Corrigendum

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Some of the data in Tables S1 and S2 of this article were originally published incorrectly. The correct versions of the tables are now available at http://dev.biologists.org/cgi/content/full/132/3/603/DC1 and replace the previous versions.

The authors apologise to readers for this mistake.

Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*

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Summary

The plant life cycle involves an alternation of generations between sporophyte and gametophyte. Currently, the genes and pathways involved in gametophytic development and function in flowering plants remain largely unknown. A large-scale mutant screen of Ds transposon insertion lines was employed to identify 130 mutants of *Arabidopsis thaliana* with defects in female gametophyte development and function. A wide variety of mutant phenotypes were observed, ranging from defects in different stages of early embryo sac development to mutants with apparently normal embryo sacs, but exhibiting defects in processes such as pollen tube guidance, fertilization or early embryo development. Unexpectedly, nearly half of the mutants isolated in this study were found to be primarily defective in post-fertilization processes dependent on the maternal allele, suggesting that genes expressed from the female gametophyte or the maternal genome play a major role in the early development of plant embryos. Sequence identification of the genes disrupted in the mutants revealed genes involved in protein degradation, cell death, signal transduction and transcriptional regulation required for embryo sac development, fertilization and early embryogenesis. These results provide a first comprehensive overview of the genes and gene products involved in female gametophyte development and function within a flowering plant.

Key words: *Arabidopsis thaliana*, Female gametophyte, Embryo sac development, Fertilization, Maternal effect, Transposon insertion mutants

Introduction

The life cycle of plants alternates between a haploid gametophyte and a diploid sporophyte. In angiosperms, the sporophyte, which corresponds to the flowering plant, is the more prominent generation. The gametophytes are reduced to a small number of cells enclosed by sporophytic tissue. The male gametophytes (the pollen grains) develop within the anthers, and the female gametophytes (the embryo sacs) develop within the ovule. Fertilization of the egg cell within the embryo sac by one of the sperm cells delivered by the pollen tube results in the diploid zygote, establishing the next sporophytic generation. Although development and function of the gametophytes are crucial for plant reproduction, relatively little was known about the genes required for, and the pathways involved in gametophytic development in flowering plants.

In the past few years, several efforts have been carried out in order to identify gametophytic mutations. Although the study of several gametophytic mutants allowed the identification of genes involved in embryo sac development (Christensen et al., 2002; Drews and Yadegari, 2002; Grini et al., 2002; Huck et al., 2003; Kwee and Sundaresan, 2003; Rotman et al., 2003), dissection of the female gametophyte developmental pathway into defined genes and functions is still an unaccomplished goal. The screening of T-DNA insertion lines for functionally important gametophytic genes has led to the identification of several T-DNA-tagged gametophytic mutants in *Arabidopsis* (Feldman et al., 1997; Bonhomme et al., 1998; Howden et al., 1998; Christensen et al., 2002). However, the analysis of T-DNA mutant collections suggested that T-DNA insertional mutagenesis can be problematic for the identification and characterization of mutations that affect female gametophyte development (Bonhomme et al., 1998). As the primary target of T-DNA integration in the mutagenesis protocols is the embryo sac, plants in which female gametophyte function, especially the egg cell, is severely compromised might be difficult to recover from T-DNA insertional knockout lines (Ye et al., 1999; Bechtold et al., 2000). Additionally, chromosomal rearrangements induced by T-DNA can also affect gametophytic transmission.

We have previously described a transposon-based gene trap system in *Arabidopsis*, which resulted in the first molecular characterization of a female gametophyte-specific mutation from plants (Sundaresan et al., 1995; Springer et al., 1995). More recently, this system was used for an analysis of 20 transposon insertion mutants affecting male gametophyte development in *Arabidopsis* (Lalanne et al., 2004). As the transposon insertions are generated in sporophytic cells and the large majority of such insertion lines carry single insertions of the *Ds* transposable element without rearrangements or truncations (Sundaresan et al., 1995), the use of transposon insertion lines is well suited for the study of genes involved in gametophytic development.

In this report we used our transposon population for a forward genetic screen to identify genes involved in female gametogenesis and early embryo development. *Ds* insertion lines (24,000) were screened for segregation distortion and more than 300 mutant lines that have defects in either female or male transmission efficiency were recognized. A large collection of mutants showing female transmission defects was studied. The phenotype of 130 female gametophytic mutants was characterized, and the genes disrupted in those mutants were identified, providing a wide-ranging assessment of the genes and functions involved in embryo sac development and the transition to the sporophytic generation.

Materials and methods

Plant growth conditions and segregation analysis

For the initial generation of the Ds-insertion lines, see Sundaresan et al. (Sundaresan et al., 1995). The lines were maintained as F4 seed stocks from selfed siliques of individual F3 plants that have been selected on kanamycin (Sundaresan et al., 1995). Typically 300-400 seeds from each line were sterilized in 20% (v/v) sodium-hypochlorite, washed with sterile water and plated on MS medium with 50 mg l-1 kanamycin in Percival growth chambers (Percival Scientific, USA), with a 16 hours light/8 hours dark cycle at 22°C. The ratio of green resistant seedlings to white sensitive seedlings was counted under a dissecting scope. Green seedlings were then transferred onto soil and grown under the conditions described above with 60% relative humidity. For crosses, flowers of the female parent were manually emasculated 2 days before anthesis and cross-pollinated 2 days later.

Cleared whole-mount preparations

Flowers from different developmental stages with at least 20 ovules per pistil were dissected and cleared overnight in Hoyers solution (Liu and Meinke, 1998). The dissected pistils were observed on a Zeiss Axioplan imaging 2 microscope under DIC optics. Images were captured on an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 3.1).

Fluorescence staining of pollen tubes

For pollen tube staining, 10 pistils containing at least 20 ovules each were manually pollinated and opened longitudinally 24 hours after pollination for each mutant. The pistils were cleared in 10% chloral hydrate at 65°C for 5 minutes and washed with H_2O, softened with 5 M NaOH at 65°C for 5 minutes and washed again with H_2O. The pistils were then treated with 0.1% Aniline Blue in 0.1 M K_3PO_4 buffer pH 8.3 for 3 hours in darkness and washed with 0.1 M K_3PO_4 buffer. The pistils were mounted in a microscope slide using a drop of glycerol and carefully squashed under a cover slip. The pistils were observed using a fluorescence microscope.

Image processing

All images were processed for publication using Adobe Photoshop CS (Adobe Systems, San Jose, USA).

Molecular analysis of the sequences flanking the Ds-insertion

The sequences flanking the Ds element were isolated with TAIL-PCR as described by Liu et al. (Liu et al., 1995) and sequenced with an Applied Biosystems (ABI) sequencer. The flanking sequences obtained were run against BLASTN to identify the genomic location of the Ds element. Specific nested primers within the identified genes surrounding the Ds insertion site were designed and used in combination with Ds-nested primers in further experiments to confirm the sequence obtained from the TAIL-PCR reaction.

