Isolation of Ethyl Methanesulfonate-Induced Gametophytic Mutants in *Arabidopsis thaliana* by a Segregation Distortion Assay Using the Multimarker Chromosome 1

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ABSTRACT

The life cycle of plants comprises two alternating generations, the diploid sporophyte (spore-bearing plant) and the haploid gametophyte (gamete-bearing plant). In contrast to animals, the postmeiotic cells give rise to haploid organisms whose function is to produce the gametes and to mediate fertilization. Analysis of gametophyte development and function has been hampered by the difficulty of identifying haplo-phase-specific mutants in conventional mutagenesis screens. Here we use a genetic strategy that is based on segregation distortion of nearby visible markers to screen for EMS-induced gametophytic mutants in *Arabidopsis thaliana*. Using the multiple marker chromosome *mm1* we have isolated seven lines that displayed an altered segregation of markers. Reciprocal backcrosses of these lines showed a marked reduction of the transmission of the male and/or female gametes. Phenotypic analysis revealed that different aspects of either gametophytic development or function were affected. Three male gametophytic lines showed specific arrests during pollen development. One male gametophytic line was specifically defective in pollen tube elongation. Three gametophytic lines showed variable defects in both male and female gametophytic development.

IN higher plants the gametophytic phase of the life cycle takes place in the reproductive tissues of the mature plant. Both the male and the female gametophytes develop from postmeiotic cells through mitotic divisions to produce haploid organisms. During male gametophytic development the microspore undergoes two mitotic divisions to produce a generative cell and two sperm cells (Mascarenhas 1989; McCormick 1993; Twell *et al.* 1998). Female gametophytic development comprises three synchronous rounds of free-nuclear mitosis followed by cellularization that results in a seven-celled embryo sac (Webb and Gunning 1990; Reiser and Fischer 1993; Grossniklaus and Schneitz 1998). Functions expressed in the male and female gametophytes as well as functions contributed by the surrounding diploid tissues are thought to be required to mediate the fusion of the gametes (Heslop-Harrison 1987; Sanders and Lord 1992; Pruitt and Hülskamp 1994; Hülskamp *et al.* 1995b; Preuss 1995).

Considering that gametophytes of higher plants have evolved from free living distinct organisms, it is conceivable that genes that are essential for important developmental processes or cellular functions are expressed during the haploid phase of the plant life cycle. This is supported by the analysis of transcriptional activity in maize pollen that suggests that gene expression overlaps substantially between the sporophytic and the gametophytic generation (Willing *et al.* 1988; Mascarenhas 1990). In addition, the examination of chromosomal deficiencies indicates the requirement of gametophytically expressed genes during male and female gametophytic development (Patterson 1978; Simcox *et al.* 1986; Zhao and Weber 1989; Kindiger *et al.* 1991; Vollbrecht and Hake 1995). A characterization of the different aspects of the gametophytic life cycle by single-gene mutational analysis is very limited. Only a few gametophytic mutants have been described previously (Singleton and Mangelsdorf 1940; Nelson and Clary 1952; Redei 1965; Kermicle 1971; Lin 1981; Springer *et al.* 1995; Chen and McCormick 1996; Ohad *et al.* 1996). More recently, two small-scale T-DNA insertional mutagenesis screens have identified additional male and female gametophytic mutants in *Arabidopsis thaliana* (Feldmann *et al.* 1997; Grossniklaus *et al.* 1998; Howden *et al.* 1998).

A genetic analysis of gametophytic mutants is challenging for several reasons. A systematic isolation of gametophytic mutants is difficult, because not all gametophytic mutations can easily be scored directly for a mutant phenotype nor do they cause sterility. It is also difficult to propagate lines carrying a gametophytic mutation without using a genetic balancing system. Moreover,

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haplo-phase-specific genes cannot be classified by complementation. Thus, the phenotype and the map position are the only criteria on which to decide whether different mutants are allelic or whether they represent different genes. One possibility for overcoming these obstacles is to identify gametophytic mutants based on the altered segregation of antibiotic resistance of T-DNAs or transposons that have inserted into genes essential for gametophytic development or function (Feldmann *et al.* 1997; Grossniklaus *et al.* 1998; Howden *et al.* 1998).

In this study we used an alternative strategy in which gametophytic mutants are recognized by the segregation distortion of nearby markers. Because this strategy enables gametophytic mutants to be identified only in close proximity to markers, we used a multiply marked chromosome 1 (mm1) that carries five visible recessive markers that are regularly distributed on the chromosome. This allowed us to simultaneously screen for all gametophytic mutations induced on this chromosome. To avoid segregation distortion caused by chromosomal rearrangements we used ethyl methanesulfonate (EMS) as a mutagen, which normally causes only point mutations. Here we present a small-scale screen in which we identified seven gametophytic mutations mapping to different marker intervals. Their genetic and morphological characterization revealed specific defects in either the development or function of mature gametophytes during the fertilization process.

MATERIALS AND METHODS

Plant strains and growth conditions: All experiments were performed using *A. thaliana* (L.) Heynh. var. *Landsberg* (*erecta* mutant) as wild type. *TH154*, a conditional male sterile mutant (Hülskamp *et al.* 1995a), was employed as the female partner in pollination experiments. Plants were grown under constant illumination at 25°.

Isolation and genetic characterization of gametophytic mutants: Plants heterozygous for the *mm1* line were EMS mutagenized as described previously (Mayer *et al.* 1991). Because M_1 plants are mosaic for potential new mutants, we collected seeds from side branches of single M_1 plants. Side branches have been shown to have a clonal origin (Misera 1993). M_2 plants were screened for >40% segregation of neighboring markers. All plants used for crosses or phenotypic analysis were rescreened for increased marker frequencies.

The map position of gametophytic mutants affecting the transmission via the male or female gametophyte was calculated from $p = 1 - 2M$, which was derived from the equation shown in Figure 1. If both gametophytic sexes are affected, plants homozygous for a marker result only from the parental gamete classes: $M = (1 - p)(1 - p)$. The map positions for this mutant class were calculated from $p = 1 \pm M^{0.5}$. These calculations are based on the assumption that the corresponding mutations are fully penetrant. An incomplete penetrance would result in an apparent increase of the genetic distance. However, the relative position of a gametophytic mutation within a given interval should be independent of the penetrance. Therefore, we based our calculations of the final map position on the relative genetic distance (RGD) of the gametophytic mutations to both flanking markers: RGD = $(p^{m1}/(p^{m1} + p^{m2}))$ INT, where INT is the size of the respective interval in centimorgans (see Figure 2).

Phenotypic characterization of gametophytic mutants: Ovule phenotypes were inspected in cleared whole-mount ovule preparations. Ovules were stained and processed as described by Schneitz *et al.* (1995).

Pollen development was studied in 4′,6-diamidino-2-phenylindole (DAPI)-stained whole-mount preparations (Coleman and Goff 1985; Jeppesen and Nielsen 1989) after a modified protocol of Regan and Moffatt (1990). Pollen from single anthers were dissected on a slide and mounted in an aqueous solution of 0.1% DAPI, 5% DMSO, and 1% Tween 20. Samples were incubated at 4° for up to 5 hr before inspection.

Scanning electron microscopy studies of mature pollen were performed as described previously (Hülskamp *et al.* 1995a). The ultrastructure of pollen at different developmental stages was analyzed by transmission electron microscopy. For transmission electron microscopy anthers were fixed and processed as described by Lukowitz *et al.* (1996). Ultrathin 50- to 80-nm sections were analyzed with a Phillips CM10 microscope.

β-Glucuronidase (GUS) assays were performed as described previously (Twell 1992).

In vitro pollen germination experiments were performed as described by Azarov *et al.* (1990). The path of the pollen tubes inside the pistil and the ability of the ovules to attract a pollen tube were visualized by staining whole pistils with aniline blue as described previously (Hülskamp *et al.* 1995b).

