DEVELOPMENTAL BIOLOGY **202,** 136–151 (1998)
ARTICLE NO. DB988980

Identification of Gametophytic Mutations Affecting Female Gametophyte Development in *Arabidopsis*

Cory A. Christensen, Santhi Subramanian, and Gary N. Drews[1]
Department of Biology, University of Utah, 257 South 1400 E, Salt Lake City, Utah 84112-0840

The female gametophyte (embryo sac or megagametophyte) plays a critical role in sexual reproduction of angiosperms. It is the structure that produces the egg cell and central cell which, following fertilization, give rise to the seed's embryo and endosperm, respectively. In addition, the female gametophyte mediates a host of reproductive processes including pollen tube guidance, fertilization, and the induction of seed development. Several major events occur during megagametogenesis, including syncitial nuclear divisions, cellularization, nuclear migration and fusion, and cell death. While these events have been described morphologically, the molecules regulating them in the female gametophyte are largely unknown. We discuss a genetic screen based on reduced seed set and segregation distortion to identify mutations affecting megagametogenesis and female gametophyte function. We report on the isolation of four mutants (*fem1, fem2, fem3,* and *fem4*) and show that the four mutations map to different locations within the genome. Additionally, we show that the *fem1* and *fem2* mutations affect only the female gametophyte, while the *fem3* and *fem4* mutations affect both the female and male gametophyte. We analyzed female gametophyte development in these four mutants as well as in the *gfa2, gfa3, gfa4, gfa5,* and *gfa7* mutants. We found that the *fem2, fem3, gfa4,* and *gfa5* mutants abort development at the one-nucleate stage, while the *fem1, fem4, gfa2, gfa3,* and *gfa7* mutants are affected in processes later in development such as polar nuclei fusion and cellularization. The establishment of a genetic screen to identify mutants and the development of a rapid procedure for analyzing mutant phenotypes represent a first step in the isolation of molecules that regulate female gametophyte development and function.

Key Words: **female gametophyte development; *Arabidopsis;* nuclear fusion; cellularization; plant reproduction; embryo sac.**

INTRODUCTION

Plants have a two-staged life cycle in which a haploid gametophyte generation alternates with a diploid sporophyte generation. Angiosperms are heterosporous and have male and female gametophytes. The male gametophyte (pollen grain) develops within the stamen's anther and consists of two sperm cells encased within a vegetative cell. The female gametophyte (embryo sac or megagametophyte) develops within the carpel's ovary and exhibits a variety of forms. The most common form, which is found in approximately 70% of the species examined, is referred to as the *Polygonum* type (Maheshwari, 1950; Willemse and van Went, 1984; Reiser and Fischer, 1993). As shown in Fig. 1, the *Polygonum*-type female gametophyte is a seven-cell structure consisting of one egg cell, two synergid cells, three antipodal cells, and one central cell.

The female gametophyte plays a central role in sexual reproduction in angiosperms. During sexual reproduction, the male gametophyte is transferred from the anther to the carpel's stigma, where it forms a pollen tube that grows through the carpel to deliver its two sperm cells to the female gametophyte. Following fertilization, the female gametophyte's egg cell and central cell give rise to the seed's embryo and endosperm, respectively. The female gametophyte plays a role in several steps of this reproductive processes including pollen tube guidance (Hulskamp *et al.,* 1995; Ray *et al.,* 1997), fertilization (van Went and Willemse, 1984; Russell, 1993), the induction of seed development (Ohad *et al.,* 1996; Chaudhury *et al.,* 1997), and gametophytic maternal control (Ray, 1997; Drews *et al.,* 1998).

Female gametophyte development consists of two phases referred to as megasporogenesis and megagametogenesis.

[1] To whom correspondence should be addressed. Fax: (801) 581-4668. E-mail: drews@bioscience.utah.edu.

0012-1606/98 $25.00

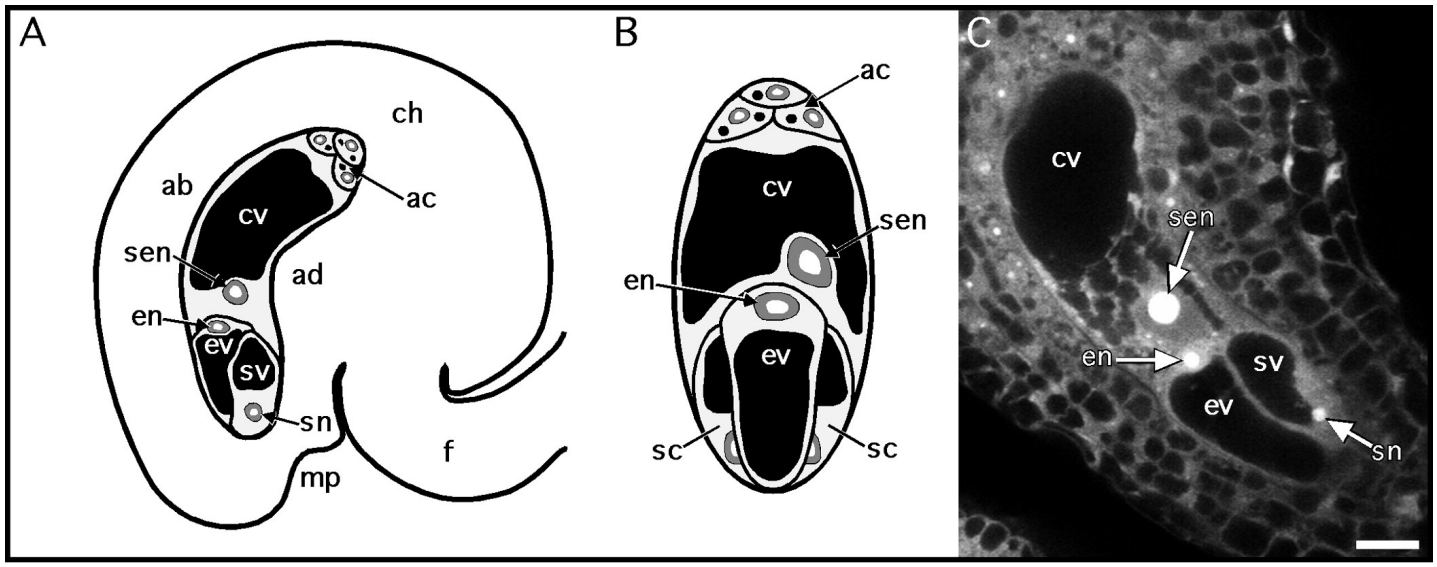

FIG. 1. The *Arabidopsis* female gametophyte. (A,B) Depictions of a wild-type female gametophyte at the seven-cell stage (stage FG6). Depicted are cytoplasm (light gray areas), vacuoles (black areas), nuclei (dark gray areas), and nucleoli (white areas within the nuclei). The female gametophyte in A is shown in longitudinal view; it is oriented with its adaxial surface (adjacent to the funiculus; ad) to the right, its abaxial surface (opposite the funiculus; ab) to the left. The female gametophyte in B is viewed from the abaxial direction. (C) CLSM image of a wild-type female gametophyte at the terminal stage (stage FG7). The second synergid cell was present but not included in this image. This image consists of a single 1.5-μm optical section. All female gametophytes are oriented with their chalazal poles up and their micropylar poles down. Abbreviations: ab, abaxial surface; ac, antipodal cells; ad, adaxial surface; ch, chalazal pole; cv, central cell vacuole; en, egg cell nucleus; ev, egg cell vacuole; f, funiculus; mp, micropylar pole; sen, secondary endosperm nucleus; sn, synergid cell nucleus; sv, synergid cell vacuole. Scale bar, 10 μm.

During megasporogenesis, a diploid megaspore mother cell undergoes meiosis and gives rise to four haploid megaspores, three of which undergo cell death. During megagametogenesis, the surviving megaspore undergoes three rounds of mitosis without cytokinesis, producing an eight-nucleate cell. Nuclear migrations, nuclear fusion, and cellularization then result in the seven-cell structure depicted in Fig. 1.

Little is known about the haploid-expressed genes that regulate and mediate the major events in megagametogenesis (mitosis, polarity establishment, nuclear migration, nuclear fusion, cellularization, and cell specification) and female gametophyte function (pollen tube guidance, fertilization, induction of seed development, and gametophytic maternal control). A number of female gametophyte mutants have been identified in both *Arabidopsis* (Redei, 1965; Castle *et al.*, 1993; Kieber *et al.*, 1993; Niyogi *et al.*, 1993; Springer *et al.*, 1995; Moore *et al.*, 1997) and maize (Singleton and Mangelsdorf, 1940; Nelson and Clary, 1952; Kermicle, 1971), indicating a requirement for haploid-expressed genes in megagametogenesis and female gametophyte function (Drews *et al.*, 1998). However, a specific function is known only for the *PRL* gene, which encodes a protein required in DNA replication and cell division (Springer *et al.*, 1995).

We have been using *Arabidopsis* as a system to identify and study haploid-expressed genes regulating and mediating female gametophyte development and function. Extensive descriptive studies of *Arabidopsis* female gametophyte structure and development have been carried out and have shown that *Arabidopsis* has a *Polygonum*-type female gametophyte (Misra, 1962; Poliakova, 1964; Webb and Gunning, 1990; Mansfield and Briarty, 1991; Mansfield *et al.*, 1991; Murgia *et al.*, 1993; Webb and Gunning, 1994; Schneitz *et al.*, 1995; Christensen *et al.*, 1997). Phenotypic analysis of female gametophyte mutants in *Arabidopsis* is facilitated by the fact that an *Arabidopsis* pistil contains a large number of ovules (50 to 60) and that the female gametophytes within these ovules develop synchronously (Christensen *et al.*, 1997). Additionally, confocal laser scanning microscopy procedures have been developed that allow for the rapid characterization of mutant phenotypes, making large-scale genetic screens practical (Christensen *et al.*, 1997).

In this paper we describe a procedure to identify female gametophyte mutants and report the isolation of four mutants, *female gametophyte1* (*fem1*), *fem2*, *fem3*, and *fem4*. We show that all of the *fem* mutations exhibit reduced transmission through the female gametophyte and that the *fem3* and *fem4* mutations also affect the male gametophyte. We also report the phenotypic characterization of these four mutants, as well as the *gfa2*, *gfa3*, *gfa4*, *gfa5*, and *gfa7* mutants (Feldmann *et al.*, 1997).