Results

Identification of gametophytic mutants

With the purpose of identifying mutants of *Arabidopsis thaliana* with defects in female gametophyte development, we performed a large-scale insertional mutagenesis screen using an Ac/Ds transposon system (Sundaresan et al., 1995). As the Ds element includes an NPTII gene conferring kanamycin resistance, plants carrying a transposed Ds element were tested for segregation ratio distortion in kanamycin resistance as a first indicative of a gametophytic defect. As the lines carry a single Ds insertion, the progeny of plants heterozygous for the insertion should segregate for kanR:kanS at 3:1. A distortion of this 3:1 segregation ratio indicates that the transposon has disrupted a gene required either for gametophyte or embryo viability. Although ratios in the order of 2:1 are indicative of embryo lethality, a ratio lower than 2:1 suggests that the Ds insertion has affected gametophyte development or function. A ratio of 1:1 would result from lethality or failure to transmit through either the male or female gametophyte, and ratios lower than 1:1 result from significant reduction in viability of both gametophytes. *Ds* insertion lines (24,000) containing either a *Ds* gene trap or an enhancer trap element were screened for segregation distortion. A total of 333 lines (1.38%) segregating at Kan^R ratios of 1.5:1 or less were identified. To determine the viability of the male and female gametophytes and the penetrance of the mutations in these lines, we crossed the heterozygous mutant lines as females using wild-type plants as sperm donors, and vice versa, and scored the number of heterozygous (kanamycin-resistant) and homozygous wild-type (kanamycin-sensitive) resulting progeny. From our analysis of 333 mutants, a total of 162 lines exhibited reduced transmission efficiency when crossed as females (Table 1). Out of the 162 female mutant lines, 28 were fully penetrant mutants (i.e. TE=0). The detailed segregation distortion data from self crosses can be found in Table S1 in the supplementary material). For this study, we selected 130 mutant lines for further characterization (see below).

Phenotypic analysis of the female mutants

In the developing wild-type ovule, a single hypodermal cell of the nucellus is specified as the archesporial cell (although occasional exceptions can be observed) (Grossniklaus and Schneitz, 1998). The archesporial cell enlarges and differentiates into the female meiocyte or megaspore mother cell (MMC). The MMC undergoes meiosis to generate four

Table 1. Transmission efficiencies detected in potential female gametophytic mutant lines

♀ × ♂	Transmission efficiency (TE)	Number of lines
Ds/+ × Wild type	⩽0.25	56
	0.25-0.5	63
	0.5-0.75	43

Plants were crossed manually and seeds of the resulting cross were collected and grown on kanamycin-containing MS plates to determine the efficiency in which the mutant allele (carrying the kanamycin resistance) was transmitted to the next generation by the females and male gametes. TE was calculated as the ratio of kanamycin-resistant plants to kanamycin-sensitive plants resulting from the crossing indicated.

spores, three of which undergo programmed cell death, leaving only the proximal (chalazal) megaspore as the functional megaspore in each ovule (see Fig. 1). The megaspore then undergoes three sequential mitotic nuclear divisions, to generate the eight nuclei of the mature embryo sac. Subsequent cellularization results in the formation of just seven cells, owing to nuclear migration and eventual fusion of two nuclei in the large central cell. In the mature embryo sac, the micropylar (distal) end of the ovule has the egg cell and two supporting cells called synergids, while the chalazal (proximal) end of the ovule has three cells of undetermined function called the antipodals that disintegrate prior to fertilization (Fig. 1A). To further characterize our female mutant lines and to determine which processes of gametophyte development or function are compromised, the terminal phenotypes of 130 lines were studied by analyzing whole-mount preparation of ovules. All these mutants displayed female TE⩽0.6, except for nine mutant lines that were characterized previous to obtaining the female transmission data. The mutant lines were analyzed at different phases of development, including post-pollination stages.

Table 2. Summary of phenotypes observed in insertion mutant lines with transmission deficiency through the female gametes

Phenotype observed	Number of lines
Embryo sac with one functional megaspore	1
Embryo sac arrested at two-nuclear stage	5
Embryo sac arrested at four-nuclear stage	3
Embryo sac with varying defects in nuclei number and position	7
Unfused polar nuclei	18
Mutants showing embryo sacs arrested at varying stages of development	8
Defects in fertilization	18
Arrested at one-cell zygotic stage	
Endosperm development abnormal or arrested	56
Endosperm development normal	6
Embryo development arrested	8
Total	130

Mutants with defects in embryo sac development

As is shown in Table 2, from 130 lines, 42 (32%) exhibited

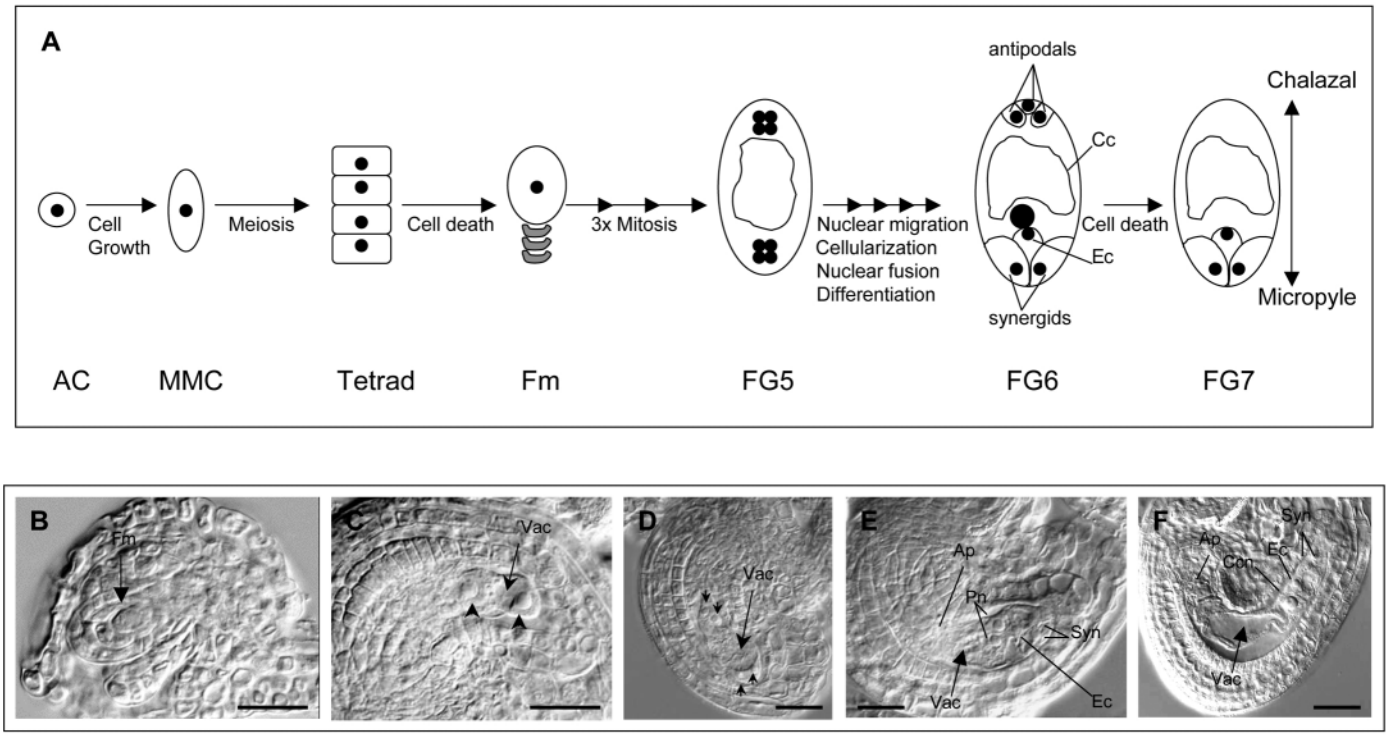

Fig. 1. Wild-type female gametophyte development in *Arabidopsis*. (A) Scheme showing the sequential developmental events leading to the wild-type female gametophyte formation in *Arabidopsis*. (B) Wild-type embryo sac containing one functional megaspore. (C) Wild-type embryo sac at the two-nuclear stage. Arrowheads indicate nuclei. (D) Wild-type embryo sac at the four-nuclear stage. Arrowheads indicate nuclei. (E) Cellularized wild-type embryo sac containing eight nuclei. (F) Wild-type embryo sac containing seven cells and seven nuclei. AC, archesporial cell; Ap, antipodal cells; Cc, central cell; Ccn, central cell nucleus; Ec, egg cell; FG5, eight-nuclear embryo sac; FG6, seven-cell embryo sac; FG7, four-cell embryo sac; Fm, functional megaspore; MMC, megaspore mother cell; Pn, polar nuclei; Syn, synergide; Vac, vacuole. Scale bar: 25 μm.