To analyze retardation of pollen tube growth of *mad4*, a temperature-sensitive male-sterile mutant, *TH154* (Hülskamp *et al.* 1995a), was pollinated with two to five pollen. After 24 hr pistils were cleared and stained with aniline blue as described in Hülskamp *et al.* (1995b). The number of germinated pollen and the number of pollen that reached the ovary were determined for each pistil.

Microscopy and graphic work: Light microscope preparations were examined using an Axiophot microscope with differential interference contrast (DIC) optics and epifluorescence attachment. Cytophotometry was done as previously described (Hülskamp *et al.* 1994). Photographs were taken on Kodak Ektachrome (Rochester, NY) color slide films. Pictures were processed using Adobe Photoshop 3.0 and Aldus Freehand 4.0 software.

RESULTS

Rationale of the screening strategy: Our screening strategy is based on the idea that a male or female gametophytic mutation on one chromosome should result in a higher apparent transmission frequency of the homologous chromosome. If the latter chromosome carried a recessive visible marker closely linked to the locus of the gametophytic mutation, the proportion of progeny plants showing the marker phenotype would increase from 25 to 50% [Figure 1, see only the parental classes (boldface square)]. However, meiotic recombination between the two loci would limit the increase in marker frequency and as the distance from the gametophytic locus increases, the apparent marker frequency would gradually decrease to the normal value of 25%. Thus, this strategy is limited by the distance at which a gametophytic mutation still causes a recognizable increase in marker frequency. We chose a marker frequency of 40% as our lower operational limit under

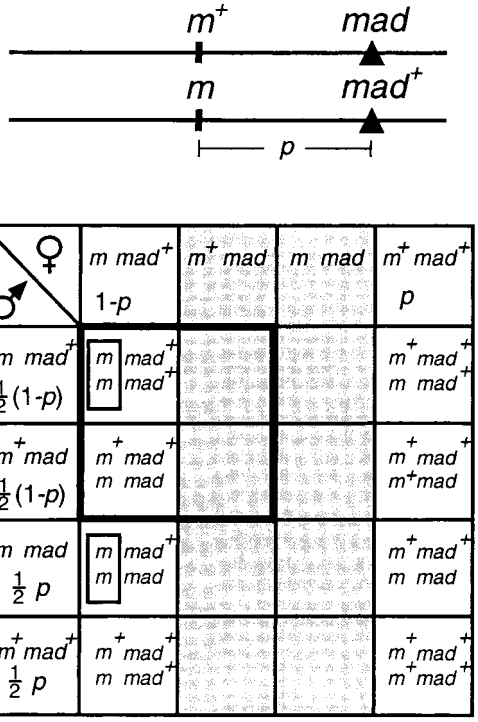

Figure 1.—Screening strategy. Detection of gametophytic mutations by increased frequency of recessive marker plants among progeny of selfed mutagenized plants. (Top) Transheterozygous plant carrying a male gametophytic defective (mad) mutation. (Middle) Progeny classes resulting from meiotic recombination and random fertilization of functional gametes (nonfunctional male gametophytes shaded). Parental classes are indicated as a boldface square. (Bottom) Proportion of recessive marker plants as a function of recombination. Distance (p) between marker (m) and mad locus. mad, mad$^+$: male gametophytic mutation and the corresponding wild-type locus. m,m+: recessive morphological marker and the corresponding wild-type locus. Marker frequency = $\frac{1}{2}(1 - p)(1 - p) + \frac{1}{2}p(1 - p) = \frac{1}{2}(1 - p)$. If the marker frequency = 0.4 the recombination distance (p is $\frac{1}{2}(1 - p) = 0.4$; $p = 0.2$. This corresponds to a genetic distance in centimorgans: $50 \ln(1/(1 - 2p)) = 25$ cM).

screening conditions. This corresponds to a distance of 25 cM (for calculation see Figure 1). We used a multiply marked chromosome 1 (*mm1*), which carries five visible recessive markers that cover the whole chromosome (Figure 2). Figure 2 shows the relative distances as calculated from the recombination frequencies between the five markers. On the basis of the above estimations any induced gametophytic defective mutation on chromosome 1 should result in an increased frequency of two neighboring markers. This has two important consequences: first, it reduces the probability of false positives and second, this strategy enables the newly identified gametophytic mutants to be mapped relative to the flanking markers.

Isolation and genetic characterization of gametophytic mutants: Out of 200 single lines screened we have isolated 7 lines that showed an increased frequency of two neighboring markers. Reciprocal backcrosses with *mm1* enabled us to determine whether the male [male gametophytic defective (mad)], the female [female gametophytic defective (fad)], or both the male and female gametes [both male and female gametophytic defective (bod)] are affected (Table 1). Transmission through the male gametes is specifically affected in 4 mutant lines, *mad1*, *mad2*, *mad3*, and *mad4*. Three lines, *bod1*, *bod2*, and *bod3*, turned out to be impaired in the transmission through both the male and the female gametes (Table 1).

The map position of each gene was calculated from the marker frequencies obtained for each line (Table 2, Figure 2). To take into account that the marker frequencies may be reduced by incomplete penetrance of the mutations, we calculated the RGD to the two flanking markers (Table 2 and Figure 2; for details see materials and methods).

Gametophytic mutations affecting the development of the male gametophyte: The male gametophyte (pollen) develops within the anther (microgametogenesis). After meiosis, the four haploid microspores begin to differentiate. The microspores enlarge and form a prominent vacuole (Figure 3, A and D, B and E). Subsequently, the nucleus moves to the pole and the microspore undergoes an asymmetric cell division, producing a large vegetative and a small generative cell (Figure 3, C and F). In whole-mount DAPI stainings the vegetative nucleus can easily be recognized as a large diffusely stained nucleus, while the generative nucleus appears smaller and more intensely stained. The generative cell is initially closely attached to the outer cell wall (bicellular stage I; Figure 3, C and F). Slightly later, this cell is detached from the outer wall and surrounded by the vegetative cell (bicellular stage II; Figure 3, G and J). The generative cell then divides to give rise to two sperm cells (tricellular stage; Figure 3, H and K; Mascarenhas 1989; McCormick 1993; Twell *et al.* 1998).

***mad1*:** In heterozygous *mad1* plants ~18% of the pollen at the tricellular stage showed no nuclear or cytoplasmic staining in whole-mount DAPI preparations and ~37% had only two nuclei ($n = 618$). In the latter class the vegetative nucleus always appeared normal in size and shape. The generative nucleus showed a variable phenotype: 62% were indistinguishable from wild type (Figure 4A), 8% were drastically increased in size (Figure 4B), and 27% had a speckled appearance (Figure 4C). Occasionally we found pollen carrying only one irregularly shaped enlarged nucleus (3%, Figure 4D). Some pollen of the two nuclear classes appeared to be viable. In *in vitro* germination experiments we occasionally found pollen tubes with one vegetative and one enlarged generative nucleus (Figure 4, E and F). DAPI stainings of pollen at the mononuclear stage revealed no deviation from wild type ($n = 400$). At the bicellular I stage ~19% of the pollen were unstained and 5% showed diffuse staining ($n = 280$). At the transmission electron microscopy (TEM) level the mutant pheno-