MATERIALS AND METHODS

Plant growth and kanamycin selection. Plant growth conditions and kanamycin selection (50 μg/ml) were performed as previously described (Christensen *et al.*, 1997; Feldmann *et al.*, 1997).

Mutant isolation. The *fem1* mutant was isolated in a screen of ethyl methanesulfonate (EMS)-mutagenized lines. Landsberg *erecta* seed was soaked in 0.1% EMS for 14 h, washed for 3 h in water, and then sown. On each of 1000 M1 plants, the M2 seed from a single silique was collected. Five M2 seeds from each M1 silique were planted and grown until at least 15 flowers had been self-pollinated. With each M2 plant, at least three siliques were cut open and scored for the 50% desiccated ovules phenotype. The *fem1* mutant was backcrossed five generations before genetic and morphological analysis was carried out.

The *fem2*, *fem3*, and *fem4* mutants were isolated in a screen of T-DNA-mutagenized lines as described under Results. The *fem2* and *fem3* mutants were isolated from lines generated by Ken Feldmann and obtained from the *Arabidopsis* Biological Resource Center in pools of 20. For these lines, we screened an average of 53 plants in each of 57 pools (pools CS2361–CS2365, CS2461–CS2494, CS2497–CS2501, CS2505, CS2507, CS2509–CS2515, CS2520, CS2522, CS2523, and CS2534). We isolated *fem4* from a screen of 195 lines generated and provided by Tom Jack.

Genetic mapping. We used simple sequence length polymorphisms (Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993) to map the mutations within the *Arabidopsis* genome. *fem1*, in the Landsberg *erecta* background, and *fem2* and *fem3*, in the Wassilewskija (Ws) background, were crossed to Columbia (Col) ecotype females. *fem4* in the Col background was crossed to a Ws female. For the *fem2*, *fem3* and *fem4* mutants, one KanR F1 plant from each cross was selected and again crossed to a Col female (*fem2* and *fem3*) or a Ws female (*fem4*). For the *fem1* mutant one F1 plant exhibiting the 50% desiccated ovules phenotype was selected and again crossed to a Col female. In all cases, the progeny resulting from the second cross constituted our mapping population.

DNA was extracted from either KanR plants (*fem2*, *fem3*, and *fem4*) or plants exhibiting the 50% seed set phenotype (*fem1*) in the mapping populations as described by Murray and Thompson (1980). Twenty-microliter polymerase chain reaction (PCR) reactions were done using 1.6 μl of template (concentration not determined), 10 pmol of each primer (Research Genetics, Huntsville, AL), 4 μmol each dNTP, 30 μmol $MgCl_2$, 0.5–1 unit of Biolase polymerase (ISC BioExpress, Kaysville, UT), 1× Biolase reaction buffer (ISC BioExpress), and either 1× tartrazine/Ficoll or 1× sucrose/creosol red (Idaho Technologies, Idaho Falls, ID). PCR cycling parameters were an initial denaturation step at 94°C for 4 min and then 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 30 s, and then a final extension at 72°C for 4 min on an MJ Research, Inc., PTC-100 Programmable Thermal Controller.

At least 52 individuals from each mapping population were tested against each marker for linkage. In all cases, the most closely linked markers had recombination frequencies that were significantly different than 0.5 by χ^2 analysis ($P < 0.025$). Recombination frequencies were calculated and converted to genetic map distances according to Koornneef and Stam (1992) and Kosambi (1944). *fem1* mapped between nga76 and LFY3 on chromosome 5. *fem1* and nga76 had a recombination frequency of 33.3 ± 7.8 (map distance = 40.2 cM, $n = 54$) and *fem1* and LFY3 had a recombination frequency of 25.0 ± 6.0 (map distance = 27.5 cM, $n = 52$). *fem2* and nga8 on the top of chromosome 2 had a recombination frequency of 9.6 ± 4.1 (map distance = 9.7 cM, $n = 52$). *fem3* and nga280 on chromosome 1 showed no recombination (LOD score = 16.3, $n = 54$). Finally, *fem4* mapped between nga280 and AthATPASE on chromosome 1. *fem4* and nga280 had a recombination frequency of 33.3 ± 7.8 (map distance = 40.2 cM, $n = 54$) and *fem4* and AthATPASE had a recombination frequency of 24.1 ± 5.8 (map distance = 26.3 cM, $n = 54$).

Confocal laser scanning microscopy. Tissue preparation, microscopy, image capture, and figure preparation were performed as previously described (Christensen *et al.*, 1997) with the following modifications. For female gametophyte analysis, carpel walls were separated from the septum prior to fixation by slicing along both sides of the pistil's replum with a 30-gauge syringe needle. After clearing, each placenta was dissected by removing the pedicel, style, and carpel walls. For male gametophyte analysis, anthers were fixed just prior to dehiscence and whole mounted.

Assignment of a female gametophyte stage to a pistil. We previously showed that female gametophyte development within a pistil is fairly synchronous (Christensen *et al.*, 1997). In most cases, a single female gametophyte stage predominates (≥75%) within a pistil. Under this circumstance, we assign one female gametophyte stage to a pistil. In some cases, a single stage does not predominate because the pistil is at a transition between two stages. Under this circumstance, we assign the pistil the two stages that together constitute ≥75% of the female gametophytes.

Reciprocal crosses. To prevent the possibility of inadvertent self-pollination or cross-contamination, single female parents were isolated in 2-liter soda bottles, all flowers on the primary inflorescence were emasculated daily before dehiscence and pollinated the following day, and secondary inflorescences were removed. In crosses between mutant males and wild-type females, the female parent was *male sterile1-1* (van der Veen and Wirtz, 1968).

RESULTS

Isolation of Female Gametophyte Mutants

We screened for lines containing female gametophyte lethal mutations. Our screen consisted of two steps. As a primary screen, we opened siliques and screened for plants with reduced seed set. On a plant heterozygous for a female gametophyte mutation, about 50% of its female gametophytes are wild type and functional, and approximately 50% are mutant and nonfunctional. Following fertilization, functional ovules develop into seeds. By contrast, ovules harboring defective female gametophytes fail to undergo seed development and desiccate instead. Thus, siliques from heterozygous female gametophyte mutants contain approximately 50% normal seeds and 50% desiccated ovules.

The secondary screen was based on the concept that female gametophyte mutants exhibit altered segregation patterns. For example, self-pollination of a heterozygous female gametophyte mutant produces heterozygous and wild-type progeny in a 1:1 ratio (if the mutation is fully penetrant and does not affect the male gametophyte). The lines we screened were mutagenized with a T-DNA containing the chimeric *nptII* gene which confers kanamycin resistance (Feldmann *et al.*, 1997). Thus, self-pollination of

TABLE 1
Mutants Discussed in This Paper

Mutant	Isolate no.	Mutagen	Mutant class[a]	Source
female gametophyte1 (fem1)	864	EMS	FGS	This paper
female gametophyte2 (fem2)	2462-5	T-DNA	FGS	This paper
female gametophyte3 (fem3)	2465-35	T-DNA	GG	This paper
female gametophyte4 (fem4)	TJ160	T-DNA	GG	This paper
gametophytic factor2 (gfa2)	102	T-DNA	GG	(Feldmann *et al.*, 1997)
gametophytic factor3 (gfa3)	13	T-DNA	GG	(Feldmann *et al.*, 1997)
gametophytic factor4 (gfa4)	84	T-DNA	GG	(Feldmann *et al.*, 1997)
gametophytic factor5 (gfa5)	114	T-DNA	GG	(Feldmann *et al.*, 1997)
gametophytic factor7 (gfa7)	21	T-DNA	GG	(Feldmann *et al.*, 1997)

[a] FGS, female gametophyte-specific; GG, general gametophytic.

a heterozygous female gametophyte mutant produces kanamycin resistant (KanR) and kanamycin sensitive (KanS) progeny in a 1:1 ratio (if the mutation is fully penetrant and does not affect the male gametophyte). By contrast, self-pollination of a heterozygous sporophytic mutant (e.g., a mutant defective in flower development) produces KanR and KanS progeny in a 3:1 ratio.

All potential mutants identified in the primary screen were allowed to self-pollinate, 100–200 progeny seeds were germinated in the presence of Kan, and Kan resistance ratios (KanR/KanS) were scored. Although we were primarily interested in lines in which KanR/KanS was 1.0, we also examined lines in which KanR/KanS was slightly higher or lower than 1.0 because, as discussed below, female gametophyte mutations can be partially penetrant and can also affect the male gametophyte.

Three lines, *fem2*, *fem3*, and *fem4*, were identified that exhibited reduced seed set and in which KanR/KanS was less than 1.5. We also identified one female gametophyte mutant (*fem1*) in an EMS screen (see Materials and Methods) and obtained five additional female gametophyte mutants (*gfa2*, *gfa3*, *gfa4*, *gfa5*, and *gfa7*) from other sources, as listed in Table 1.

Linkage Analysis

To determine whether the *fem2–fem4* mutations were linked to the *nptII* gene, we crossed heterozygous female gametophyte mutants with wild type, germinated the F1 progeny in the presence of Kan, transferred 100 KanR F1 progeny to dirt, and scored these plants for reduced seed set. With the *fem2–fem4* mutants, all 100 of the F1 progeny scored exhibited reduced seed set, indicating that in each of these lines, the *nptII* gene was linked to the female gametophyte mutation within 3.7 cM at the 95% confidence level. The tight linkage between the *nptII* gene and the female gametophyte mutations allowed us, in subsequent experiments, to use the KanR trait as a marker for the presence of the mutations.

We determined the genetic map positions of the *fem* mutations. All four mutations mapped to locations in the genome that were different from each other and from the *gfa1–gfa7* mutations (Feldmann *et al.*, 1997), indicating that they are not allelic (see Materials and Methods). Of the other mutants discussed in this paper, only *gfa3* and *gfa7* map to similar positions (Feldmann *et al.*, 1997).