obvious defects in embryo sac development, which were called EDA mutants (for embryo sac development arrest). This category includes mutants that have defects during the nuclear division phase of megagametogenesis (Fig. 2B-D), mutants presenting abnormal nuclear numbers and positions (Fig. 2E), and mutants that became cellularized, but fail in polar nuclei fusion (Fig. 2F).

Within the mutants exhibiting defects during the nuclear division phase of megagametogenesis, we found a mutant arrested at stage FG1 in which the functional megaspore persisted during ovule development (Fig. 2B). We also found five mutants that progressed beyond stage FG1 but were arrested at the two-nuclear stage (Fig. 2C). All of them presented a vacuole between the two nuclei. Three mutants were found able to progress until the four-nuclear stage. Although nuclei position was normal, the mutants were arrested at this stage and the nuclei either persisted (Fig. 2D) or degenerate (not shown) during ovule development. We found another seven mutants with defects in nuclear number and positions (Fig. 2E). These mutants are characterized by presenting unusual numbers of nuclei (i.e. five nuclei, Fig. 2E), or normal number of nuclei but with aberrant distribution along the developing embryo sac (not shown). Eighteen mutants became cellularized but had defects in fusion of the polar nuclei. In this category of mutants, the polar nuclei migrated properly, but failed to fuse (Fig. 2F). Eight mutants exhibited embryo sac arrested at varying stages of megagametogenesis, showing variations within the same silique (not shown). Thus, a single silique from this class of mutant can present embryo sacs with diverse defects ranging from embryo sacs arrested at two- or four-nuclear stage, to embryo sacs with defects in polar nuclei fusion (not shown).

Mutants presenting defects in fertilization

A large fraction of mutants was found to be affected during post-pollination processes. Among these, we found 18 mutants that appeared normal at stage FG7, but were found to be unfertilized, suggesting that the mutation is affecting embryo sac functions such as pollen tube guidance or fertilization. These mutants were called UNE mutants (for unfertilized embryo sac mutants). When post-pollination stages were analyzed, some of these lines showed two intact synergids, suggesting that ovules were not attracting pollen tubes or that they failed to undergo synergid cell death (Fig. 3C).

To examine the nature of the fertilization failure further, we analyzed pollen tube growth patterns in the unfertilized mutants by Aniline Blue staining. The lines were classified according to the presence of pollen tubes at the micropyle of mutant ovules (i.e. unfertilized ovules) in comparison with the wild-type ovules present in the same pistil. When the pollen tubes were only found at the micropyle of wild-type ovules but not in the mutant ovules, the line was classified as mutant that fails to attract pollen tubes. On the one hand, the lines classified as unfertilized mutants with normal pollen tube guidance presented pollen tubes at the micropyle of at least 90% of the mutant ovules. Of 18 lines analyzed, 12 had pollen tubes at the micropyle, indicating that pollen tube guidance was not affected significantly in these cases, suggesting that failure of synergid cell death might have occurred in these mutants (Fig. 3A). On the other hand, four mutants failed to attract pollen

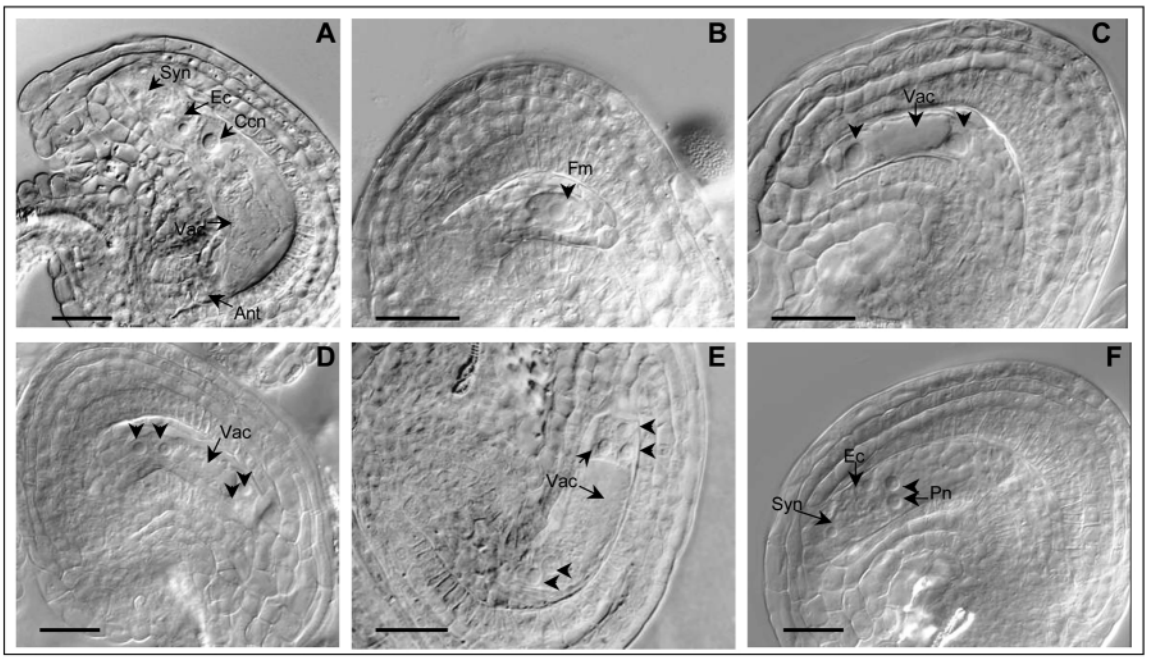

Fig. 2. Phenotype of mutants with defects in embryo sac development. (A) Mature wild-type embryo sac. (B) Arrested embryo sac containing a functional megaspore and degenerating spores (EDA 8). (C) Mutant presenting an embryo sac arrested at the two-nuclear stage (EDA 1). The vacuole is indicated and the arrowheads indicate the nuclei. (D) Mutant with an embryo sac arrested at the four-nuclear stage (EDA 4). The vacuole is indicated and the arrowheads indicate the nuclei. (E) Embryo sac showing abnormal number and position of nuclei (EDA 13). The arrowheads indicate the nuclei positions. (F) Polar nuclei fail to fuse (EDA 27). Ant, antipodal; Syn, synergide; Ec, egg cell; Ccn, central cell nucleus; Vac, vacuole; Fm, functional megaspore; Pn, polar nuclei. Scale bar: 25 μm.

tubes (Fig. 3B) and two mutant lines exhibited abnormal pollen tube growth patterns, which are currently under characterization. These defects in pollen tube attraction and guidance were also observed when wild-type plants were used as pollen donors (not shown), confirming that the defects arise due to disruption of female gametophytic functions.