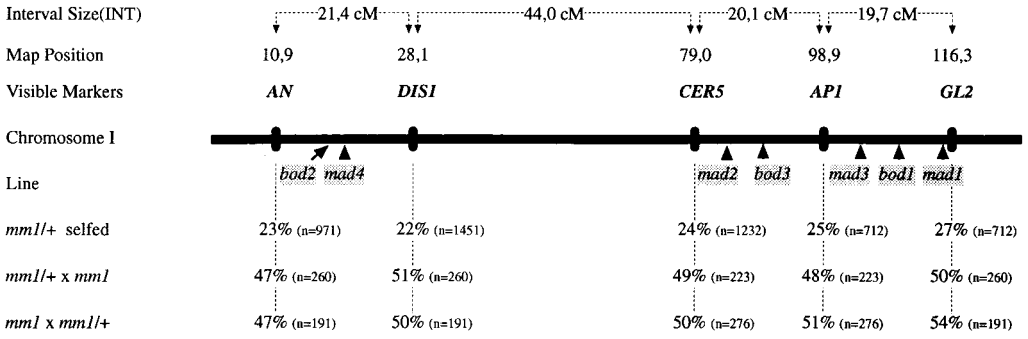

Figure 2.—Schematic representation of the multiple marker chromosome 1 (*mm1*). The mm1 chromosome carries five visible markers: *angustifolia* (*an*), *distorted-1* (*dis1*), *eceriferum-5* (*cer5*), *apetala-1* (*ap1*), and *glabra-2* (*gl2*) as described in Meyerowitz and Ma (1994). The genetic distances between the five morphological markers have been calculated from the F_1 progeny of selfed heterozygous *mm1* plants and the reciprocal crosses are shown in the lower part of this figure. The total number of F_1 plants analyzed (*n*) are shown in brackets. Map positions are from the Unified Genetic Map, Unified-1 (AtDB). The map positions of gametophytic mutations are indicated relative to their flanking markers. For mapping details see materials and methods.

types at the bicellular II stage are likely to correspond to aborted pollen (Figure 4G, left) and pollen with retarded growth (Figure 4G, right). Approximately 5% of all pollen exhibited a thick cell wall subdividing the pollen into two large cells with the smaller cell encompassing approximately one-third of the total area in individual sections (Figure 4G, left pollen, and 4H). We did not observe nuclei in these cells ($n = 20$), suggesting that cytokinesis occurred in the absence of a nuclear division. In addition, we found pollen with one normal vegetative nucleus and one smaller nucleus that otherwise resembled the vegetative nucleus (Figure 4I). Although the smaller nucleus is surrounded by a plasma membrane similar to a typical generative cell at the bicellular I stage, no small vacuoles accumulate around this cell and the nucleolus is clearly condensated (Figure 4I). At the tricellular stage several pollen, in which the two generative cells and sometimes also the vegetative cell are closely associated, were found (Figure 4, J and K). These complexes looked like aggregations of nuclear material and cell membranes without a clear distinction between individual cells (Figure 4, J and K) and they may correspond to the class of mutants that appeared speckled in DAPI stainings. In summary, the range of phenotypic aspects in *mad1* mutants suggests that *MAD1* is involved in the cell and/or nuclear division during mitosis II of pollen development.

***mad2*:** In DAPI stainings of mature pollen of heterozygous *mad2* plants, ~49% ($n = 503$) of all pollen showed morphological aberrations of different strength: 17% showed no nuclear or cytoplasmic staining, 17% had one generative and one vegetative nucleus, in 14% the generative nucleus had a speckled appearance, 1% showed only one highly enlarged nucleus (Figure 5D), and 1% had two diffuse, large nuclei that both resembled the vegetative nucleus (Figure 5C). The frequency of the latter phenotype was variable, ranging between 1 and 10% in single anther preparations even in the same plant. The earliest deviation from wild type was seen at the bicellular I stage. Approximately 9% of all pollen had two small nuclei (Figure 5A). In addition, 1% exhibited two equally sized diffuse nuclei, suggesting that the failure of the generative nucleus to differentiate can be traced back to this stage (Figure 5B). A more

TABLE 1

Segregation of flanking markers upon self-pollination and reciprocal backcrosses

Mutant line	Selfed				♀ × ♂ *mm1* × line				♀ × ♂ Line × *mm1*			
	m1(%)	Σ[a]	*m2*(%)	Σ[a]	*m1*(%)	Σ[a]	*m2*(%)	Σ[a]	*m1*(%)	Σ[a]	*m2*(%)	Σ[a]
mad1[b]	43	961	50	1341	75	110	89	133	44	133	43	133
mad2[c]	46	207	39	324	70	145	57	145	52	190	48	190
mad3[b]	48	1305	46	1068	97	181	94	106	54	493	56	296
mad4[d]	41	693	41	1417	86	184	85	391	47	392	44	810
bod1[b]	57	411	62	411	81	121	93	121	62	270	72	270
bod2[d]	46	1140	40	903	64	188	61	220	61	272	59	259
bod3[c]	56	397	51	422	88	140	77	235	62	214	66	143

[a] Total number of plants analyzed. Only data from individual crosses yielding >50 plants were pooled.
[b] Flanking markers: *m1* = *ap1*, *m2* = *gl2* (see Figure 2).
[c] Flanking markers: *m1* = *cer5*, *m2* = *ap1* (see Figure 2).
[d] Flanking markers: *m1* = *m2* = *dis1* (see Figure 2).

TABLE 2

Mapping of gametophytic mutations relative to flanking markers

Mutant line	Marker segregation selfed (%)[a]		Recombinational distance (*p*) from flanking markers		Relative genetic distance from flanking markers (cM)[a]	
	m1	*m2*	*m1*	*m2*	*m1*	*m2*
mad1	43	50	0.14	0	19.7	0
mad2	46	39	0.08	0.22	5.4	14.7
mad3	48	46	0.04	0.08	6.6	13.1
mad4	41	41	0.18	0.18	10.7	10.7
bod1	57	62	0.25	0.21	10.5	9.2
bod2	46	40	0.32	0.37	10.0	11.4
bod3	56	51	0.25	0.29	9.4	10.7

[a] For flanking markers and calculated marker intervals see Figure 2.

detailed analysis of slightly older pollen at the bicellular II stage by TEM provided evidence for cell differentiation defects of the generative cell. We frequently found pollen in which the generative cell was still attached to the pollen wall and exhibited three characteristics of a vegetative cell (Figure 5, E and F). First, the generative cell appeared to be surrounded by two intact plasma membranes but lacked the accumulation of small vacuoles. Second, no cell wall material/intine accumulates around the generative cell. Third, we often found relatively large vacuoles in the generative cell that are normally found only in the vegetative cell. In summary, this suggests that *mad2* pollen are affected in the differentiation of the generative cell. This interpretation would also be in agreement with the late phenotypes found in DAPI stainings, which all affect the differentiation of the generative cell.

***mad3*:** In heterozygous *mad3* plants ~49% of the pollen are reduced in size and are often collapsed (n = 918; Figure 6, A and C). DAPI staining of mature pollen revealed three phenotypic classes: The most frequent aberrant class has one vegetative and one generative nucleus (53%; Figure 6A). The generative nucleus appeared to be larger and more intensely stained than generative nuclei in wild type (compare left and right pollen in Figure 6A), suggesting that the generative nucleus has undergone DNA replication but failed to divide. This visual impression was confirmed by cytophotometric measurements of nuclear DNA amounts. For determination of the relative DNA content the relative fluorescence intensity of whole-mount DAPI-stained pollen was compared between mutant and wild-type pollen within the same sample preparation. While generative nuclei of wild-type pollen showed a relative staining intensity of 5.5 ± 0.97 (n = 50), mutant generative nuclei exhibited 9.9 ± 1.25 (n = 50). Thus, mutant generative nuclei have approximately twice the DNA content as wild type. In a second class (31%), only one vegetative nucleus was found (Figure 6B). A small fraction of pollen showed no nuclear or cytoplasmic staining (16%). The earliest deviation from wild-type development was observed at the bicellular II stage. Of all pollen, 49% was significantly smaller and could be divided into three classes (n = 912): In ~74% the generative nucleus was still attached to the cell wall (Figure 6E, similar to the bicellular I stage). In 22% the generative nucleus was found in the center of the pollen (similar to bicellular II stage, Figure 6D) and 4% of the pollen showed no staining. This finding suggests that a significant fraction of mutant pollen is delayed in development. This view is supported by the observation that mutant pollen that appeared to be arrested in the bicellular I stage still had extranuclear DNA that is normally not visible at bicellular II stage (compare Figures 6E and 3, C and G). A closer inspection by TEM revealed that virtually no intine layers were detectable in *mad3* mutant pollen at bicellular I stage. The generative cell, when still attached to the outer pollen wall, clearly showed a cell membrane but lacked the intine layers (compare Figure 6, G and H). After separation from the pollen wall the generative cell appeared to be intact; however, no cell wall and no vesicle accumulation around the generative cell was observed (compare Figure 6, I–J and K–L). Thus, by morphological criteria, the primary defect appears to be in the formation of the intine layers. This suggests that an intact intine layer is important for further differentiation of the generative cell.