Penetrance in the Female Gametophyte

To determine the penetrance of the *fem* mutations in the female gametophyte, we crossed heterozygous mutant plants as female parents with wild-type males and scored the number of F1 progeny that were heterozygous (KanR or exhibited 50% seed set) and homozygous wild type (KanS or exhibited 100% seed set). The data from these crosses are summarized in Table 2. With all mutants tested, the ratio of heterozygous to homozygous wild-type (KanR/KanS) progeny was <1.0, indicating that these mutations exhibited reduced transmission through the female gametophyte and thus affected the female gametophyte. With the *fem1* and *fem4* mutants, no transmission of the mutation was observed, indicating that the *fem1* and *fem4* mutations were 100% penetrant in the female gametophyte. By contrast,

TABLE 2
Results from Crosses of Heterozygous Female Gametophyte Mutants as Female Parents with Wild-Type Males

Mutant	No. of progeny		Het/Homo[a]	Penetrance in FG
	Heterozygous	Homozygous		
fem1	0	206	0	100
fem2	273	486	0.56	44
fem3	225	388	0.58	42
fem4	0	111	0	100

[a] Ratio of heterozygous to homozygous progeny.

TABLE 3

Results from Crosses of Heterozygous Female Gametophyte Mutants as Male Parents with Wild-Type Females

Mutant	No. of progeny		Het/Homo[a]	Penetrance in MG
	Heterozygous	Homozygous		
fem1	219	221	0.99	1[b]
fem2	342	303	1.13	0
fem3	340	606	0.56	44
fem4	238	412	0.58	42

[a] Ratio of heterozygous to homozygous progeny.
[b] Not significantly different than 0%.

with the *fem2* and *fem3* mutants, at least 56% transmission of the mutation through the female gametophyte was observed, indicating that the corresponding mutations were partially penetrant in the female gametophyte. We, in addition, carried out crosses with the *gfa2, gfa3, gfa4, gfa5,* and *gfa7* mutants and obtained penetrance values similar to those reported previously (Feldmann *et al.,* 1997).

Female Gametophyte Specificity

To determine whether the *fem* mutations also affect the male gametophyte, we crossed heterozygous mutant plants as male parents with wild-type females and scored the number of F1 progeny that were heterozygous (KanR or exhibited 50% seed set) and homozygous wild type (KanS or exhibited 100% seed set). The data from these crosses are summarized in Table 3. With the *fem1* and *fem2* mutants, heterozygous and homozygous wild-type progeny were present in approximately equal proportions in the F1 generation, indicating that the *fem1* and *fem2* mutations do not affect the male gametophyte. By contrast, with the *fem3* and *fem4* mutants, the ratio of heterozygous to homozygous wild-type (KanR/KanS) progeny was <0.6, indicating that the corresponding mutations exhibited reduced transmission through the male gametophyte and thus affected the male gametophyte.

In summary, we have identified two classes of female gametophyte mutations. One class affects the female gametophyte but not the male gametophyte and the second class affects both gametophytes. We refer to these two classes as female gametophyte-specific mutations and general gametophytic mutations, respectively. The class that each of the female gametophyte mutations falls within is listed in Table 1.

Megagametogenesis in the fem and gfa Mutants

Female gametophyte mutations could affect the female gametophyte either by disrupting megagametogenesis or by interfering with one of the female gametophyte's functions. To distinguish between these possibilities, we analyzed the terminal phenotypes of the female gametophyte mutants. To do this, we allowed the female gametophytes within heterozygous pistils to progress to the terminal developmental stage (stage FG7, Fig. 2A) and examined at least 80 female gametophytes for each mutant. The *fem1, fem2, fem3, fem4, gfa2, gfa3, gfa4, gfa5,* and *gfa7* mutants had morphologically abnormal female gametophytes at the terminal developmental stage, indicating that the mutations in these lines affect megagametogenesis. The terminal phenotypes of the female gametophyte mutants are summarized in Table 4 and are discussed in detail below.

To determine the developmental stage at which megagametogenesis departs from wild type in the *fem* and *gfa* mutants, we analyzed megagametogenesis at all developmental stages in heterozygous pistils. We previously showed that female gametophyte development within a pistil is synchronous (Christensen *et al.,* 1997), which allowed us to use wild-type female gametophytes to determine the developmental stage of mutant female gametophytes within the same pistil. Each developmental stage was analyzed by confocal laser scanning microscopy (CLSM). Using this procedure, nucleoli appear white, cytoplasm appears gray, and vacuoles appear black (Figs. 1C, 3, and 4) (Christensen *et al.,* 1997). The results of this analysis are summarized in Table 5. CLSM images of mutant female gametophytes are shown in Figs. 3 and 4.

Megagametogenesis in wild type. Megagametogenesis in wild-type *Arabidopsis* has been described (Misra, 1962; Poliakova, 1964; Webb and Gunning, 1990; Mansfield *et al.,* 1991; Murgia *et al.,* 1993; Webb and Gunning, 1994; Schneitz *et al.,* 1995; Christensen *et al.,* 1997) and has been divided into seven morphologically distinct stages that are depicted in Fig. 2A (Christensen *et al.,* 1997). First, the surviving megaspore (stage FG1) undergoes mitosis to produce a two-nucleate cell (stage FG2). Shortly thereafter, the two nuclei separate to the chalazal and micropylar poles and a vacuole forms in the center (stage FG3). The developing female gametophyte then undergoes second (stage FG4) and third rounds of mitosis. This results in an eight-nucleate cell with four nuclei at each of two poles separated by a large central vacuole (beginning of stage FG5). Next, one nucleus from each pole (the polar nuclei) migrates toward the female gametophyte's center and the embryo sac becomes cellularized. The polar nuclei eventually fuse and the female gametophyte consists of seven cells and seven nuclei (stage FG6; Figs. 1A and 1B). As a final developmental step, the three antipodal cells undergo cell death. Thus, the mature *Arabidopsis* female gametophyte (stage FG7) consists of one haploid egg cell, two haploid synergid cells, and one diploid central cell (Figs. 1C and 2B). Megagametogenesis in *Arabidopsis* occurs over a period of approximately 3.5 days (C. A. Christensen and G. N. Drews, unpublished results).

To determine the extent to which abnormal female gametophyte development occurs in wild type, we analyzed >1000 wild-type female gametophytes encompassing all developmental stages. Under our growth conditions (Chris-

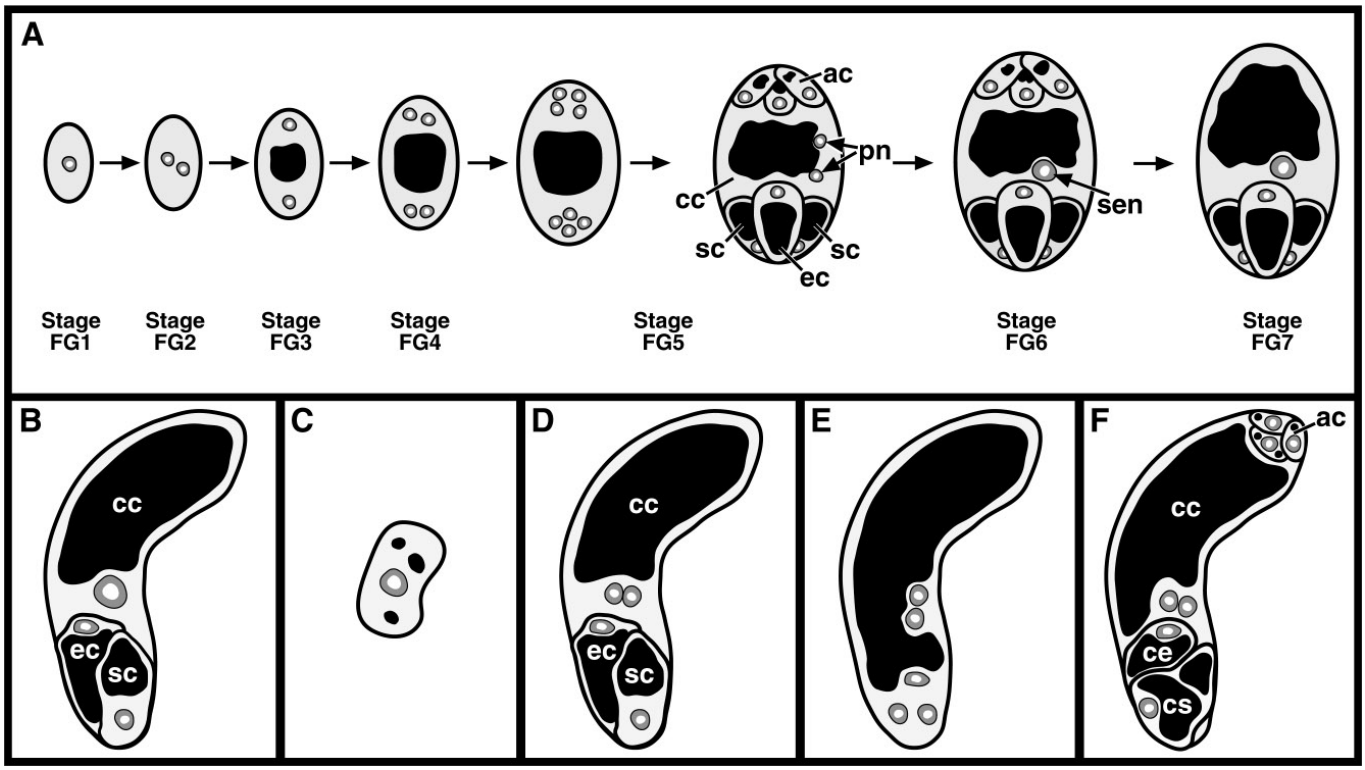

FIG. 2. Depictions of megagametogenesis in wild-type (A,B) and female gametophyte mutant phenotypes at the terminal stage (C–F). (A) Megagametogenesis stages (Christensen *et al.*, 1997). (B) Longitudinal view of a wild-type female gametophyte at the terminal stage (stage FG7). (C) Terminal phenotype of *fem2, fem3, gfa4,* and *gfa5* female gametophytes. (D) Terminal phenotype of *gfa2, gfa3,* and *gfa7* female gametophytes. (E) Terminal phenotype of *gfa3* and *gfa7* female gametophytes. (F) Terminal phenotype of the *fem4* mutant. Depicted are cytoplasm (light gray areas), vacuoles (black areas), nuclei (dark gray areas), and nucleoli (white areas within the nuclei). All female gametophytes are oriented with their chalazal poles up and their micropylar poles down. Abbreviations: ac, antipodal cells; cc, central cell; ce, cell in similar position and polarity to egg cell; cs, cell in similar position and polarity to synergid cell; ec, egg cell; pn, polar nucleus; sc, synergid cell; sen, secondary endosperm nucleus.

tensen *et al.*, 1997), less than 1% of the ovules we observed contained a female gametophtye that had undergone abnormal development. Of those that developed abnormally, all aborted at the one-nucleate stage and none had a defect in a later developmental step (i.e., polar nuclei migration, cellularization, polar nuclei fusion, or antipodal cell death).

gfa2. At the terminal developmental stage (stage FG7) approximately half of the embryo sacs in *gfa2/GFA2* pistils were phenotypically mutant (Table 5), consistent with the 100% penetrance of the *gfa2* mutation in the female gametophyte (Feldmann *et al.*, 1997). The terminal phenotype of *gfa2* female gametophytes is shown in Fig. 3B and depicted in Fig. 2D. In mature *gfa2* female gametophytes, the overall morphology of the egg cell, central cell, and synergid cells was normal (Fig. 3B and data not shown). However, in contrast to wild-type female gametophytes at stage FG7, the nucleoli of the two polar nuclei were unfused (Fig. 3B). Because Fig. 3B is a projection of several CLSM optical sections, the nuclear membrane boundary was not distinguishable. Figures 3C and 3D are single optical sections of mature *gfa2* female gametophytes showing that the two polar nuclei, as well as the nucleoli, were separate. Thus, the *gfa2* mutation appears to specifically affect fusion of the polar nuclei.