Mutants presenting defects in embryogenesis

An unexpectedly large number of mutants, represented by 62 lines (48%), exhibited fertilized embryo sacs in which the embryo development was arrested at the one-cell zygotic stage (Table 2, Fig. 3D). In general, endosperm development was also arrested in these mutants, containing one to four big nuclei (Fig. 3D). However, six of the zygotic-arrest mutants showed normal endosperm development (not shown). To determine if these mutants were due to defects in the female gametophyte, we crossed all 62 mutant lines as females using wild-type plants as pollen donors and we examined the resulting progeny by scoring the number of mutant embryo sacs per silique at 24 to 48 hours after pollination. In all the cases, the ratio of mutant to wild-type embryo sacs found was at least 35%, demonstrating that the zygotic arrest arises from the female gametophyte and is not rescued by wild-type pollen.

Eight mutants were found arrested at later stages of embryo development, ranging from two-cell stage to globular and early heart stages (Fig. 3E). These mutants are characterized by a slight general delay in embryo sac development and fertilization (up to 12 hours), with various levels of endosperm development. When these lines were crossed as females using wild-type plants as pollen donors, the ratio of mutant to wild-type embryos in the silique was 35-50%, indicating that the embryo arrest phenotype in this class also arises from a mutation of the female gametophyte.

These mutants with defects in embryogenesis were called MEE mutants (for maternal effect embryo arrest).

Identification of the genes involved in female gametophytic mutations

Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995) was performed to isolate the flanking sequence surrounding the Ds element using Ds-specific primers and arbitrary degenerated primers. The chromosomal location of the flanking sequences was determined by nucleotide BLAST searches and further confirmed by PCR using gene-specific primers in combination with a Ds element primer. Using the current annotation of the *Arabidopsis* genome, two out of the 127 (1.5%) insertion sites sequenced showed to be within a predicted gene (hypothetical protein), while 19 (15%) are within genes encoding proteins with an EST match but without any protein match (unknown proteins). The rest of the tagged genes were classified according to their biological role or biochemical function as described by Lin et al. (Lin et al., 1999). A distribution of the genes disrupted in the female gametophytic mutants is shown in Fig. 4, while the identity of

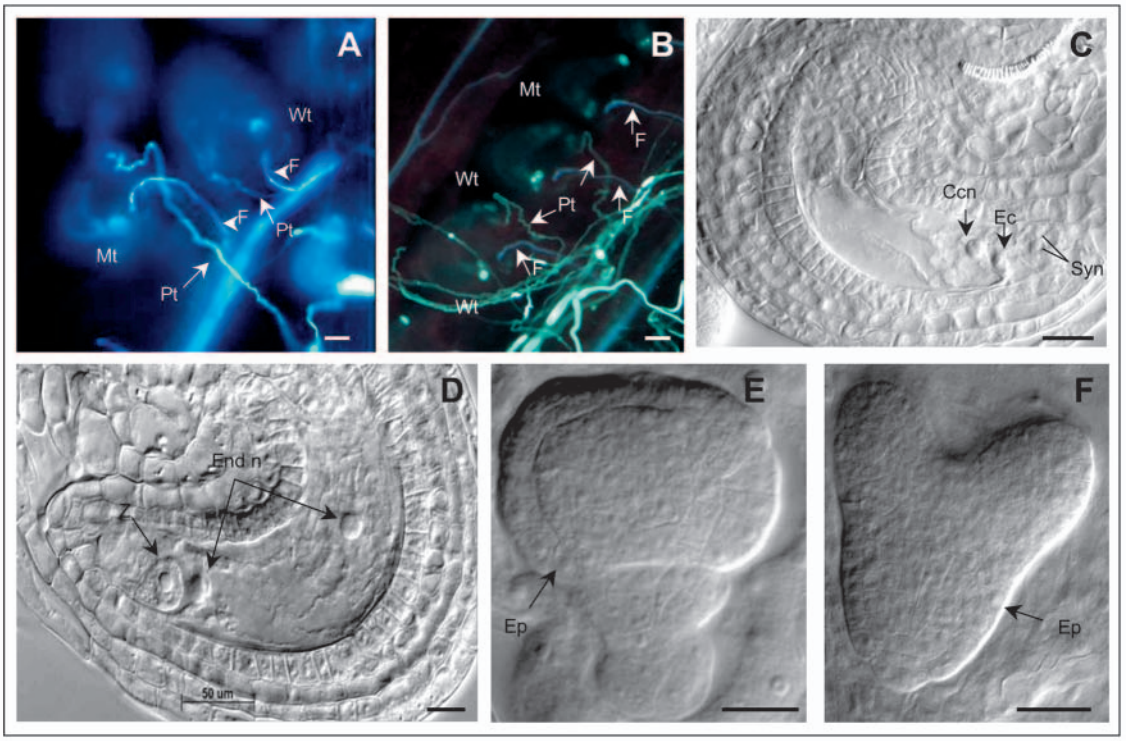

Fig. 3. Phenotype of mutants showing defects in fertilization and embryo development. (A) Silique showing a wild-type (Wt) and one mutant embryo (Mt) sac, both with pollen tubes (Pt) at the micropyle (UNE 10). (B) Silique showing two wild-type embryo sacs with pollen tubes at their micropyles and one mutant embryo sac that failed to attract a pollen tube to its micropyle (UNE 14). (C) Mutant embryo sac presenting two intact synergids after pollination (UNE 9). (D) Mutant showing embryo development arrested at the one-cell stage presenting two big endosperm nuclei (End n) (MEE 31). A single arrow indicates the zygote. (E) Mutant showing an abnormal embryo proper (Ep) and suspensor (MEE 70). (F) Wild-type control embryo at early heart stage. Scale bar: 25 μm. Syn, synergide; Ec, egg cell; Ccn, central cell nucleus; F, filament.

the disrupted genes classified by phenotypic category can be found in Table 3 (a fuller description of the genes based on the current annotation in the TAIR database can be found in Table S2 in the supplementary material). As unlikely, but possible, excision and nearby insertions of the Ds element could have occurred prior to the establishment of the stable insertion line, it is probable that although the mutants are tightly linked to the Ds insertion, some of the mutants are not tagged. The first class of mutant lines represents mutants with obvious defects in the development of the embryo sac. A large fraction (13%) of these mutants represented disruptions of protein degradation pathways. However, these were restricted to mutants that are arrested during the nuclear division phase of megagametogenesis or in mutants presenting embryo sacs with abnormal nuclear numbers and positions (Fig. 4A). Genes implicated in secondary metabolism, transcriptional regulation and signal transduction were also found to be involved both in the nuclear division phase and in the nuclear fusion phase. A transmembrane receptor protein was identified (line EDA 23, Table 3) as well as a guanine nucleotide exchange protein (Line EDA 10, Table 3). The latter protein is an interactor of PRK1 (Park et al., 2000), which is a receptor-like kinase with serine/threonine kinase activity isolated from petunia and involved in embryo sac development (Lee et. al, 1997). Genes encoding proteins involved in electron-transferring systems (classified as proteins involved in energy metabolism) were found disrupted in those mutants exhibiting un-fused polar nuclei. Genes for calmodulin-binding proteins and calcium-binding proteins were also found related to polar nuclei fusion (line EDA 39 and EDA 34, Table 3). Different classes of transcription factors were identified including basic-helix-loop-helix-type transcription factors and CCAAT-binding factors (Fig. 4A, Table 3).