Gametophytic mutations affecting the development of the male and female gametophyte: Three mutant lines, *bod1*, *bod2*, and *bod3*, showed a reduced transmission of the gametophytic mutation via both the male and the female side (Table 1). This suggests that the corresponding genes are involved in more general functions that are required for the development of both gametophytes. The penetrance of the mutant phenotype was generally low, as expected from the transmission frequencies in reciprocal backcrosses (Table 1). In particular the penetrance of female gametophytic defects was fairly low. In the following we therefore

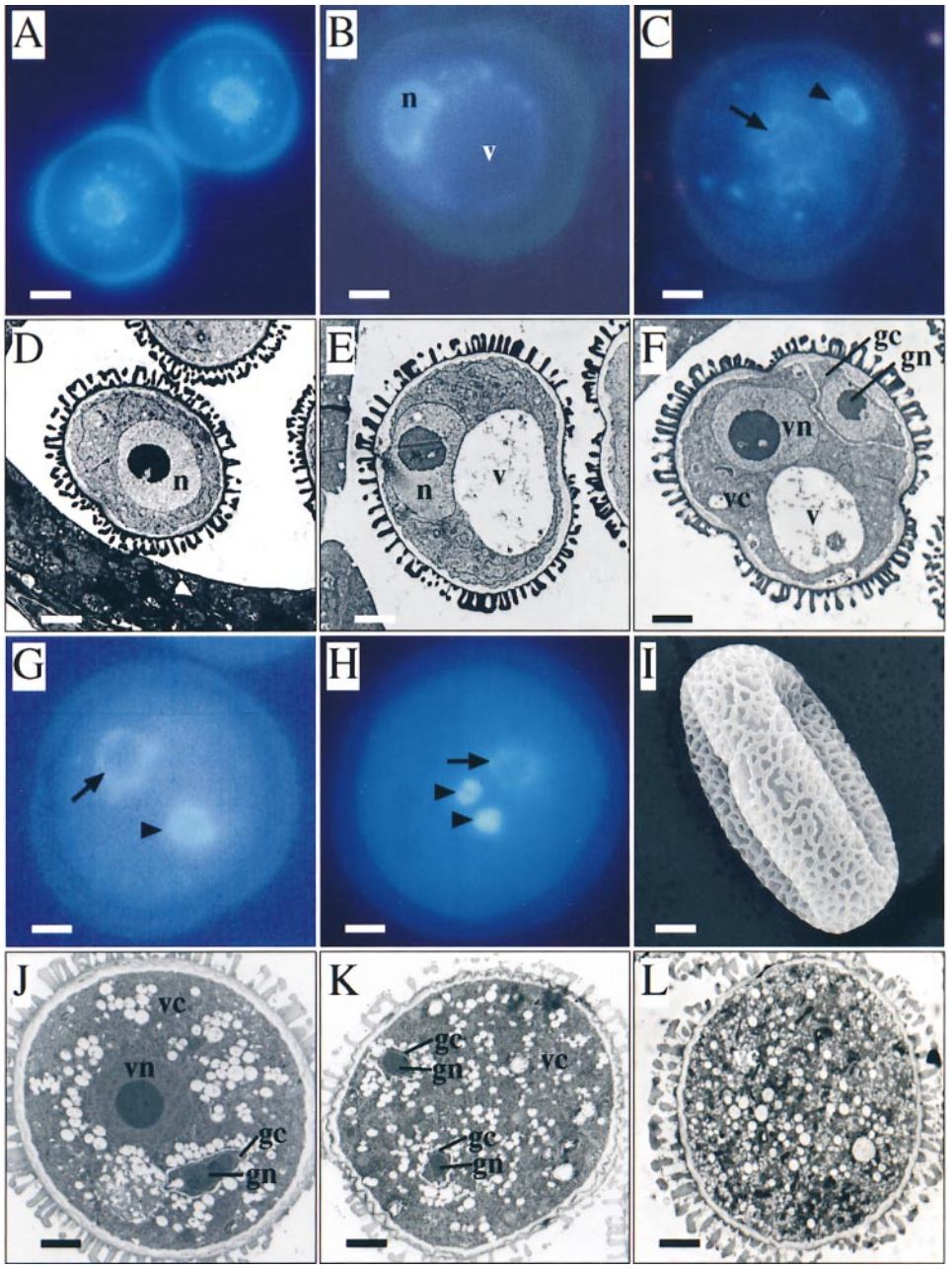

Figure 3.—Microspore development in wild type. (A–C, G and H) Fluorescent micrographs of DAPI-stained whole-mount pollen. (D–F and J–K) Transmission electron micrographs of sectioned anthers. (I) Scanning electron micrograph of a mature pollen grain. Bar, 3 μm unless indicated otherwise. (A and D) Released microspores. The nucleus is large and centrally located. At this stage extrachromosomal DNA is found around the nucleus. (B and E) Vacuolated microspore. (C and F) Bicellular I pollen after pollen mitosis I (PMI). Note that the generative cell is in immediate contact with the pollen wall and surrounded by a cell wall continuous with the intine layer of the outer wall. (G and J) Bicellular II pollen. The generative cell is disengaged from the intine wall and migrated to a central position. Note accumulation of vesicles at the surface of the generative cell. (H and K) Tricellular pollen after pollen mitosis II (PMII). The generative cell has divided to produce two sperm cells. (I and L) Mature pollen. Note the cytoplasm is more dense than in earlier stages. n, nucleus; gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell; v, vacuole.

focus primarily on the description of male gametophytic defects. The female gametophytic defects are described separately thereafter.

***bod1*:** In heterozygous *bod1* plants 48% of the mature pollen showed a mutant phenotype (n = 817). The majority exhibited no nuclear or cytoplasmic staining (42%). A total of 24% showed one vegetative and one smaller nucleus with a speckled appearance and ~30% contained two generative nuclei and one additional small and intensely stained nucleus (Figure 7B). This suggests that in this mutant class the vegetative cell is either defective or has adopted the identity of a generative cell. To distinguish between these two possibilities we introduced a nuclear-targeted vegetative cell-specific marker, Lat52::*GUS/NIA*, into the *bod1* background (Twell 1992). Mutant pollen displaying three generative-like nuclei were selected in DAPI stainings and inspected for the expression of the GUS/NIA marker gene. While wild-type pollen showed strong GUS staining in the vegetative nucleus (Figure 7D) most mutant pollen of this class did not show any localized GUS staining. A few pollen that showed a spot of GUS expression inside a large nucleus were found (Figure 7C). This observation suggests a defect in the nuclear integrity rather than a switch in cell fate. In accord with this a closer inspection at the TEM level revealed a highly aberrant morphology of the vegetative nucleus (Figure 7, J and K). The vegetative nucleus looked lobed rather than round and appeared fragmented inside (Figure 7K). Despite the apparent defects of the vegetative nu-