To determine the stage in which female gametophyte development departs from wild type, we analyzed *gfa2/GFA2* pistils containing female gametophytes between stages FG1 and FG7. In *gfa2/GFA2* pistils containing female gametophytes between stages FG6 and FG7 (pistil stages FG6/7 and FG7), approximately half (109/207) of the female gametophytes observed were abnormal (Table 5). By contrast, in *gfa2/GFA2* pistils containing female gametophytes at stage FG5 and earlier (pistil stages FG1-FG5/6), essentially all (283/291) female gametophytes observed were morphologically wild type (Table 5). We therefore conclude that *gfa2* female gametophytes begin to develop abnormally beginning at the transition from stage FG5 to stage FG6, which is the time when the polar nuclei fuse (Fig. 2A).

In our analysis of developing female gametophytes, the major defect observed was a failure of the polar nuclei to fuse. In wild-type female gametophytes, antipodal cell degeneration occurs subsequent to polar nuclei fusion. In *gfa2*

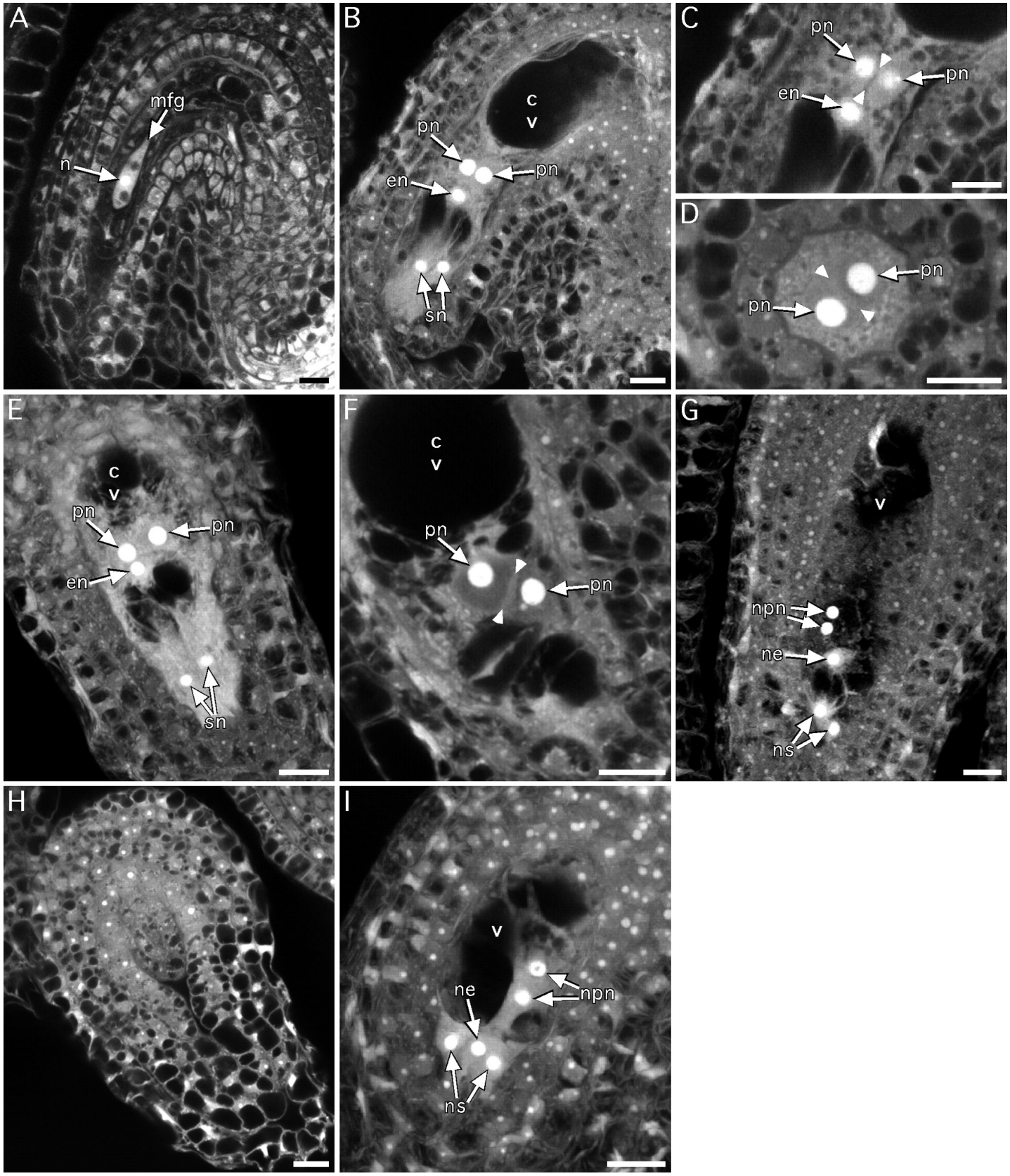

FIG. 3. CLSM images of *fem3*, *gfa2*, and *gfa3* female gametophytes. (A) Terminal phenotype of a *fem3* female gametophyte. This image is a projection of two 1.5-μm optical sections. (B) Terminal phenotype of a *gfa2* female gametophyte. This image is a projection of four 1.5-μm optical sections. (C,D) Single optical sections showing a magnified view of the unfused polar nuclei in two *gfa2* female gametophytes. The image in C is a single optical section of the projection shown in B. Arrowheads show the position of the nuclear

TABLE 4
Terminal Phenotypes of Female Gametophyte Mutants

Mutant	Phenotype
fem1	Female gametophyte absent. Embryo sac cavity filled with highly autofluorescent material.
fem2	One-nucleate female gametophyte.
fem3	One-nucleate female gametophyte.
fem4	At the micropylar pole, cell morphology, including nuclear positioning, vacuolar and cellular shape, and vacuole number was irregular. Nuclear and cell numbers correspond to those of stages FG5, FG6, or FG7.
gfa2	Polar nuclei fail to fuse.
gfa3	In most (59%), the polar nuclei fail to fuse. In some (22%), the embryo sac has five nuclei in approximately the correct positions (to be the egg, synergid and unfused polar nuclei), but appears to be uncellularized. In some (19%), the ovules lacked female gametophytes.
gfa4	One-nucleate female gametophyte.
gfa5	One-nucleate female gametophyte.
gfa7	In most (58%), the polar nuclei fail to fuse. In some (28%), the embryo sac has five nuclei in approximately the correct positions (to be the egg, synergid and unfused polar nuclei), but appears to be uncellularized. In some (14%), the ovules lacked female gametophytes.

female gametophytes, antipodal cell degeneration also occurs, although it appeared to occur more slowly than in wild type (data not shown).

gfa3. Under our growth conditions, the penetrance of the *gfa3* mutation in the female gametophyte was 82%; thus, the expected frequency of phenotypically mutant embryo sacs in *gfa3*/*GFA3* pistils was 41%. However, at the terminal developmental stage (stage FG7), approximately 30% of the female gametophytes in *gfa3*/*GFA3* pistils were phenotypically mutant (Table 5), suggesting that some nonfunctional *gfa3* female gametophytes were morphologically normal at the resolution of CLSM.

The phenotypes exhibited by *gfa3* female gametophytes at the terminal developmental stage are shown in Figs. 3E–3H and depicted in Figs. 2D and 2E. The predominant (54/92) defect observed was failure of the polar nuclei to fuse (Figs. 2D and 3E). As with *gfa2* mutants, individual optical sections showed that the polar nuclei were adjacent to one another but remained separate (Fig. 3F). Two less frequent terminal phenotypes were also observed at the terminal stage (Table 4). The first was female gametophytes that were uncellularized but contained five nuclei that were in approximately the correct positions to be the egg nucleus, synergid nuclei, and unfused polar nuclei (Figs. 2E and 3G). The second was an ovule lacking a female gametophyte (Fig. 3H).

The defects exhibited by developing *gfa3* female gametophytes are summarized in Table 6. The major defect observed occurred during stage FG6. During stage FG6 in wild type, the polar nuclei are fused and the antipodal cells are undergoing cell death. By stage FG7, the antipodal cells are completely degenerated (Fig. 2A). In *gfa3*/*GFA3* pistils at stage FG7, 304 female gametophytes were observed that lacked antipodal cells, and of these 54 had unfused polar nuclei ("unfused polar nuclei" column in Table 6, Fig. 3E). Prior to stage FG6, two less frequent defects were observed. The first was compressed embryo sacs at the four-nucleate stage ("Four nuclei and compressed" column in Table 6) and the second was uncellularized female gametophytes at late stage FG5 ("Uncellularized" column in Table 6, Fig. 3I). Based on frequency and developmental progression, these two defects probably gave rise to the ovules lacking female gametophytes ("No FG" column in Table 6, Fig. 3H) and the uncellularized female gametophytes observed at the terminal stage ("Uncellularized" column in Table 6, Fig. 3G), respectively.

gfa7. The *gfa7* mutant exhibited the same three terminal phenotypes that were exhibited by the *gfa3* mutant in

boundaries. (E) Terminal phenotype of a *gfa3* female gametophyte. This image is a projection of five 1.5-μm optical sections. (F) Single optical section showing a magnified view of the unfused polar nuclei in a *gfa3* female gametophyte. Arrowheads show the position of the nuclear boundaries. (G) Terminal phenotype of a *gfa3* female gametophyte. This image is a projection of eight 1.5-μm optical sections. (H) Terminal phenotype of a *gfa3* female gametophyte. This image consists of a single optical section. (I) Phenotype of a *gfa3* female gametophyte at pistil stage FG5. This image is a projection of eight 1.5-μm optical sections. All female gametophytes are oriented with their chalazal poles up and micropylar poles down and their abaxial surface to the left and adaxial surface to the right. Abbreviations: en, egg cell nucleus; cv, central cell vacuole; mfg, mutant female gametophyte; n, nucleus; ne, nucleus in similar position to egg nucleus; npn, nucleus in similar position to polar nucleus; ns, nucleus in similar position to synergid nucleus; pn, polar nucleus; sn, synergid cell nucleus; v, vacuole. Scale bars, 10 μm.