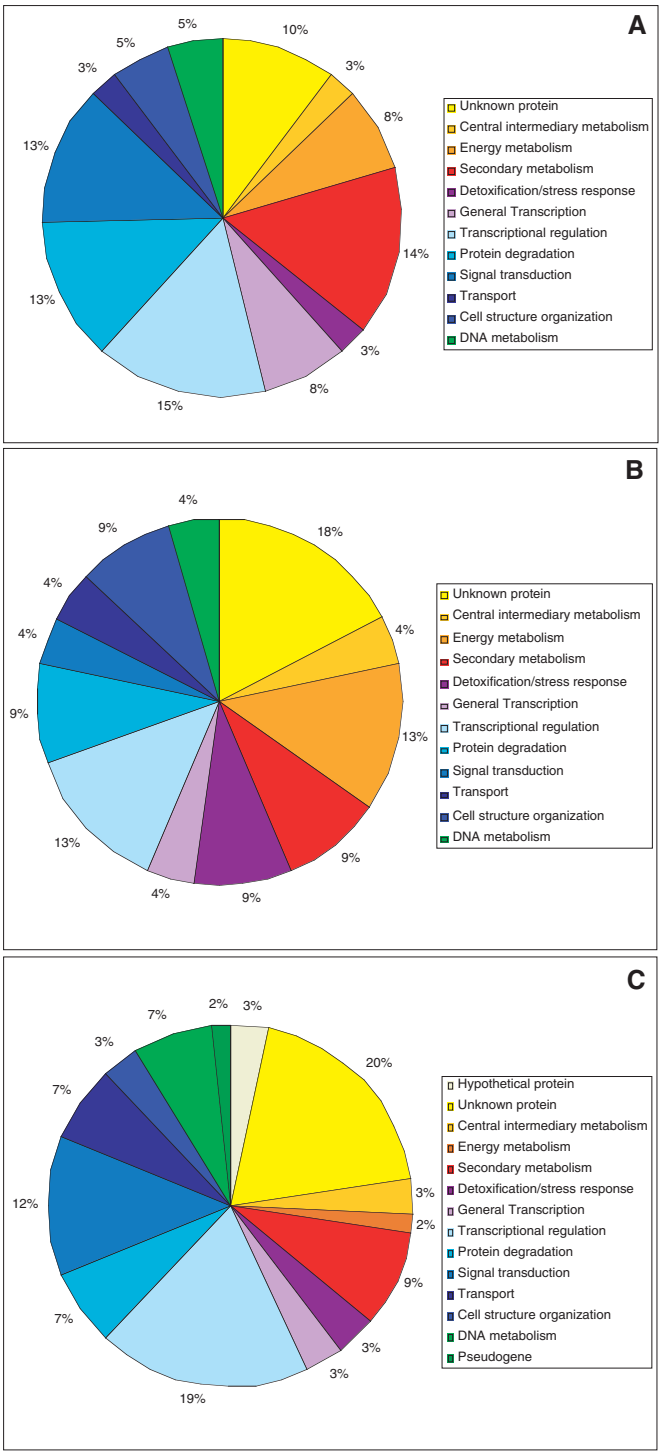

Fig. 4. Distribution and classification of the genes disrupted in mutants with defects in embryo sac development (A), fertilization process (B) and early stages of embryo development (C).

A diverse set of genes were also found to be essential for fertilization, with a moderate prevalence of transcriptional regulators and proteins involved in energy metabolism (13%, Fig. 4B). Among these, proteins involved in cytochome c maturity (line MEE 30, Table 3) and with cytochrome P450 (line UNE 9, Table 3) were observed. A gene encoding an oxysterol binding protein, which is involved in oxysterol-directed apoptosis in animals (Christ et al., 1993; Schroepfer, 2000) was also detected (line UNE 18, Table 3). On the other hand, a gene encoding for an antioxidant enzyme was found (MEE 42, Table 3). For the class of mutants that showed embryo development arrested at the one-cell zygotic stage, a large and diverse collection of genes was found. A high percentage of the genes was found to encode proteins with unknown functions (20%, Fig. 4C). However, among all the genes that appear to be essential for post-zygotic development, transcriptional regulators appear to be predominant (19%, Fig. 4C). Transcription factors belonging to different families such as MYB family, WRKY family and TCP family were found. Proteins involved in signal transduction pathways also appear to be crucial for post-zygotic development. Among them, we could find receptor-like protein kinases (line MEE 39, Table 3), a phospholipase D (line MEE 54, Table 3) and a response regulator of the two-components signal transduction pathways (line MEE 7, Table 3). A gene encoding an enzyme from the porphyrin pathway was also found disrupted in one of these mutants with embryo development defects (line MEE 61, Table 3), consistent with a recent report showing that most of the enzymes from the porphyrin pathway are continuously expressed through seed development (Henning et al., 2004).

In the case of seven genes (At2g34790, At2g47470, At2g47990, At3g23440, At4g00020, At4g00310 and At4g13890), we found two independent insertion lines per gene, with the Ds element insertions at two different positions within the gene. Although, in general, similar or overlapping

phenotypes were obtained when the terminal phenotype of each pair of mutant lines was compared, in the case of At3g23440 and At4g00020, the phenotype of the mutants was very different (Table 3). These divergences might arise from differences in the insertion sites of the Ds element into these genes, resulting in alleles that are not nulls but have different levels of residual expression. In the case of genes whose expression is required early for normal embryo sac development, as well as later for progression of embryogenesis, the observed differences in the terminal phenotypes might reflect such allelic variation. For example, two insertion mutants were recovered for the gene At3g23440: EDA 7 and MEE 37. Although for EDA 7 the insertion site is localized in the intergenic region downstream of the 3′ UTR, in MEE 37 the Ds element is localized in the coding region of the gene.

Discussion

The development and reproductive functions of the embryo sac are affected by a large and diverse set of genes

With the aim of identifying the genes involved in female gametophyte development and function, we isolated and characterized *Arabidopsis* gametophytic mutants on a genome-wide scale. From an original Ds insertion mutant population of 24,000 lines, 333 gametophytic mutant lines (1.38%) were recovered. This overall frequency is similar to the ones reported by other groups obtained in comparable screens using both T-DNA and transposable elements as insertional mutagens (Moore et al., 1997; Bonhomme et al., 1998; Howden et al., 1998). However, we were able to isolate a more significant fraction of female gametophytic mutants, when compared with T-DNA based screens, where the recovery of male gametophytic mutants seems to predominate (Bonhomme et al., 1998). Forty-six percent of the mutations identified affect both the female and the male gametophyte, suggesting that many of the major events of gametogenesis could be general and regulated in the male and the female gametophytes by the same gene products. Some of the essential cellular processes required in both gametophytes include mitosis, vacuole formation, cellularization, nuclear migration and cell expansion. Thus, mutations involving genes required for some of these events are likely to affect both female and male gametophyte development or function as was previously suggested (Christensen et al., 1998). When the terminal phenotype of the insertion lines that presented low transmission efficiency as females was studied, a wide array of gametophytic defects was recovered. We observed phenotypes ranging from those that affect different stages of embryo sac development to mutants with apparently normal embryo sacs but whose functions such as pollen tube guidance, fertilization or early embryo development support are affected. Among 130 female gametophyte mutants studied, 89 (i.e. 68%) did not exhibit obvious defects in embryo sac development, a proportion considerably higher than previously reported (Christensen et al., 2002). In that study of 39 female gametophyte mutants, most of the lines (~95%) displayed defects in different steps of megagametogenesis. In this larger scale study, the majority of the mutants showed normal mature embryo sacs at the end of FG7. Even though these female gametophytes appear normal, functions such as pollen tube guidance, fertilization or early embryo development were observed to be affected. As these mutant phenotypes were dependent on the female gametophyte, the wild-type genes or gene products are required in the embryo sac for the achievement of those reproductive functions. Thus, our data suggest that a large class of female gametophyte-expressed genes control reproductive functions from fertilization to early embryo development.