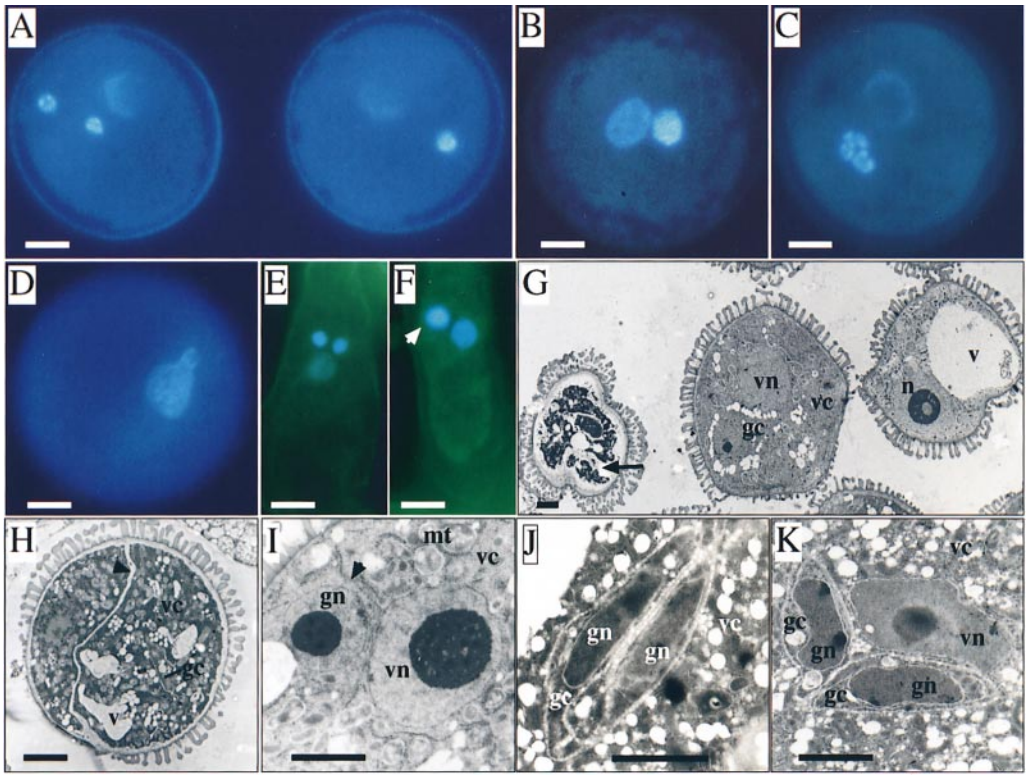

Figure 4.—Phenotype of *mad1* pollen. (A–D) Fluorescent micrographs of DAPI-stained whole-mount pollen. (E–F) Fluorescent micrographs of DAPI and Aniline Blue double-stained pollen tubes of *in vitro* germinated pollen. (G–K) Transmission electron micrographs of sectioned anthers. Bar, 3 μm if not indicated otherwise. (A–D) Pollen from mature anthers. (A) Comparison of wild-type (left) and mutant pollen (right). The mutant pollen shows one apparently normal generative nucleus. (B) Mutant pollen with large generative nucleus. Compare size of generative nucleus in A. (C) Generative nucleus with speckled appearance. (D) Pollen with one large nucleus. (E–F) Migrating nuclei in wild-type (E) and mutant (F) pollen tubes. The mutant pollen tube shows one large generative nucleus (arrowhead, compare with wild type). (G–I) Mutant and wild-type pollen at bicellular II stage. (G) Comparison of wild-type and mutant pollen. Left, pollen with internal wall (arrow); middle, wild-type pollen; right, pollen arrested at vacuolate stage. Note difference in cytoplasmic density. (H) Mutant pollen with internal cell wall (arrowhead). (I) High magnification of the generative and vegetative nuclei in mutant pollen (compare with wild type, Figure 3J). The nucleolus of the generative nucleus appears more dense than in wild type and the nucleus is surrounded by cell membranes (arrowhead). Bar, 1 μm. (J and K) Pollen from mature anthers. Note that cell membranes do not clearly separate distinct cells. (J) Two closely attached generative cells. (K) Two generative cells in close contact with vegetative nucleus. Bar, 1 μm. gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell; v, vacuole; mt, mitochondrion.

cleus, vegetative cell-specific functions still appear to be present because pollen of this mutant class are able to germinate and to grow a pollen tube *in vitro* (Figure 7, G and H). A second phenotypic aspect we frequently observed associated with this mutant class was that the two generative nuclei appeared to be located together in a distinct compartment (compare Figure 7E with 7F). At the TEM level this phenotype is likely to correspond to generative cells that contain two separate nuclei (Figure 7, I and L). This phenotype suggests that nuclear division has taken place but cytokinesis failed. A third phenotypic aspect was noted when analyzing sections at the TEM level. We often found large densely stained structures that were surrounded by stacks of membranes (Figure 7, L and M). To determine the earliest stage in which *bod1* mutant pollen differs from wild type we analyzed pollen at earlier stages. Pollen at the one nuclear stage (n = 600) and the bicellular II stage (n = 1011) revealed no deviation from wild type, suggesting that *BOD1* is not required before the second mitotic division. Although the wide range of phenotypes does not allow one to draw conclusions on the role of the *BOD1* gene, it is conceivable that defects of the vegetative nucleus and thus the vegetative cell is a primary event, which in turn may affect the division of the generative cell.

***bod2*:** Of the mature pollen, 38% of heterozygous *bod2* plants shows morphological defects (n = 1522). Mutant pollen show different size classes with smaller as well as larger pollen (Figure 8, A, B, and D). The nuclear phenotype is quite variable and the relative frequency of different classes differed in separate anthers of a single flower. Pollen often showed no nuclear or cytoplasmic staining or only one generative and one vegetative nucleus. Compared to wild type, the most striking difference was that mature pollen frequently still had a large vacuole (Figure 8, A and B). Some of these pollen appeared to be viable, as we found some that germinated and grew a pollen tube in *in vitro* germination assays (Figure 8C). The first deviation from wild-type development could be traced back to the bicellular II stage where 40% of the microspores were highly vacuolated and often showed no nuclear staining (n = 486). The TEM analysis of mutant microspores at the bicellular II stage revealed a large fraction that appeared delayed in development compared to the surrounding

wild-type pollen. The cytoplasm was less dense (Figure 8F), which is typical for earlier stages of wild-type development, and we often found a prominent vacuole (Figure 8E). In addition we observed differentiation defects of the vegetative and generative nuclei/cells. The generative cell did not show an accumulation of small vacuoles at the cell membrane and the cytoplasm was darker than that in wild type. The nucleolus of the vegetative nucleus often showed a round light area that, in serial sections, appeared like an invagination. In summary, the wide range of general defects in *bod2* mutant pollen suggests that *BOD1* is involved in general cellular processes rather than in the regulation of distinct differentiation processes.

***bod3*:** In plants heterozygous for *bod3* ~37% of the mature pollen showed no nuclear or cytoplasmic staining (n = 880). However, slightly younger three-celled pollen exhibited a clear defect in the development of the generative cell (n = 518). A total of 25% displayed five distinct intensely DAPI-stained spots (Figure 9B). The number of these spots was always five, suggesting that these spots represent condensed chromosomes. Of the pollen 12% showed one vegetative and only one enlarged generative nucleus (Figure 9A). DNA measurements revealed that the enlarged nuclei had approximately twice the DNA content of generative cells of wild-type pollen in the same sample preparation [relative staining intensities: 18 ± 2.97 (n = 50) *vs.* 9.4 ± 2.27 (n = 50)]. The two phenotypic aspects, the "chromosome phenotype" and the enlarged DNA content of the generative cell, suggest that *bod3* mutants undergo replication, but fail to complete cell division at different stages. Consistent with this interpretation is the finding that no deviation from wild-type development was found in DAPI stainings at the bicellular II stage. Our ultrastructural analysis at the TEM level generally supported this interpretation. The earliest deviation from wild type was found at the bicellular I stage. Mutant pollen were found with unusually large and irregularly shaped generative cells that were still attached to the pollen wall (Figure 9C). At the bicellular II stage generative cells frequently did not accumulate vacuoles at the cell membrane (Figure 9D). In mature pollen we often found very elongated generative cells (Figure 9, E and G) or up to three small generative cells (Figure 9, F and H). It is, however, unclear whether the latter correspond to the chromosome-like DAPI phenotype or the class of enlarged generative nuclei.