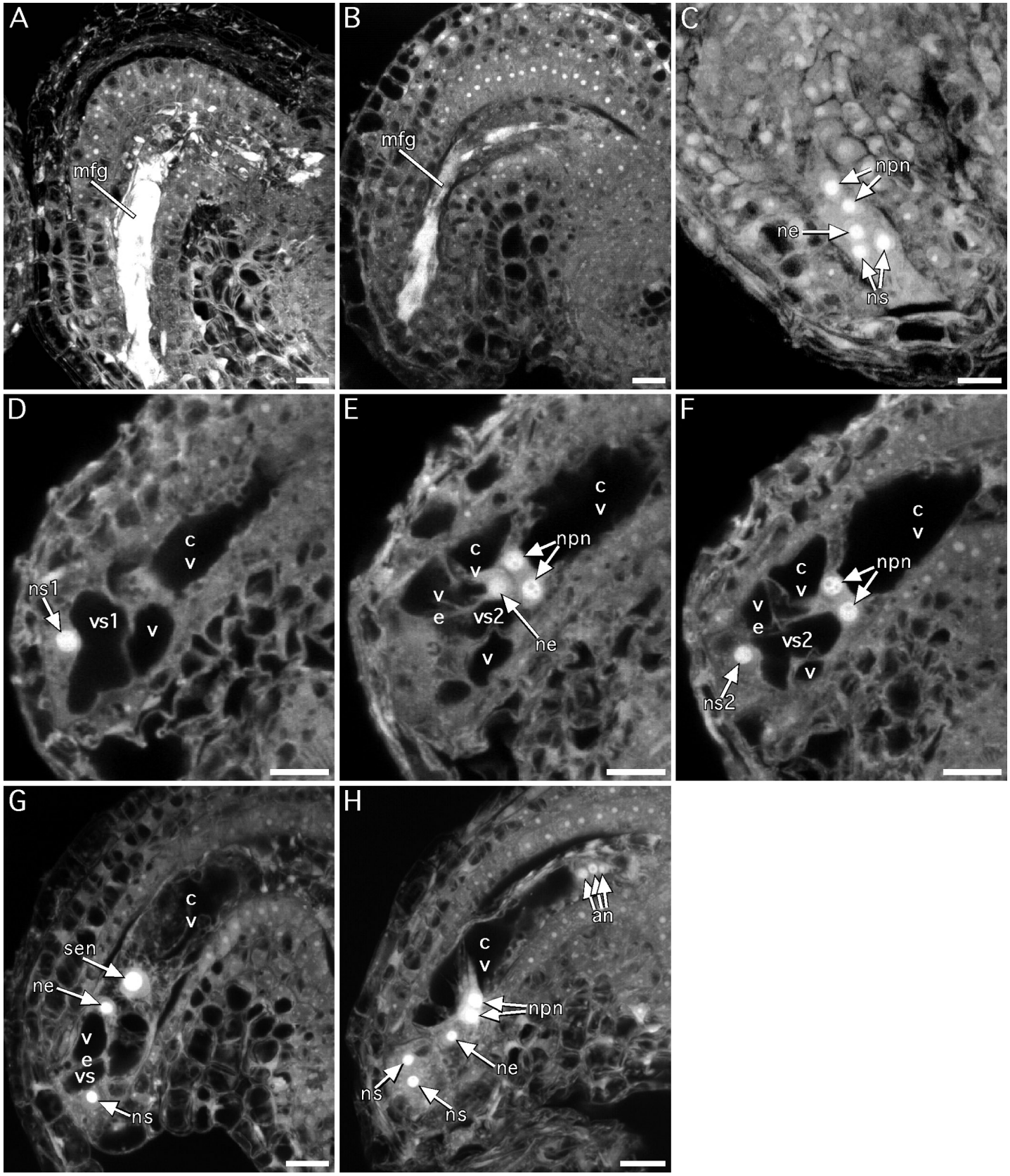

FIG. 4. CLSM images of *fem 1* and *fem 4* female gametophytes. (A) Terminal phenotype of a *fem 1* female gametophyte. This image is a projection of six 1.5-μm optical sections. (B,C) Phenotypes of two *fem 1* female gametophytes at pistil stage FG5/6. Each image is a projection of four 1.5 μm optical sections. (D–H) Phenotypes of *fem 4* female gametophytes at the terminal stage. Due to abnormal cellular morphologies, distinct cellular identities could not be assigned. The position and overall polarity of cell e are similar to those of the egg cell.

TABLE 5
Summary of CLSM Analysis of Female Gametophyte Mutants

Mutant	Pistil stage[a]	No. of pistils analyzed	No. of normal FGs observed	No. of abnormal FGs observed	% Abnormal
fem 1	FG3–FG4/5	9	108	2	2
	FG5	4	60	0	0
	FG5/6	4	41	33	45
	FG6/7	9	74	77	51
	FG7	5	38	42	53
fem 4	FG1–FG3/4	11	158	1	< 1
	FG4	4	51	4	7
	FG4/5	4	37	7	16
	FG5	5	45	13	22
	FG5/6	2	19	13	41
	FG6	2	11	10	48
	FG6/7	4	18	17	49
	FG7	10	86	86	50
gfa2	FG1–FG4/5	15	197	3	2
	FG5	6	60	1	2
	FG5/6	2	26	5	16
	FG6/7	3	21	19	48
	FG7	11	77	90	54
gfa3	FG1–FG4	7	83	0	0
	FG4/5	4	77	3	4
	FG5	5	44	10	19
	FG5/6	7	66	12	15
	FG7	18	212	92	30

[a] Assignment of a pistil stage is described under Materials and Methods.

approximately the same frequencies (Table 4). Since the *gfa3* and *gfa7* mutants have an identical terminal phenotype (Table 4) and also have the same map position (Feldmann *et al.*, 1997), they most likely harbor mutations in the same gene. For this reason, we examined earlier developmental stages only in the *gfa3* mutant.

fem1. At the terminal developmental stage (stage FG7), approximately half of the female gametophytes in *fem1*/*FEM1* pistils were phenotypically mutant (Table 5), consistent with the 100% penetrance of the *fem1* mutation. As shown in Fig. 4A, *fem1* female gametophytes at the terminal stage were completely degenerated and their embryo sac cavities were filled with autofluorescent material.

As shown in Table 5, *fem1* female gametophytes begin to develop abnormally at approximately late stage FG5 or stage FG6, which is about the time when the polar nuclei fuse (Fig. 2A). The most commonly observed (27/33) phenotype at this developmental time is shown in Fig. 4B. As with later stages (Fig. 4A), the female gametophyte was completely degenerated and the embryo sac cavity was

The positions and overall polarities of cells s1 and s2 are similar to those of the synergids. (D–F) Serial optical sections of the micropylar half of one *fem4* female gametophyte. (D) A single optical section showing cell s1 (ns1 and vs1). (E) A projection of two 1.5-μm optical sections showing cell e (ne and ve). (F) A projection of two 1.5-μm optical sections showing cell s2 (ns2 and vs2). The vacuole labeled v was in addition to the three vacuoles normally seen in the egg apparatus. The two vacuoles labeled cv were connected (not shown). (G) In this *fem4* female gametophyte, the polar nuclei have fused (sen) and the antipodal cells have degenerated. This image is a projection of three 1.5-μm optical sections. An additional synergid cell was present but not included in the projection. (H) In this *fem4* female gametophyte, the polar nuclei (npn) were unfused and the antipodal cells (an) were present. This image is a projection of five 1.5-μm optical sections. All female gametophytes are oriented with their chalazal poles up and micropylar poles down and their abaxial surface to the left and adaxial surface to the right. Abbreviations: an, antipodal cell nucleus; cv, central cell vacuole; mfg, mutant female gametophyte; ne, nucleus in similar position to egg nucleus; npn, nucleus in similar position to polar nucleus; ns, nucleus in similar position to synergid nucleus; ns1, nucleus of cell s1; ns2, nucleus of cell s2; sen, secondary endosperm nucleus; v, vacuole; ve, vacuole in similar position to egg cell vacuole; vs, vacuole in similar position to synergid cell vacuole; vs1, vacuole of cell s1; vs2, vacuole of cell s2. Scale bars, 10 μm.