Mutants arrested during embryo sac development

Within the category of mutants with embryo sac developmental defects, we could find mutants arrested during the nuclear division phase of megagametogenesis, mutants with abnormal nuclear numbers and positions and mutants that became cellularized, but fail in polar nuclei fusion. An analysis of the genes disrupted in these mutant lines showed that 13% of these encode proteins involved in proteolysis, including components and regulators of ubiquitin-mediated proteolytic pathways (At1g59680, At2g18080, At2g34920, At2g48140, Table 3; Fig. 4A; see Table S2 in the supplementary material for all gene descriptions). However, genes in this functional category were limited to mutants with defects in the nuclear division phase of megagametogenesis, in accordance with the essential role of proteolysis in cell division control (reviewed by King et al., 1996). These findings are also consistent with the isolation of two gametophytic mutants, *nomega* (Kwee and Sundaresan, 2003) and *apc2* (Capron et al., 2003), in genes encoding components of the anaphase promoting complex/cyclosome (APC/C), a large protein complex with ubiquitin ligase activity. Receptor-protein kinases and known receptor kinase interactors were also found to be involved during the nuclear division phase of megagametogenesis (Fig. 4A; At5g44700 and At1g01960, Table 3), as well as different classes of transcription factors including basic helix-loop-helix-type transcription factors and CCAAT-binding factors (Fig. 4A, Table 3). An interesting finding was the fact that genes encoding proteins located in endomembrane and some of them involved in electron-transferring systems were found disrupted in mutants that fail in polar nuclei fusion (Fig. 4A; At1g70540, At1g72440, At2g34790, At3g03810, At4g14040, Table 3). Previously, GFA2 – a member of the DnaJ protein involved in oxidative phosphorylation and ATP production by the mitochondria – was found to be required for the fusion of the polar nuclei in *Arabidopsis* (Christensen et al., 2002). As a disruption of ATP-producing systems would cause pleiotropic effects on other energy-requiring processes, not observed in these plants, other functions than energy production can be disrupted in these mutants. As suggested for *gfa2* mutation, nuclear membrane fusion may require diffusible factors, including hemes, cytochromes, carbon skeletons and thymidylates (Mackenzie and McIntosh, 1999; Kushnir et al., 2001; Christensen et al., 2002); membrane contact may be required to shuttle lipids between different compartments (Staehelin, 1997).

Calmodulin-binding proteins and Ca^{2+}-binding proteins were also found related to polar nuclei fusion (At4g33050 and At4g00140, Table 3). The involvement of Ca^{2+} ions in membrane fusion has been reported earlier in somatic protoplast fusion (Keller and Melchers, 1973). Furthermore,

Table 3. Identity of the genes disrupted by Ds::KanR in female gametophytic mutants grouped by phenotypic category with the transmission ratios for each mutant gene

General defect observed	Mutant line ID	DS element location	Kan^R:Kan^S crossed as female	Phenotype observed
In nuclear division phase of megagametogenesis	EDA 1	At1g59680	0.0092	Arrested at two-nuclear stage
	EDA 2	At2g18080*	0.0000	Arrested at two-nuclear stage
	EDA 3	At2g34860	0.0029	Arrested at two-nuclear stage
	EDA 4	At2g48140	0.0000	Arrested at four-nuclear stage
	EDA 5	At3g03650	0.1900	Arrested at four-nuclear stage
	EDA 6	At3g23440	0.3500	Arrested at two-nuclear stage
	EDA 7	At3g56990	0.0090	Arrested at four-nuclear stage
	EDA 8	At4g00310*	0.3300	Arrested at stage FG1
	EDA 9	At4g34200	0.0000	Arrested at two-nuclear stage
Abnormal nuclear numbers and positions	EDA 10	At1g01960	0.4700	Variant nuclear number or not embryo sac
	EDA 11	At1g55420	0.1700	Variant nuclear number and positions
	EDA 12	At2g35950	0.5200	Variant nuclear number and positions
	EDA 13	At2g47990	0.0000	Variant nuclear number and positions
	EDA 14	At3g60360	0.0000	Abnormal positions and size four-nuclear stage
	EDA 15	At4g14790	0.4400	Variant nuclear number and positions
Arrested at varying stages of embryo sac development	EDA 16	At1g61140	0.4600	Aberrant embryo sacs and with unfused polar nuclei
	EDA 17	At1g72970	0.1800	Aberrant embryo sac and arrested at two-nuclear stage
	EDA 18	At2g34920	0.3500	Aberrant embryo sacs and with unfused polar nuclei
	EDA 19	At2g47990	0.0380	Aberrant embryo sacs and with unfused polar nuclei
	EDA 20	At4g00020	0.2300	Aberrant embryo sacs and unfertilized ovules
	EDA 21	At4g13235	0.4900	Aberrant embryo sacs and with unfused polar nuclei
	EDA 22	At5g05920	0.3000	Arrested at four-nuclear stage and unfused polar nuclei
	EDA 23	At5g44700	0.1974	Arrested at two-nuclear stage and unfused polar nuclei
Unfused polar nuclei	EDA 24	At1g70540	0.0208	Unfused polar nuclei
	EDA 25	At1g72440	0.5846	Unfused polar nuclei
	EDA 26	At2g01730	0.4100	Unfused polar nuclei
	EDA 27	At2g20490	0.0000	Unfused polar nuclei
	EDA 28	At2g34790	0.4100	Unfused polar nuclei
	EDA 29	At2g35940	0.5100	Unfused polar nuclei
	EDA 30	At3g03810	0.1200	Unfused polar nuclei
	EDA 31	At3g10000	0.4600	Unfused polar nuclei
	EDA 32	At3g62210	0.4773	Unfused polar nuclei
	EDA 33	At4g00120	0.5200	Unfused polar nuclei
	EDA 34	At4g00140	0.1573	Unfused polar nuclei
	EDA 35	At4g05450	0.0350	Unfused polar nuclei
	EDA 36	At4g13890	0.2500	Unfused polar nuclei
	EDA 37	At4g13890	0.2500	Unfused polar nuclei
	EDA 38	At4g14040	0.6840	Unfused polar nuclei
	EDA 39	At4g33050	0.6200	Unfused polar nuclei
	EDA 40	At4g37890	0.5844	Unfused polar nuclei
	EDA 41	At5g52460	0.2900	Unfused polar nuclei
Defects in fertilization	UNE 1	At1g29300	0.2000	Defects in pollen tube attraction
	UNE 2	At1g78130	0.2900	Unfertilized ovules but normal pollen tube attraction
	UNE 3	At2g01110	0.7143	Unfertilized ovules but normal pollen tube attraction
	UNE 4	At2g12940	0.3500	Defects in pollen tube attraction
	UNE 5	At2g47470	0.7813	Unfertilized ovules but normal pollen tube attraction
	UNE 6	At3g03340	0.8200	Unfertilized ovules but normal pollen tube attraction
	UNE 7	At3g03690	0.2800	Unfertilized ovules but normal pollen tube attraction
	UNE 8	At3g05690	0.1800	Unfertilized ovules but normal pollen tube attraction
	UNE 9	At3g10560	0.3400	Defects in pollen tube attraction
	UNE 10	At4g00050	0.4500	Unfertilized ovules but normal pollen tube attraction
	UNE 11	At4g00080	0.6417	Defects in pollen tube attraction
	UNE 12	At4g02590	0.5200	Unfertilized ovules but normal pollen tube attraction
	UNE 13	At4g12620	0.3700	Unfertilized ovules but normal pollen tube attraction
	UNE 14	At4g12860	0.5800	Defects in pollen tube attraction
	UNE 15	At4g13560	0.4900	Defects in pollen tube attraction
	UNE 16	At4g13640	0.2400	Unfertilized ovules but normal pollen tube attraction
	UNE 17	At4g26330	0.2300	Unfertilized ovules but normal pollen tube attraction
	UNE 18	At5g02100	0.1400	Unfertilized ovules but normal pollen tube attraction
Arrested at one-cell zygotic stage	MEE 1	ND	0.3800	Endosperm development arrested
	MEE 2	ND	0.5600	Endosperm development arrested
	MEE 3	At2g21650*	0.0610	Endosperm development arrested
	MEE 4	At1g04630	0.0050	Endosperm development arrested
	MEE 5	At1g06220	0.4100	Endosperm development arrested
	MEE 6	At1g07890	0.0420	Endosperm development arrested