Female gametophytic development in *bod1*, *bod2*, and *bod3* mutants: In reciprocal backcrosses the three lines,

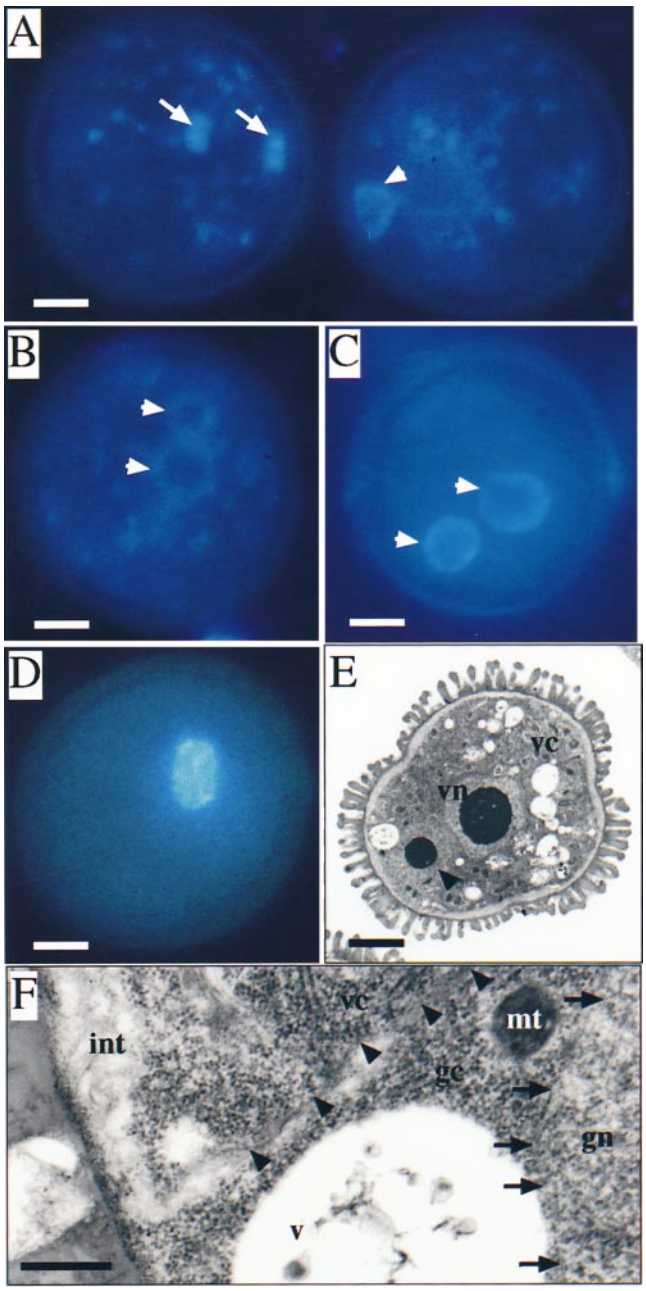

Figure 5.—Phenotype of *mad2* pollen. (A–D) Fluorescent micrographs of DAPI-stained whole-mount pollen. (E–F) Transmission electron micrographs of sectioned anthers. Bar, 3 μm if not indicated otherwise. (A) Comparison of wild-type (right) and mutant pollen (left) in anthers at bicellular I stage. Mutant pollen shows two equally stained regions (arrows). (B) Mutant pollen from same anther as A. Two vegetative-like nuclei are visible (arrowheads). (C–D) Mutant pollen from mature anthers. (C) Two vegetative-like nuclei. (D) One large intensely stained nucleus. (E) Bicellular II stage. Note prominent nucleolus in generative nucleus (arrowhead). Small vacuoles normally surrounding the generative cell are absent (compare Figure 3J). Large vacuoles are found in the vegetative cell. (F) High magnification of the generative cell in E. The plasma membranes around the generative cell are intact (arrowheads). Accumulation of intine at the pollen wall appears to be normal while no cell wall material and/or intine was found around the generative cell (compare Figure 3F). Nuclear membrane is indicated by arrows. Bar, 200 nm. (×40.000). gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell; int, intine; v, vacuole; mt, mitochondrion.