TABLE 6
Summary of Defects Observed in Developing *gfa3* Female Gametophytes

Pistil stage[a]	No. of pistils analyzed	No. of FGs analyzed	No. of abnormal female gametophytes observed				
			Four nuclei and compressed	No FG	Uncellularized	Unfused polar nuclei	Total
FG4/5	4	80	1	0	2	0	3
FG5	5	54	5	1	3	1	10
FG5/6	7	78	2	6	3	1	12
FG7	18	304	0	18	20	54	92

[a] Assignment of a pistil stage is described under Materials and Methods.

filled with autofluorescent material. Occasionally (6/33) the less severe phenotype shown in Fig. 4C was observed. In such female gametophytes, the nuclei were present in the correct numbers and at the appropriate positions to be the polar nuclei, egg nucleus, and synergid nuclei, but a prominent central vacuole was absent (compare Fig. 4C with Fig. 1C). In some (3/6) of these, the "polar nuclei" were adjacent to each other but were unfused (e.g., the female gametophyte shown in Fig. 4C). Generally, one or more of the nuclei had an irregular shape and thus was probably in the process of degeneration (data not shown). Taken together, these data suggest that development of *fem1* female gametophytes begins to depart from wild type just before polar nuclei fusion (late stage FG5) and that an early event appears to be degeneration of the central vacuole.

fem4. At the terminal developmental stage (stage FG7), 50% of embryo sacs in *fem4*/*FEM4* pistils were abnormal (Table 5), corresponding to the 100% penetrance of the *fem4* mutation (Table 2). The terminal phenotype of *fem4* female gametophytes is shown in Figs. 4D–4H and depicted in Fig. 2F. Essentially all (85/86) of the *fem4* female gametophytes we observed at the terminal developmental stage had defects in cellular morphology in the cells at the embryo sac's micropylar pole (Table 7). In wild type, the egg cell has a distinctive pear shape and a highly polarized cytoplasm; its nucleus and most of its cytoplasm are at its chalazal end and a large vacuole occupies the remaining three-fourths of the cell (Fig. 1). Likewise, wild-type synergid cells have a distinctive shape and a highly polarized cytoplasm, which has the opposite polarity of the egg cell cytoplasm (Fig. 1). As with wild type, *fem4* female gametophytes at the terminal developmental stage had three cells at the micropylar pole. However, in contrast to wild type, these cells had irregular shapes and polarities. An example of a *fem4* female gametophyte at the terminal developmental stage is shown in Figs. 4D–4F. In this example, the egg cell is not pear shaped and is tilted relative to the female gametophyte's long axis (Fig. 4E), and the synergid cells were abnormally shaped and their nuclei occupied positions more chalazal and abaxial than those of wild type (Figs. 4D and 4F).

In addition to the cellular morphology defects exhibited by all *fem4* female gametophytes, some mature *fem4* fe-

TABLE 7
Summary of Defects Observed in Developing *fem4* Female Gametophytes

Pistil stage[a]	No. of pistils analyzed	No. of FGs analyzed	No. of abnormal female gametophytes observed					
			Abnormal nuclear position at stage FG4	Developmental delay	Abnormal cellular morphology at stage FG5	Abnormal cellular morphology at stage FG6	Abnormal cellular morphology at stage FG7	Total
FG4	4	55	2	0	1	1	0	4
FG4/5	4	44	4	0	2	1	0	7
FG5	5	58	3	3[b]	7	0	0	13
FG5/6	2	32	4	0	9	0	0	13
FG6	2	21	0	10	0	0	0	10
FG6/7	4	35	0	0	15	2	0	17
FG7	10	172	0	1	35	23	27	86

[a] Assignment of a pistil stage is described under Materials and Methods.
[b] One of these female gametophytes was at stage FG3, and the others were at stage FG4.

male gametophytes also exhibited defects in polar nuclei fusion and antipodal cell degeneration, which are developmental steps that follow cellularization (Fig. 2A). Of the 86 abnormal female gametophytes observed at the terminal developmental stage, 27% had intact antipodal cells and 41% had unfused polar nuclei and intact antipodal cells (Fig. 4H, Table 7).

To determine the developmental stage at which cellular morphology becomes abnormal, we analyzed female gametophyte development in *fem4*/*FEM4* pistils at all developmental stages. These data are summarized in Table 7. In wild type, cellularization begins immediately following the third mitosis (early stage FG5) and by late stage FG5, the egg and synergid cells have distinct polarities and morphologies as described above (Fig. 2A; Christensen *et al.*, 1997). In *fem4* female gametophytes at late stage FG5, the egg and synergid cells already exhibited defects in cell shape, vacuolar shape, and cell position, indicating that cellular morphology was abnormal as soon as these cells were formed (Table 7).

Analysis of female gametophyte development in the *fem4* mutant also revealed two subtle defects that occurred before stage FG5 and were not manifested at the terminal developmental stage (Table 7). The first was abnormal nuclear position at stage FG4 ("Abnormal nuclear position at stage FG4" column in Table 7). In *fem4*/*FEM4* pistils, some four-nucleate embryo sacs were observed in which one of the two nuclei at each pole was closer to the center of the embryo sac than in wild type (data not shown). The second defect was apparent developmental asynchrony ("Developmental delay" column in Table 7). We previously showed that megagametogenesis in wild type is fairly synchronous and that female gametophytes at stage FG4 are not present in pistils containing female gametophytes at stage FG6 (Christensen *et al.*, 1997). However, some *fem4*/*FEM4* pistils contained both stage FG4 and stage FG6 female gametophytes (Table 7). Nevertheless, *fem4* female gametophytes do progress beyond stage FG4 because by the terminal stage nearly all (85/86) *fem4* female gametophytes observed had undergone the third mitosis and had become cellularized (Table 7).

fem2, fem3, gfa4, and gfa5. The phenotype of the *fem2*, *fem3*, *gfa4*, and *gfa5* mutants is depicted in Fig. 2C. In this group, megagametogenesis did not progress beyond stage FG1 (one-nucleate stage; Fig. 2A). As shown in Fig. 3A, ovule morphology (integument length and ovule shape) was similar to ovules containing mature, wild-type female gametophytes. However, female gametophyte morphology was similar to female gametophytes at stage FG1 [tear drop shape, one nucleus, and small vacuoles (Webb and Gunning, 1990; Christensen *et al.*, 1997)]. We did not examine earlier developmental stages in this group.

Within *fem2*/*FEM2*, *fem3*/*FEM3*, *gfa4*/*GFA4*, and *gfa5*/*GFA5* pistils, the frequencies of morphologically abnormal female gametophytes were 51, 47, 10, and 18%, respectively. The fact that the these frequencies were higher than expected for the *fem2* and *fem3* mutants (based on the genetic transmission data in Table 2) suggests that these mutations may be linked to chromosomal rearrangements (Ray *et al.*, 1997). The fact that these frequencies were higher than expected for the *gfa4* and *gfa5* mutants [based on the 42% penetrance of the *gfa4* mutation and the 79% penetrance of the *gfa5* mutation reported by Feldmann *et al.* (1997)] may suggest that some morphologically normal female gametophytes were nonfunctional.

Microgametogenesis in the fem and gfa Mutants

As shown in Table 1, the *fem3*, *fem4*, *gfa2*, *gfa3*, *gfa4*, *gfa5*, and *gfa7* mutations also affect the male gametophyte. To determine whether they affect microgametogenesis, we analyzed the terminal phenotypes of these mutants. To do this, we used CLSM to analyze the male gametophytes within anthers from late stage 12 (just prior to dehiscence) flowers (Smyth *et al.*, 1990). The results of this analysis are shown in Fig. 5. Within *fem3*/*FEM3*, *gfa3*/*GFA3*, *gfa4*/*GFA4*, and *gfa5*/*GFA5* anthers, morphologically abnormal pollen grains were observed (Figs. 5B–5E). With these mutants, abnormal pollen grains were collapsed (arrows) or highly vacuolate (arrowheads). These data indicate that the *fem3*, *gfa3*, *gfa4*, and *gfa5* mutations affect microgametogenesis. By contrast, only morphologically normal pollen grains (over 75 pollen grains scored) were observed in *fem4*/*FEM4* and *gfa2*/*GFA2* anthers (Figs. 5F and 5G), suggesting that the *fem4* and *gfa2* mutations may affect some aspect of male gametophyte function.

DISCUSSION

Isolation of Female Gametophyte Mutants

Molecules mediating and regulating the major events of megagametogenesis (mitosis, polarity establishment, vacuole formation, nuclear migration, cellularization, cell specification and differentiation, nuclear fusion, and cell death) and female gametophyte function (pollen tube guidance, fertilization, induction of seed development, and gametophytic maternal control) have not been identified. To begin to identify these molecules, we have initiated a genetic screen for female gametophyte mutants that is based on two criteria, seed set and segregation distortion. Each of these criteria alone is ineffective in definitively identifying female gametophyte mutants. Reduced seed set screens are limited because this phenotype also can be caused by a variety of other factors including adverse environmental conditions (e.g., high growth temperature or water stress), chromosomal rearrangements (e.g., reciprocal translocations and large inversions), and sporophytic mutations (e.g., a female-sterile mutation with partial penetrance). Segregation distortion screens are limited because they identify both female gametophyte and male gametophyte mutations. However, when combined, these two criteria are effective in identifying female gametophyte mutants. Using