Table 3. *Continued*

General defect observed	Mutant line ID	DS element location	Kan^R:Kan^S crossed as female	Phenotype observed
	MEE 7	At1g10470	0.4400	Endosperm development arrested
	MEE 8	At1g25310	0.1642	Endosperm development arrested
	MEE 9	At1g60870	0.3100	Endosperm development arrested
	MEE 10	At2g01200	0.5000	Endosperm development arrested
	MEE 11	At2g01620	0.0000	Endosperm development arrested
	MEE 12	At2g02955	0.4200	Endosperm development arrested
	MEE 13	At2g14680	0.0000	Endosperm development arrested
	MEE 14	At2g15890	0.0240	Endosperm development arrested
	MEE 15	At2g16970	0.4300	Endosperm development arrested
	MEE 16	At2g18650	0.0190	Endosperm normal
	MEE 17	At2g22250	0.3333	Endosperm normal
	MEE 18	At2g34090	0.2900	Endosperm development arrested
	MEE 19	At2g34130	0.4800	Endosperm development arrested
	MEE 20	At2g34220	0.1800	Endosperm development arrested
	MEE 21	At2g34570	0.3800	Endosperm development arrested
	MEE 22	At2g34780	0.4900	Endosperm development arrested
	MEE 23	At2g34790	0.3913	Endosperm development arrested
	MEE 24	At2g34830	0.2900	Endosperm development arrested
	MEE 25	At2g34850	0.3000	Endosperm development arrested
	MEE 26	At2g34870	0.4600	Endosperm development arrested
	MEE 27	At2g34880	0.4800	Endosperm development arrested
	MEE 28	At2g35210	0.4083	Endosperm development arrested
	MEE 29	At2g35340	0.4100	Endosperm development arrested
	MEE 30	At2g47470	0.2600	Endosperm development arrested
	MEE 31	At3g02570	0.5300	Endosperm development arrested
	MEE 32	At3g06350	0.5328	Endosperm development arrested
	MEE 33	At3g10920	0.0556	Endosperm normal
	MEE 34	At3g11270	0.2353	Endosperm normal
	MEE 35	At3g15030	0.0837	Endosperm development arrested
	MEE 36	At3g16440	0.0000	Endosperm development arrested
	MEE 37	At3g23440	0.3000	Endosperm development arrested
	MEE 38	At3g43160	0.4800	Endosperm development arrested
	MEE 39	At3g46330	0.4700	Endosperm development arrested
	MEE 40	At3g53700	0.4300	Endosperm development arrested
	MEE 41	At3g62670	0.1800	Endosperm development arrested
	MEE 42	At3g63080	0.5900	Endosperm development arrested
	MEE 43	At4g00020	0.5100	Endosperm normal
	MEE 44	At4g00060	0.2500	Endosperm development arrested
	MEE 45	At4g00260	0.3519	Endosperm development arrested
	MEE 46	At4g00310	0.2800	Endosperm development arrested
	MEE 47	At4g00950	0.3400	Endosperm development arrested
	MEE 48	At4g14080*	0.2200	Endosperm development arrested
	MEE 49	At4g01560	0.1900	Endosperm development arrested
	MEE 50	At4g00231	0.5700	Endosperm development arrested
	MEE 51	At4g04040	0.1000	Endosperm development arrested
	MEE 52	At4g04160	0.0435	Endosperm development arrested
	MEE 53	At4g10560	0.1100	Endosperm normal
	MEE 54	At4g11850	0.5500	Endosperm development arrested
	MEE 55	At4g13345	0.4600	Endosperm development arrested
	MEE 56	At4g13380	0.4300	Endosperm development arrested
	MEE 57	At4g13610	0.5800	Endosperm development arrested
	MEE 58	At4g13940	0.6593	Endosperm development arrested
	MEE 59	At4g37300	0.3093	Endosperm development arrested
	MEE 60	At5g05950	0.2500	Endosperm development arrested
	MEE 61	At5g14220	0.4000	Endosperm development arrested
	MEE 62	At5g45800	0.7714	Endosperm development arrested
Embryo development defects	MEE 63	At1g02140	0.8300	Embryos arrested at various stages of development
	MEE 64	At1g79860	0.4500	Arrested at the two-cell embryo stage
	MEE 65	At2g01280	0.6852	Arrested at early embryo stages
	MEE 66	At2g02240	0.2000	Arrested at the two-cell embryo stage
	MEE 67	At3g10110	0.3400	Arrested at the two-cell embryo stage
	MEE 68	At4g24660	0.0972	Arrested at early embryo stages
	MEE 69	At4g37140	0.2300	Arrested at the two-cell embryo stage
	MEE 70	At5g58230	0.0000	Abnormal embryo development

*Indicates sequences identified by TAIL-PCR but not confirmed yet by gene-specific primers.
ND, not determined.

the requirement of Ca^{2+} ions has previously been shown for the fusion of sperm with central cell protoplasts in maize (Kranza et al., 1998), as well as in the fusion of sperm with egg cell protoplasts (Kranz and Lorz, 1994), suggesting that Ca^{2+} ions might also be involved in membrane union during polar nuclei fusion.