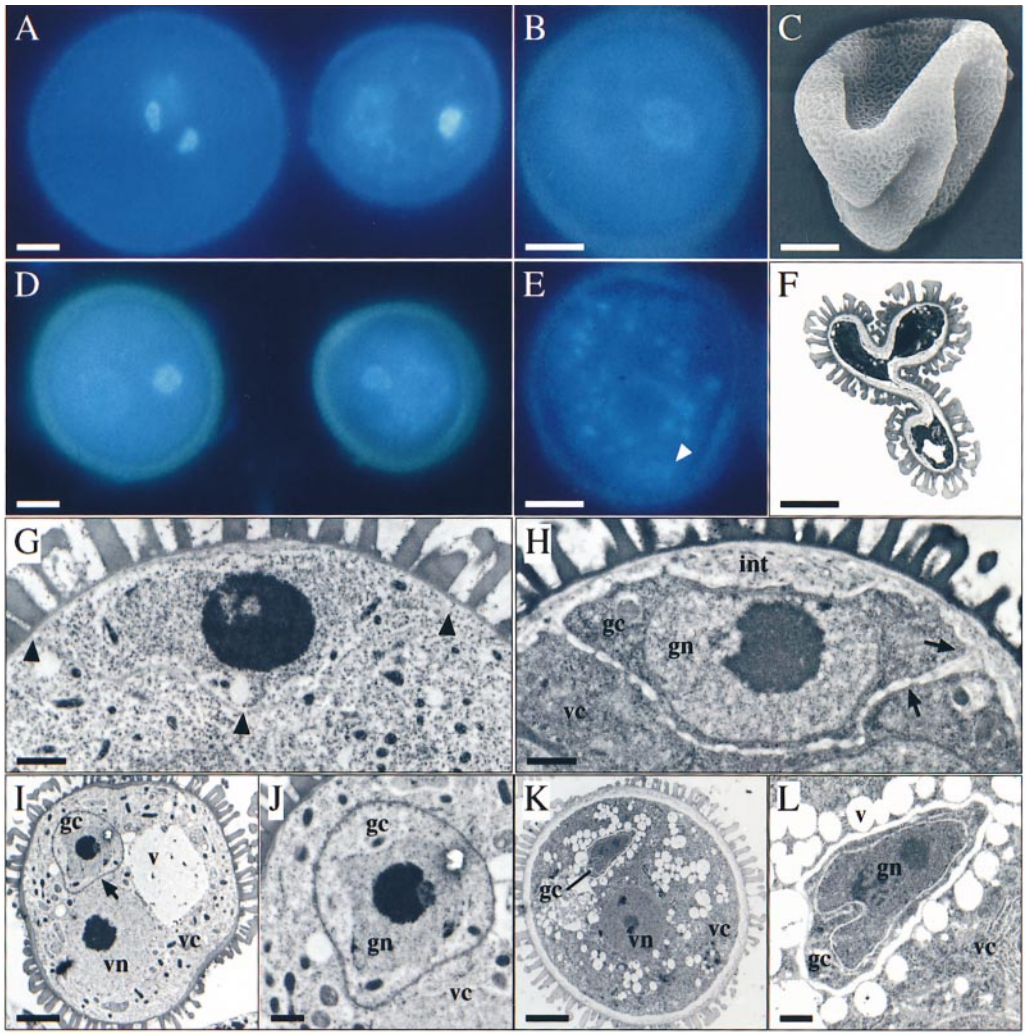

Figure 6.—Phenotype of *mad3* pollen. (A, B, D, and E) Fluorescent micrographs of DAPI-stained whole-mount pollen. (C) Scanning electron micrograph of mutant pollen. (F–L) Transmission electron micrographs of pollen and sectioned anthers. Bar, 3 μm if not indicated otherwise. (A) Comparison of wild-type (left) and mutant pollen (right) in mature anthers. The mutant pollen is significantly smaller than wild type. The generative nucleus is larger and more intensely stained. (B) Mature pollen with one diffusely stained nucleus. (C) Scanning electron micrograph of a collapsed mutant pollen (compare Figure 3G). (D) Bicellular II stage, comparison of mutant (right) and wild-type pollen (left). Mutant pollen is smaller. (E) Mutant pollen at bicellular I stage. Generative cell is attached to the pollen wall (arrowhead, compare Figure 6D). Note extrachromosomal DNA (compare Figure 3D). (F) Mutant pollen collapsed. (G and H) Bicellular I stage. Comparison of generative cells in mutant (G) and wild type (H; compare Figure 3I). No intine or cell wall is seen in the mutant (see arrowheads). Note vegetative and generative cytoplasm is less dense in the mutant. Bar, 1 μm. (I–L) Bicellular II stage. Comparison of mutant (I and J) and wild type (K and L). The cytoplasm of mutant pollen is less dense than in wild type. (J and L) Higher magnification of generative cells in mutant and wild type. Nuclear membranes and cell membranes appear to be intact in mutant pollen; however, no accumulation of lipid bodies and small vacuoles around the generative cell surface is observed. Bar, 1 μm. n, nucleus; gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell; int, intine; v, vacuole.

bod1, *bod2*, and *bod3*, showed a weak reduction in the transmission of the gametophytic mutation via the female gametes (Table 1). To determine whether embryo sac development is affected in these mutants we studied cleared whole-mount preparations of mature ovules (Schneitz *et al.* 1995). The development of the female gametophyte (megagametogenesis) takes place inside the ovules. Megagametogenesis consists of four mitotic cycles that yield an eight-nucleate, seven-celled embryo sac (Webb and Gunning 1990; Reiser and Fischer 1993; Grossniklaus and Schneitz 1998). Eventually the embryo sac differentiates to produce an egg cell, two synergids, three antipodal cells, and a large central cell (Figure 10A). Female gametogenesis was found to be moderately affected in all three mutant lines (Table 3). *bod2* appeared to express the weakest phenotype. Of all embryo sacs ~17% either were reduced in size (Figure 10D) or appeared to be collapsed (Figure 10E). In plants heterozygous for *bod3* ~12% of all ovules showed a collapsed embryo sac (Figure 10C). *bod1* mutants exhibited the strongest phenotype. On average, 23% of all mature ovules appeared to lack an embryo sac completely (Figure 10B). A more detailed analysis of mutant phenotypes in earlier stages was not attempted because the frequency of mutant phenotypes was low and also varied considerably even between different flowers from the same plant.

***mad4*, a gametophytic mutant affecting pollen tube growth:** In *mad4* mutants transmission is specifically impaired via the pollen. *mad4* does not show any obvious defect during pollen development (Table 4). Thus, this mutant affects the function of the pollen or pollen tube during the fertilization process. To examine the stage at which the mutant pollen / pollen tube fails to proceed,

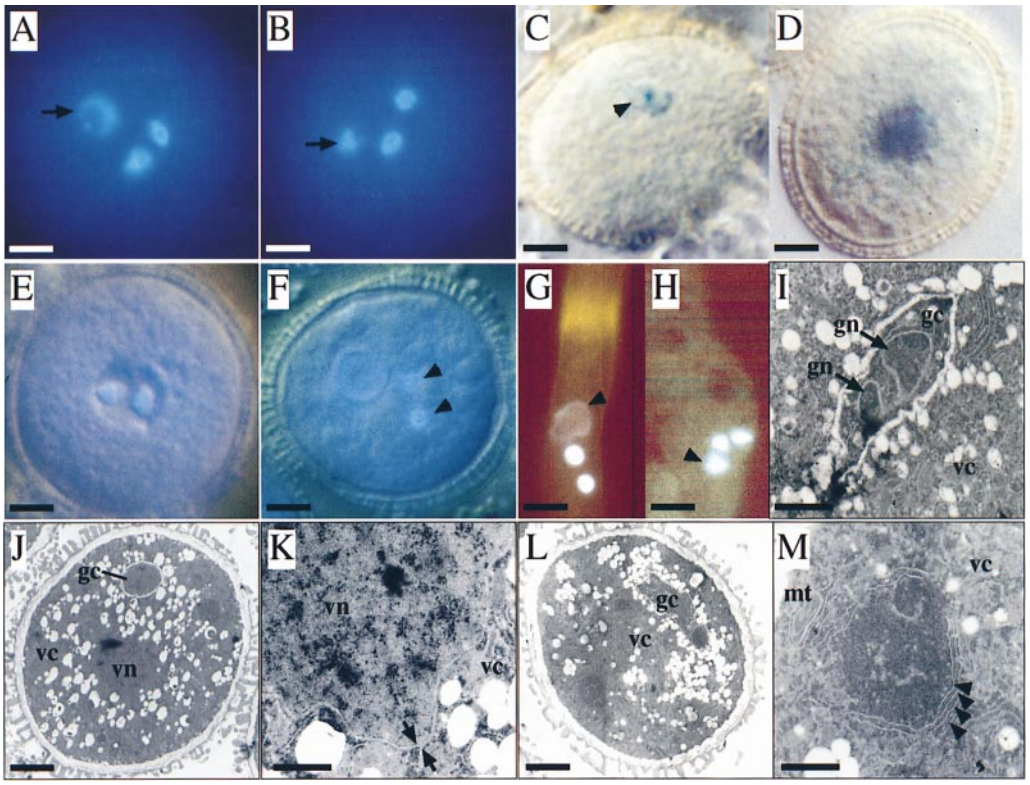

Figure 7.—Phenotype of *bod1* pollen. (A–B) Fluorescent micrographs of DAPI-stained whole-mount pollen. (C–D) DIC micrographs of GUS-stained whole-mount pollen. (E–F) Fluorescent/DIC micrographs of DAPI-stained whole-mount pollen. (G–H) Fluorescent micrographs of DAPI/Aniline Blue double-stained pollen tubes from *in vitro* germinated pollen. (I–M) Transmission electron micrographs of sectioned anthers. Bar, 3 μm if not indicated otherwise. (A–F) Pollen from mature anthers. (A–B) Comparison of wild-type (A) and mutant pollen (B). The mutant vegetative nucleus is smaller and more condensed (arrows; C–D). Comparison of GUS-stained pollen from heterozygous Lat52::GUS/Nia plants (D) and heterozygous Lat52::GUS/Nia *bod1*-plants (C). Note the nucleus in the mutant pollen is clearly visible, but GUS staining is restricted to a small region (arrowhead). (E–F) Comparison of wild type (F) and mutant (E). The generative nuclei in the mutant seems to reside in a common compartment (compared to wild type in 7F, arrowheads). The vegetative nucleus in the mutant is not in the focal plane. (G–H) Migrating nuclei in *in vitro* germinated wild-type (G) and *bod1* (H) pollen tubes. Compare vegetative nuclei (arrowheads). (I–M) Mutant pollen at the tricellular stage. (I) High magnification of one generative cell with two nuclei. The two nuclei were clearly separate in serial sections. Bar, 1 μm. (J–K) Mutant pollen with degenerated vegetative nucleus (J) and high magnification of the vegetative nucleus (K). The vegetative nuclear membrane appears to be intact (arrows). (L) Mutant pollen with large generative cell. (M) High magnification of L. Note densely stained region surrounded by several membrane layers (arrowheads). Bar, 200 nm. gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell; mt, mitochondrion.

we analyzed different steps during the fertilization process. The first steps, the recognition of the pollen grain as judged by the hydration of pollen grains and the germination rate of pollen from *mad4*, were indistinguishable from wild type (Table 4). Further pollen tube growth, however, was retarded relative to wild type. In pollination experiments with wild-type pollen we found that after 24 hr pollen tubes of 98% of all pollen applied to the stigma had arrived in the ovary (Table 4). In contrast only 51% of the pollen tubes from *mad4* mutant plants had traveled down to the ovary in the same time period (Table 4). We often observed pollen tubes that had stopped growth even before penetrating the stigma surface (data not shown). These findings indicate that in *mad4* mutants, pollen tube growth is affected.