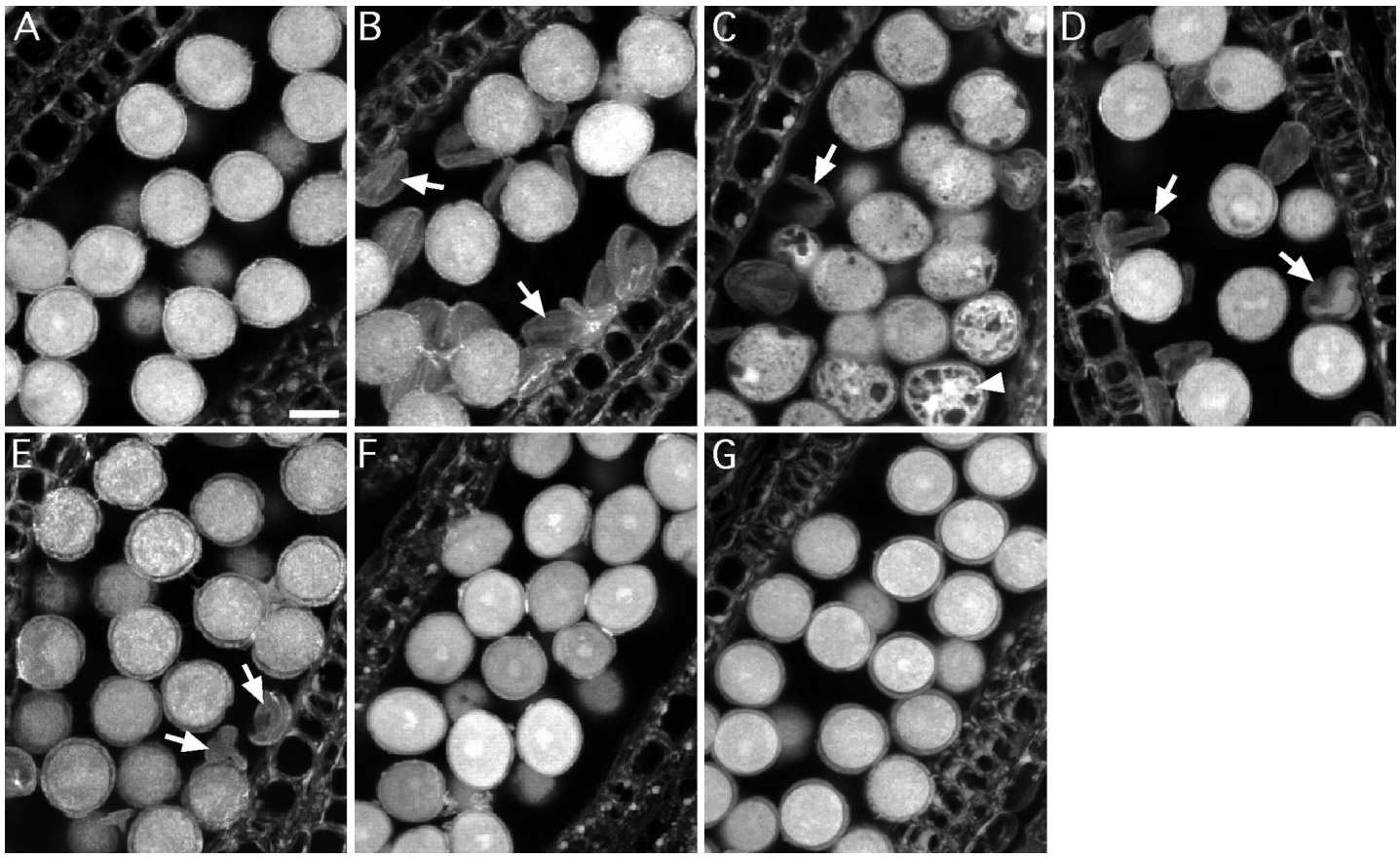

FIG. 5. CLSM images of mature pollen grains from wild type and the *fem3, fem4, gfa2, gfa3, gfa4,* and *gfa5* mutants. (A) Wild type. (B) *fem3*. (C) *gfa3*. (D) *gfa4*. (E) *gfa5*. (F) *fem4*. (G) *gfa2*. Arrows show collapsed pollen grains; arrowhead shows hypervacuolate pollen grain. All images are single 1.5-μm optical sections. Scale bar, 10 μm.

this screening procedure, we identified four lines (*fem1, fem2, fem3,* and *fem4*) containing female gametophyte mutations. Several female gametophyte mutants were previously identified based on reduced seed set or segregation distortion including the *Arabidopsis ctr1, emb173, Gf, gfa1 − gfa7, hdd, prl,* and *trp1;trp4* mutants (Redei, 1965; Castle *et al.*, 1993; Kieber *et al.*, 1993; Niyogi *et al.*, 1993; Springer *et al.*, 1995; Feldmann *et al.*, 1997; Moore *et al.*, 1997) and the maize *ig, lo1, lo2, sp1,* and *sp2* mutants (Manglesdorf, 1931; Singleton, 1932; Rhoades and Rhoades, 1939; Singleton, 1940; Singleton and Mangelsdorf, 1940; Nelson and Clary, 1952; Kermicle, 1971). The isolation of these mutants, as well as the mutants described in this paper, demonstrates that the criteria of reduced seed set and segregation distortion should be effective in large-scale screens for female gametophyte mutants.

Female Gametophyte-Specific and General Gametophytic Mutations

The mutants discussed in this paper fall into two classes. The *fem1* and *fem2* mutations affect the female gametophyte but not the male gametophyte and, thus, fall into the female gametophyte-specific class. The *fem3, fem4, gfa2, gfa3, gfa4, gfa5,* and *gfa7* mutations affect both the female and male gametophyte and, therefore, are general gametophytic (Feldmann *et al.*, 1997). Female gametophyte-specific mutations are important because the affected genes encode functions required by the female gametophyte but not the male gametophyte including the establishment of female gametophyte polarity, specification and differentiation of the female gametophyte cells, controlled cell death of the antipodal and synergid cells, central vacuole formation and maintenance, pollen tube guidance, induction of seed development, and gametophytic maternal control (Drews *et al.*, 1998). General gametophytic mutations are also an important mutation class because many important cellular processes are required in both gametophytes including mitosis, vacuole formation, cell expansion, subcellular migration, nuclear migration and fusion, and cellularization. Thus, mutations affecting some of the major events of megagametogenesis are likely to fall into the general gametophytic class (Drews *et al.*, 1998). With all of the mutants described in this paper, we do not yet know

whether the corresponding mutations also affect the sporophyte.

The fem1, fem2, fem3, fem4, gfa2, gfa3, gfa4, gfa5, and gfa7 Mutations Affect Megagametogenesis

All of the female gametophyte mutations we analyzed affect megagametogenesis. These nine mutations affect a range of steps. In the *fem2, fem3, gfa4,* and *gfa5* mutants, megagametogenesis does not progress beyond the one-nucleate stage (stage FG1; Figs. 2C and 3A). Developmental arrest at the one-nucleate stage could result from defects in a variety of processes including mitosis, cell cycle regulation, cellular metabolism, or developmental control. From the genetic and morphological data presented here, there is no way to determine which of these processes is affected by the *fem2, fem3, gfa4,* and *gfa5* mutations.

The *gfa2* mutation affects fusion of the polar nuclei (Figs. 2D and 3B–3D). In this mutant, the polar nuclei migrate together, come to lie side-by-side, but fail to fuse. Polar nuclei fusion has been described in plants and has been shown to consist of two steps. In the first step, the endoplasmic reticulum (ER) membranes that are continuous with the outer nuclear membranes of the two nuclei fuse, resulting in a continuous outer membrane surrounding unfused inner nuclear membranes; and in the second step, the inner nuclear membranes come in contact and merge to complete the fusion process (Jensen, 1964). We do not yet know which of these steps is affected by the *gfa2* mutation. Nuclear fusion has also been studied in yeast and has been found to occur via a process similar to that in plants (Kurihara *et al.,* 1994). Yeast nuclear fusion mutants have been identified and some of the affected genes encode components of the translocon, which is a protein complex embedded in the ER membrane that is responsible for protein translocation into the ER (Marsh and Rose, 1997).

Polar nuclei fusion is also affected by the *gfa3* and *gfa7* mutations (Figs. 2D, 3E, and 3F). However, unlike the *gfa2* mutation, the *gfa3* and *gfa7* mutations are pleiotropic and also result in uncellularized female gametophytes (Figs. 2E, 3G, and 3I). Pleiotropy of mutations affecting polar nuclei fusion is not surprising because, in yeast, some of the molecules required for nuclear fusion are also required for other cellular processes and, as a consequence, some of the yeast nuclear fusion mutations are pleiotropic (Marsh and Rose, 1997). For example, the yeast Sec63 protein is required for both nuclear fusion and protein translocation into the ER lumen (Ng and Walter, 1996). Thus, by analogy, the pleiotropy exhibited by the *gfa3* and *gfa7* mutations could reflect additional functions of the *GFA3* and *GFA7* gene products.

fem1 female gametophytes develop normally until late during stage FG5 (Table 5), which is just before polar nuclei fusion but after three rounds of mitosis and cellularization. Thus, the *fem1* mutation does not affect many fundamental cellular processes such as mitosis, vacuole formation, nuclear migration, and cellularization. In some (6/33, Table 5) *fem1* embryo sacs, most aspects of morphology are normal except that the central vacuole is not present (Fig. 4C). Thus, FEM1 may be required for maintenance of the central vacuole and the *fem1* mutation may cause disintegration of this vacuole. If this is true, then the more severe phenotype observed (Figs. 4A and 4B) could be caused by the release of vacuolar contents. Additional analysis using transmission electron microscopy is required to determine if that is the case.

fem4 female gametophytes exhibit defects in cellular morphology (Figs. 2F and 4D–4H). The mechanism of cellularization during megagametogenesis is not well understood (Russell, 1993). One model is that following the third mitosis, microtubules emanate radially from individual nuclei, phragmoplasts form where the microtubules meet, and cell plates form at the equator of the phragmoplasts (Brown and Lemmon, 1991; Russell, 1993). In support of this model, phragmoplasts have been observed in *Arabidopsis* female gametophytes between sister and nonsister nuclei following the third mitosis (Webb and Gunning, 1994). In this model, the location of the cell walls is ultimately determined both by the position of the nuclei and the organization of the cytoskeleton. If this model is correct, then the *fem4* mutation could affect cellular morphology by disrupting nuclear position or the organization or function of the cytoskeleton. Cytoskeletal organization defects also could explain the aberrant nuclear position and apparent developmental delay observed in *fem4* female gametophytes at stage FG4 (Table 7). Further analysis of cellularization and cytoskeletal organization in *fem4* female gametophytes is required to address these issues. Some *fem4* embryo sacs also exhibit defects in polar nuclei fusion and antipodal cell degeneration (Table 7); however, because these developmental steps occur after cellularization, these defects probably occur as a secondary consequence of the earlier cellularization defect.

Of the previously identified female gametophyte mutants, morphological analysis throughout megagametogenesis has been carried out only with the *Gf* (Redei, 1965), *hdd* (Moore *et al.,* 1997), *ig* (Kermicle, 1971), *lo2* (Nelson and Clary, 1952), and *prl* (Springer *et al.,* 1995) mutations. The *Gf* mutation causes a phenotype identical to that of the *fem2, fem3, gfa4,* and *gfa5* mutants, developmental arrest at the one nucleate stage (stage FG1). By contrast, the phenotypes of the *hdd, ig, lo2,* and *prl* mutants do not resemble those of the mutants presented here. The *hdd, lo2,* and *prl* mutations cause developmental arrest at the one-, two-, four-, or eight-nucleate stage (Moore *et al.,* 1997; Sheridan and Huang, 1997), and the *ig* mutation causes extra cycles of nuclear divisions to occur (Kermicle, 1971; Lin, 1978; Lin, 1981; Huang and Sheridan, 1996). In addition, the *hdd* and *ig* mutations cause asynchrony of nuclear divisions at the micropylar and chalazal poles (Huang and Sheridan, 1996; Moore *et al.,* 1997). These data suggest that the *HDD, IG, LO2,* and *PRL* genes may play a role in controlling nuclear division. Consistent with this idea, the *PRL* gene

is related to the yeast MCM2-3-5 genes that are required for initiation of DNA replication (Springer *et al.*, 1995).

