath (f, l). Fluorescence (left) and bright field images (right) are shown. Scale bar, 20 μm.

Figure 2. Pollen phenotypes in open flowers of wild-type and homozygous *gem3* mutant plants after DAPI-staining. Fluorescence (above) and bright field images (below) are shown. Tricellular (a–c), binucleate (d–f), uninucleate (g–i), or aborted (j–l) pollen grains. Complete or partial dividing cell walls are present in some binucleate or uninucleate pollen grains. Two arrowheads indicate a complete dividing wall (e,f,i). Scale bar, 20 μm.

Figure 3. Pollen development in wild-type and *gem3* mutants. DAPI-stained developing spores from wild-type (a-h), *gem3*$^{-/-}$ (i-l), and *gem3* $^{+/-}$ (m-p) are shown at the following stages: tetrad (a, i-l), microspore (b–d), early bicellular (e, m–p), mid bicellular (f), late bicellular (g), tricellular (h). Pollen grains at early bicellular stage were double-stained with aniline blue and DAPI (e, m–p). All images

are at the same magnification, scale bar, 10 μm. (q,r) the distribution of phenotypes observed at the tetrad stage (q), or the percentage of normal spores at different developmental stages (r) are shown for wild-type and *gem3* mutants. Abbreviations: Mon, monad; Di, dyad; Tri, triad; Tet, normal tetrad; Tet*, abnormal tetrad with unequal spore sizes; Pen, pentad; >Pen, polyad with more than five nuclei/micronuclei; UMS, unpolarized microspore (b–c); PMS, polarized microspore (d); EBC, early bicellular pollen (e); LBC, late bicellular pollen (g); TC, tricellular pollen (h); MP, mature pollen.

Figure 4. Cytology of DAPI-stained chromosomes and nuclei in dividing pollen mother cells. Two examples of pollen mother cells undergoing typical meiosis are shown for wild-type and *gem3*$^{+/-}$ (a, c, e, g, i, k, m, left and right, respectively) and *gem3*$^{-/-}$ (b, d, f, h, j, l, n) plants. (a-b) Late prophase I, (c–d) Metaphase I, (e–f) Telophase I, (g–h) Dyad stage of prophase II, (i–j) Meta-anaphase II, (k–l) Telophase II, (m–n) Tetrad stages before cytoplasmic separation. Scale bar (a-n), 10μm.

Figure 5. Analyses of seed-set and female gametogenesis. (a–c) Dissected siliques from wild-type (a), *gem3*$^{+/-}$ (b), and *gem3*$^{-/-}$ (c) plants. Aborted ovules in *gem3* mutants are indicated by arrows (b–c). (d–f) Mature seeds of wild-type (d), *gem3*$^{+/-}$ (e) and *gem3*$^{-/-}$ (f) plants. Shriveled seeds are marked by asterisks (f). (g) Frequencies of aborted ovules, aborted seeds, and mature seeds are shown for wild-type (+/+) and *gem3* mutant genotypes. Note: mature seeds in the *gem3*$^{-/-}$ plant are larger than those in wild-type and *gem3*$^{+/-}$ plants. (h–m) Images of ovules from wild-type (h–j) and *gem3*$^{-/-}$ mutant (k–m) plants. (h) FG5: female gametophyte stage 5, 8-nucleate stage before the fusion of two polar nuclei. (i–j) FG7: female gametophyte stage 7, 4-cell stage with a fused central cell nucleus and degeneration of three antipodal cells. Note: two optical sections are shown for a wild-type ovule at FG7 stage. (k–l) FG1: female gametophyte stage 1, one-cell stage with a single survival megaspore with three degenerating megaspores. (m) FG2-3, female gametophyte stage 2 to 3, two nuclei stages with or without vacuoles. An, antipodal nuclei; Pn, polar nuclei; Cn, central cell nucleus; En, egg cell nucleus; Sn, synergid cell nuclei; Fm, functional megaspore nucleus; Dm, degenerating megaspore nuclei. Scale bar (h-m), 25μm.

Figure 6. Identification and expression of *GEM3/AUG6*. (a) *GEM3/AUG6* is shown with 13 exons (red boxes) and 12 introns (lines). Arrows indicate the location of mutations with changes shown underneath. (b) RT-PCR analysis of *GEM3*/*AUG6* and *H2B* using inflorescence RNA samples from wild-type (+/+), *gem3*$^{+/-}$ (+/-), and *gem3*$^{-/-}$ (-/-) plants. Control genomic DNA fragments amplified with the same primers are indicated, gDNA. (c) Two alternative AG splice acceptors induced by the *gem3* mutation in the intron 5 are shown in blue (MtDNA). Two types of mutant transcripts are shown with a premature stop codon in red (MtmRNA_1) and a five amino acid deletion in-frame in blue (MtmRNA_2). (d) Complementation analysis. Frequencies of mutant pollen grains from *gem3*$^{+/-}$ (+/-) and *gem3*$^{-/-}$ (-/-) plants with or without complementing T-DNA (*AUG6*) are shown. (e-g) *gem3*$^{-/-}$ seedlings, without (e–f), or with (g), a complementing T-DNA. (h) Wild-type seedling. (i, j) An inflorescence and one-week-old proAUG6-GUS seedlings. (k) RT-PCR analysis. Total RNA from samples enriched for tetrads and microspores (F2.1), microspores and bicellular pollen (F2.2), bicellular pollen (F2.3), tricellular pollen (F2.3), and mature pollen (MP) were used. Primers for the *Histone H3* (At5g10980) were used as a control. (l–m) Native *AUG6* promoter driven H2B-mCherry fusion protein expression. (l) Left to right, developing microspores, bicellular and tricellular pollen. (m) A root at the seedling stage. Note: red signals on the wall of microspores and pollen grains are autofluorescence.

Figure 7. Visualization of MT arrays. (a–j) Developing spores at or immediately after pollen mitosis I in wild-type (a-e) and *gem3*$^{+/-}$ (f-j) plants. (a,f) Anaphase, (b–c, g–j) Telophase, (d–e) Bicellular pollen after pollen mitosis I. (k–y) Dividing pollen mother cells in wild-type (k–o) and *gem3*$^{-/-}$ (p–y) plants. (k,p,u) Late prophase I. (l,q,v) Meta-anaphase I. (m,r,w) Telo-interphase I. (n,s,x) Meta-anaphase II. (o,t,y) Telophase II. Numbers indicate separating nuclei (m,r) or groups of chromosomes (o,t,y). Asterisks (w) mark separated chromosomes. Lines with arrowheads (x) show displaced spindles that are abnormally close to each other. An arrowhead (t,y) indicates lagging chromosomes positioned away from the four separate groups. Scale bar (a-j, k-s, t, u-x, y), 10μm.