Female gametophytic mutants affecting the fertilization process

Fertilization in *Arabidopsis* requires controlled growth of the pollen tube until it enters the micropyle to penetrate the female gametophyte (Ray et al., 1997; Shimizu and Okada, 2000) (reviewed by Higashiyama, 2003). From the analysis of pollen tube growth pattern in *Arabidopsis* mutants defective in embryo sac development, the female gametophyte was suggested to play a key role in pollen tube guidance (Hülskamp et al., 1995; Ray, 1997; Shimizu and Okada, 2000). The pollen tube enters the female gametophyte by growing into one of the synergid cells, where it ruptures at its tip releasing its contents. The synergid cell penetrated by the pollen tube undergoes cell death, apparently at the time of pollen tube discharge, or very shortly before pollen tube arrival (Faure et al., 2002; Huck et al., 2003). According to previous observations, synergid cell death may be a requisite for normal fertilization, decreasing the resistance to sperm cell migration during fertilization (Willemse and van Went, 1984; Huang and Russell, 1992) and by facilitating the male gamete transfer from the pollen tube to the egg and the central cell (Russell, 1993; Fu et al., 2000). An analysis of the genes disrupted in our mutant collection with fertilization defects showed a wide spectrum of functional categories (Fig. 4B). Among these, mitochondrial and endomembrane gene products, which are predominantly related to electron transport systems, were found (e.g. At1g29300, At2g47470, At3g03690, At3g10560; Table 3). In mammals, the mitochondrial intermembrane space contains several cell death activators that are released in response to a variety of cell death stimuli (Green and Reed, 1998; Hengartner, 2000; Lam et al., 2001). Although much less is known in plants, several reports support a role for mitochondria in cell death (Balk et al., 1999; Lacomme and Santa Cruz, 1999; Navarre and Wolpert, 1999; Stein and Hansen, 1999; Sun et al., 1999; Balk and Leaver, 2001). The release of cytochrome c from mitochondria has been shown to be a primary cellular trigger for programmed cell death in plants (Balk et al., 1999; Stein and Hansen, 1999; Sun et al., 1999; Balk and Leaver, 2001). However, several reports support a role for the endoplasmic reticulum during programmed cell death in plants (Hayashi et al., 2001; Danon et al., 2004). In the present study, several genes related to cell death pathways were isolated in mutants defective in synergid cell death. Among these, proteins related to cytochome c maturity and with cytochrome P450, another protein associated to plant cell death (Godiard et al., 1998) were found (At2g47470 and At3g10560, Table 3). A gene encoding an oxysterol-binding protein was also detected (At5g02100, Table 3), suggesting that a pathway similar to the oxysterol-directed apoptosis, a well known mechanism of cell death in animals (Christ et al., 1993; Schroepfer, 2000) and recently reported associated to the hypersensitive response in barley (Hein et al., 2004) might be involved in synergid cell death in the female gametophyte.

Additionally, a gene encoding an antioxidant enzyme was shown to be disrupted in a mutant with defects during the fertilization process (At3g63080, Table3). Antioxidant pathways might be active during this process, probably limiting the oxidative stress that cell-death signals, such as reactive oxygen species (ROS), can generate in the gametophyte.

Genes predicted to encode transcriptional factors were also found disrupted in lines with fertilization defects, both in pollen tube-attracting lines and in those lines that fail in pollen tube attraction. These genes belong to several different families of transcription factors, including the bHLH family, the MYB family and the bZIP family (e.g. At3g05690, At4g00050, At2g12940, At4g02590, At4g13640; Table 3) that might be responsible for the regulation of the specific processes required for fertilization, including pollen tube guidance and reception.

Female gametophytic mutants affecting embryo development

A large proportion (50.4%) of the gametophyte mutants isolated in our collection exhibited embryo sacs that were fertilized, but with very early arrest of embryo development (Fig. 3C). In general, delayed embryo sac development and a disruption in endosperm development were also observed in this class of mutants. In all the cases the mutation showed reduced transmission through the female gametophyte, indicating that these lines are female-gametophyte mutants displaying maternal effects on embryo and endosperm development. Maternal effects can be caused by mutations in genes that are expressed during the gametophyte development and whose gene products are required for embryo and endosperm development. Examples of this kind of maternal gametophytic mutants include the *Arabidopsis capulet* (*cap1*) and *cap2* mutants (Grini et al., 2002), the *Arabidopsis proliferaI* (*prI*) mutant (Springer et al., 1995; Springer et al., 2000) and the maize *maternal effect lethal* (*mell*) mutant (Evans and Kermicle, 2001). Maternal effects can be also caused by mutations in genes whose paternally contributed alleles are imprinted. This is the case of the female gametophyte mutants *fie*, *mea* and *fis2*, which present endosperm development in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998). The FIE, MEA and FIS2 proteins are related to the polycomb group proteins (Ohad et al., 1996; Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 2000) and were shown to be expressed in the central cell before fertilization as well as in the developing endosperm (Luo et al., 2000). No fertilization-independent endosperm development was observed in our mutant collection. On the contrary, endosperm development is generally arrested in these lines, showing only one to four large nuclei (Fig. 3C). These observations are in accordance with the phenotype observed in *prl* and *mel1* mutants where the development of the embryo or endosperm is severely affected very early in seed development (Springer et al., 1995; Springer et al., 2000; Evans and Kermicle, 2001). The set of genes disrupted in these lines contains many potential signal transduction components. Among them, receptor-like protein kinases, phospholipase D and a response regulator of the two-component signal transduction pathways were identified (At3g46330, At4g11850, At1g10470, Table 3). Mutants defective in early embryo development showed to have disrupted genes encoding transcription factors that belong to different families such as MYB family, WRKY family and

TCP family (At2g21650, At2g34830, At3g15030, At1g25310, Table 3; Fig. 4C).

An interesting issue is whether the mutants with embryo and endosperm phenotypes observed herein are due to a true maternal effect of the female gametophyte (i.e. maternal factors) or due to delayed paternal genome activation (Vielle-Calzada et al., 2000). Although some of the phenotypes observed in mutants with early arrest (1 cell zygote) of embryo development resemble those from female gametophytic mutants exhibiting maternal effects (Springer et al., 1995; Springer et al., 2000; Evans and Kermicle, 2001), much more detailed investigation will be needed in order to distinguish between these two possibilities.

For those mutants exhibiting embryo development arrested at later stages (from two-cell to globular stage), the defects might be ascribed to a disrupted gene whose expression is required after fertilization or in specific cells of the embryo. The phenotypes observed would be consistent with genes described previously that are required to be zygotically transcribed from the maternal allele only and regulated by genomic imprinting (Vielle-Calzada et al., 2000). The fact that some of these mutants showed a slight delay in embryo sac development and fertilization might indicate that the wild-type genes are expressed during the female gametophyte development, though they are not essential for normal embryo sac development. The imprinted genes *MEA*, *FIS2* and *FIE* were shown to be expressed before pollination in the female gametophyte. While *MEA* and *FIS2* products are found in the two polar nuclei and in the central cell nuclei, the *FIE* product occurs in the central cell before pollination (Luo et al., 2000), consistent with their proposed functions in the repression of endosperm development.

In summary, our analysis provides a comprehensive overview of the genes and functions that are required in the female gametophyte for its development into an embryo sac, and post-fertilization for the progression of embryogenesis in *Arabidopsis*. This study should serve as a basis for future studies addressed towards the elucidation of the molecular pathways involved in these crucial steps of plant reproduction.

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Supplementary material

Supplementary material for this article is available at
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