The fem3, gfa3, gfa4, and gfa5 Mutations Affect Microgametogenesis

As discussed above, the *fem3, fem4, gfa2, gfa3, gfa4, gfa5,* and *gfa7* mutations affect both the female and male gametophyte. Thus, information about the pollen defects caused by these mutations may help to understand the functions of the corresponding genes during megagametogenesis. As a first step in this direction, we analyzed the terminal pollen phenotypes of these mutants. We found that *fem3, gfa3* (and presumably also *gfa7*), *gfa4,* and *gfa5* affect microgametogenesis. Further analysis is required to determine the specific steps of microgametogenesis affected. By contrast, the *gfa2* and *fem4* mutations do not appear to affect microgametogenesis and may instead affect some aspect of pollen function such as pollen germination, pollen tube growth, or pollen tube guidance.

ACKNOWLEDGMENTS

We thank Ken Feldmann for providing the *gfa2, gfa3, gfa4, gfa5* and *gfa7* mutants, Tom Jack for providing T-DNA mutagenized lines, and the *Arabidopsis* Biological Resource Center for making available the Ken Feldmann collection and *ms1-1*. We also thank Diana Lee for helpful comments on the manuscript. This work was supported by National Science Foundation Grant IBN-9630371.

REFERENCES

Bell, C. J., and Ecker, J. R. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. *Genomics* **19,** 137–144.

Brown, R. C., and Lemmon, B. E. (1991). Pollen development in orchids. 1. Cytoskeleton and the control of division plane in irregular patterns of cytokinesis. *Protoplasma* **163,** 9–18.

Castle, L. A., Errampalli, D., Atherton, T. L., Franzmann, L. H., Yoon, E. S., and Meinke, D. W. (1993). Genetic and molecular characterization of embryonic mutants identified following seed transformation in *Arabidopsis*. *Mol. Gen. Genet.* **241,** 504–514.

Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S., and Peacock, W. J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **94,** 4223–4228.

Christensen, C. A., King, E. J., Jordan, J. R., and Drews, G. N. (1997). Megagametogenesis in *Arabidopsis* wild type and the *Gf* mutant. *Sex. Plant Reprod.* **10,** 49–64.

Drews, G. N., Lee, D., and Christensen, C. A. (1998). Genetic control of female gametophye development and function. *Plant Cell* **10,** 1–15.

Feldmann, K. A., Coury, D. A., and Christianson, M. L. (1997). Exceptional segregation of a selectable marker (KanR) in *Arabidopsis* identifies genes important for gametophytic growth and development. *Genetics* **147,** 1411–1422.

Huang, B.-Q., and Sheridan, W. F. (1996). Embryo sac development in the maize *indeterminate gametophyte1* mutant: Abnormal nuclear behavior and defective microtubule organization. *Plant Cell* **8,** 1391–1407.

Hulskamp, M., Schneitz, K., and Pruitt, R. E. (1995). Genetic evidence for a long-range activity that directs pollen tube guidance in *Arabidopsis*. *Plant Cell* **7,** 57–64.

Jensen, W. A. (1964). Observations on the fusion of nuclei in plants. *J. Cell Biol.* **23,** 669–672.

Kermicle, J. L. (1971). Pleiotropic effects on seed development of the indeterminate gametophyte gene in maize. *Am. J. Bot.* **58,** 1–7.

Kieber, J. J., Rothenberg, M., Roman, G., Feldman, K. A., and Ecker, J. R. (1993). *CTR1,* a negative regulator of the ethylene response pathway in *Arabidopsis,* encodes a member of the Raf family of protein kinases. *Cell* **72,** 427–441.

Konieczny, A., and Ausubel, F. M. (1993). A procedure for maping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* **4,** 403–410.

Koornneef, M., and Stam, P. (1992). Genetic analysis. In "Methods in *Arabidopsis* Research" (C. Koncz, N.-H. Chua, and J. Schell, Eds.), pp. 83–99. World Scientific Publishing, Singapore.

Kosambi, D. D. (1944). The estimation of map distances from recombination values. *Ann. Eugen.* **12,** 172–175.

Kurihara, L. J., Beh, C. T., Latterich, M., Schekman, R., and Rose, M. D. (1994). Nuclear congression and membrane fusion: Two distinct events in the yeast karyogamy pathway. *J. Cell Biol.* **126,** 911–923.

Lin, B.-Y. (1978). Structural modifications of the female gametophyte associated with the *indeterminate gametophyte* (*ig*) mutant in maize. *Can. J. Genet. Cytol.* **20,** 249–257.

Lin, B.-Y. (1981). Megagametogenetic alterations associated with the indeterminate gametophyte (*ig*) mutation in maize. *Rev. Brasil Biol.* **41,** 557–563.

Maheshwari, P. (1950). "An Introduction to the Embryology of Angiosperms." McGraw-Hill, New York.

Manglesdorf, P. C. (1931). Modification of mendelian ratios in maize by mechanical separation of gametes. *Proc. Natl. Acad. Sci. USA* **17,** 698–700.

Mansfield, S. G., and Briarty, L. G. (1991). Early embryogenesis in Arabidopsis thaliana. II. The developing embryo. *Can. J. Bot.* **69,** 461–476.

Mansfield, S. G., Briarty, L. G., and Erni, S. (1991). Early embryogenesis in *Arabidopsis thaliana*. I. The mature embryo sac. *Can. J. Bot.* **69,** 447–460.

Marsh, L., and Rose, M. D. (1997). The pathway of cell and nuclear fusion during mating in *S. cerevisiae*. In "The Molecular and Cellular Biology of the Yeast *Saccharomyces*" (J. R. Pringle, J. R. Broach, and E. W. Jones, Eds.), pp. 827–888. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Misra, R. C. (1962). Contribution to the embryology of *Arabidopsis thaliana* (Gay and Monn.). *Agra Univ. J. Res. Sci.* **11,** 191–199.

Moore, J. M., Calzada, J.-P. V., Gagliano, W., and Grossniklaus, U. (1997). Genetic characterization of *hadad,* a mutant disrupting female gametogenesis in *Arabidopsis thaliana*. *Cold Spring Harbor Symp. in Quant. Biol.,* in press.

Murgia, M., Huang, B.-Q., Tucker, S. C., and Musgrave, M. E. (1993). Embryo sac lacking antipodal cells in *Arabidopsis thaliana* (Brassicaceae). *Am. J. Bot.* **80,** 824–838.

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8,** 4321–4325.

Nelson, O. E., and Clary, G. B. (1952). Genetic control of semi-sterility in maize. *J. Hered.* **43,** 205–210.

Ng, D. T. W., and Walter, P. (1996). ER membrane protein complex required for nuclear fusion. *J. Cell Biol.* **132,** 499–509.

Niyogi, K. K., Last, R. L., Fink, G. R., and Keith, B. (1993). Suppressors of *trp1* fluorescence identify a new *Arabidopsis* gene, *TRP4,* encoding the anthranilate synthase beta subunit. *Plant Cell* **5,** 1011–1027.

Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P., and Fischer, R. L. (1996). A mutation that allows endosperm development without fertilization. *Proc. Natl. Acad. Sci. USA* **93,** 5319–5324.

Poliakova, T. F. (1964). Development of the male and female gametophytes of *Arabidopsis thaliana* (L) Heynh. *Issledov Genet. USSR* **2,** 125–133.

Ray, A. (1997). Three's company: Regulatory cross-talk during seed development. *Plant Cell* **9,** 665–667.

Ray, S., Park, S.-S., and Ray, A. (1997). Pollen tube guidance by the female gametophyte. *Development* **124,** 2489–2498.

Redei, G. P. (1965). Non-mendelian megagametogenesis in *Arabidopsis*. *Genetics* **51,** 857–872.

Reiser, L., and Fischer, R. L. (1993). The ovule and the embryo sac. *Plant J.* **5,** 1291–1301.

Rhoades, M. M., and Rhoades, V. H. (1939). Genetic studies with factors in the tenth chromosome in maize. *Genetics* **24,** 302–314.

Russell, S. D. (1993). The egg cell: Development and role in fertilization and early embryogenesis. *Plant Cell* **5,** 1349–1359.

Schneitz, K., Hulskamp, M., and Pruitt, R. E. (1995). Wild-type ovule development in *Arabidopsis thaliana:* A light microscope study of cleared whole-mount tissue. *Plant J.* **7,** 731–749.

Sheridan, W. F., and Huang, B.-Q. (1997). Nuclear behavior is defective in the maize (Zea mays L.) *lethal ovule2* female gametophyte. *Plant J.* **11,** 1029–1041.

Singleton, W. R. (1932). Complete elimination of certain classes of gametes in *Zea*. *Proc. Sixth Int. Cong. Genet.* **2,** 182–184.

Singleton, W. R. (1940). Influence of female stock on the functioning of small pollen male gametes. *Proc. Natl. Acad. Sci. USA* **26,** 102–104.

Singleton, W. R., and Mangelsdorf, P. C. (1940). Gametic lethals on the fourth chromosome of maize. *Genetics* **25,** 366–389.

Smyth, D. R., Bowman, J. L., and Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis*. *Plant Cell* **2,** 755–767.

Springer, P. S., McCombie, W. R., Sundaresan, V., and Martienssen, R. A. (1995). Gene trap tagging of *PROLIFERA,* an essential *MCM2-3-5*-like gene in *Arabidopsis*. *Science* **268,** 877–880.

van der Veen, J. H., and Wirtz, P. (1968). EMS induced genic male sterility in *Arabidopsis thaliana:* A model selection experiment. *Euphytica* **17,** 371–377.

van Went, J. L., and Willemse, M. T. M. (1984). Fertilization. In "Embryology of Angiosperms" (B. M. Johri, Ed.), pp. 273–317. Springer-Verlag, Berlin.

Webb, M. C., and Gunning, B. E. S. (1990). Embryo sac development in *Arabidopsis thaliana*. I. Megasporogenesis, including the microtubular cytoskeleton. *Sex. Plant Reprod.* **3,** 244–256.

Webb, M. C., and Gunning, B. E. S. (1994). Embryo sac development in *Arabidopsis thaliana*. II. The cytoskeleton during megagametogenesis. *Sex. Plant Reprod.* **7,** 153–163.

Willemse, M. T. M., and van Went, J. L. (1984). The female gametophyte. In "Embryology of Angiosperms" (B. M. Johri, Ed.), pp. 159–196. Springer-Verlag, Berlin.

Received for publication April 20, 1998
Revised June 10, 1998
Accepted June 10, 1998