DISCUSSION

Because of a variety of properties *A. thaliana* has emerged as a genetic and molecular plant model system. In particular the genetic dissection of developmental processes by systematic mutagenesis screens is a powerful method with which to identify relevant genes (Meyerowitz and Pruitt 1985; Schiefelbein and Somerville 1990; Coen and Meyerowitz 1991; Jürgens *et al.* 1991; Hülskamp *et al.* 1994). A similar strategy for analysis of developmental aspects of the gametophytic life phase, however, is not readily applicable for several reasons. The screening for gametophytic mutants affecting any step during the fertilization process by direct inspection for mutant phenotypes is extremely tedious. Also the search for gametophytic mutants affecting ontogenesis of the gametophytes is complicated by a high background of false positives. M_1 screens suffer from the fact that plants resulting from freshly mutagenized seeds are often severely affected in growth and fertility, which would lead to the identification of many false positives (P. E. Grini, unpublished results). Screening of M_2 populations is complicated because of a high background caused by sporophytic mutants that affect gametophytic development with a variable penetrance (M. Hülskamp, unpublished results). Another drawback is that gametophytic mutants cannot be sorted in complementation groups and thus the only criterion

for demonstrating that mutations affect different genes is the difference in map position.

To circumvent these limitations several laboratories have designed strategies that enable the identification of gametophytic mutants by the segregation distortion of markers linked to the gametophytic mutation. One possibility is to screen for gametophytic mutants resulting from T-DNA or transposable element insertions. These mutants can be identified by an altered segregation ratio of the resistance gene of the T-DNA or the transposon (Feldmann *et al.* 1997; Moore *et al.* 1997; Howden *et al.* 1998). This strategy also facilitates subsequent cloning and molecular characterization of the affected genes (Springer *et al.* 1995; Grossniklaus *et al.* 1998). However, the current limitation of these strategies is that the efficiencies do not yet afford saturation mutagenesis, but only characterization of a subset of gametophytic mutants.

In this article we present an alternative strategy that is based on conventional mutagenesis. The recognition of gametophytic mutations by a distortion of marker frequencies enables us not only to identify genes required during the haploid life phase irrespective of the phenotype but also to balance these lines. Using EMS as a mutagen we largely avoid selecting for chromosomal arrangements that may also cause segregation distortions. The use of several linked markers for monitoring the transmission of gametophytic mutations automatically yields an approximate map position for the newly identified gametophytic mutants. In a small-scale mutagenesis screen we have isolated seven gametophytic mutations that map to specific marker intervals and affect either development or function of the pollen or the embryo sacs. Although the small number of lines tested in our pilot screen does not allow us to estimate the number of gametophytic genes, the strategy used is in principle suitable for a saturation screen. EMS mutagenesis is generally considered to mutate genes randomly and hence the allele frequency in such screens can be used to estimate the saturation level. However, because complementation tests are not possible with gametophytic mutants, an estimation of the saturation level would require an independent calibration of the screen with known recessive mutants (*e.g.*, embryo lethal mutants and trichome mutants).

Specificity of male gametophytic defects varies between different mutants: Pollen development has been shown to be influenced by sporophytically acting genes including those that function during premeiotic and postmeiotic development (Chaudhury 1993). This sug-

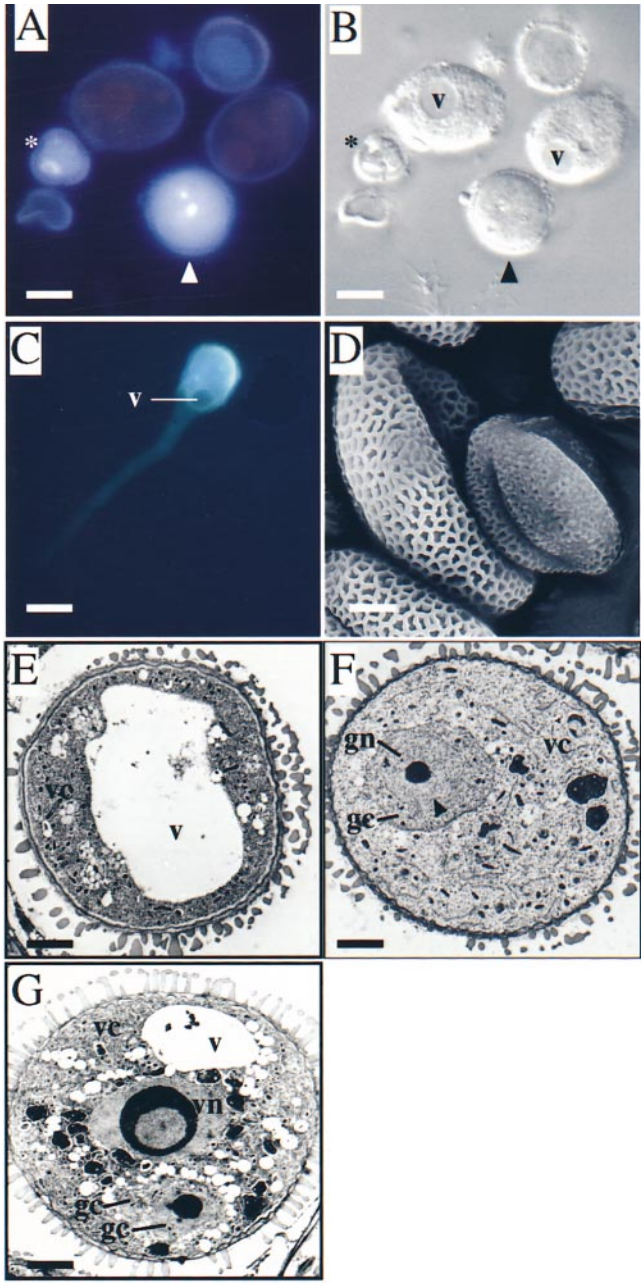

Figure 8.—Phenotype of *bod2* pollen. (A) Fluorescent micrograph of DAPI-stained whole-mount pollen. (B) DIC micrograph of whole-mount pollen. (C) Fluorescent micrograph of DAPI/Aniline Blue double-stained pollen tubes from *in vitro* germinated pollen. (D) Scanning electron micrograph of whole-mount pollen. (E–G) Transmission electron micrographs of sectioned anthers. Bar, 3 μm if not indicated otherwise. (A–B) Comparison of wild-type (arrowheads) and mutant pollen (asterisks) in mature anthers. Note the size difference between mutant and wild type. The enlarged mutant pollen shows no cytoplasmatic staining and has a large vacuole (compare DIC image in B). The smaller mutant pollen (asterisk) has one generative and one vegetative nucleus. Bar, 10 μm. (C) Germinated mutant pollen (left) with large vacuole. Bar, 20 μm. (D) Comparison of wild-type (left) and mutant pollen (right). (E–G) Mutant pollen from same anther at bicellular II stage. (E) Note the large vacuole in vegetative cell. (F) Note that the cell and nuclear membranes of the generative cell are intact. Arrowhead indicates nuclear membrane. (G) Note the cavity in nucleolus of the vegetative cell. The large vacuole is not typical for this stage. gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell; v, vacuole.

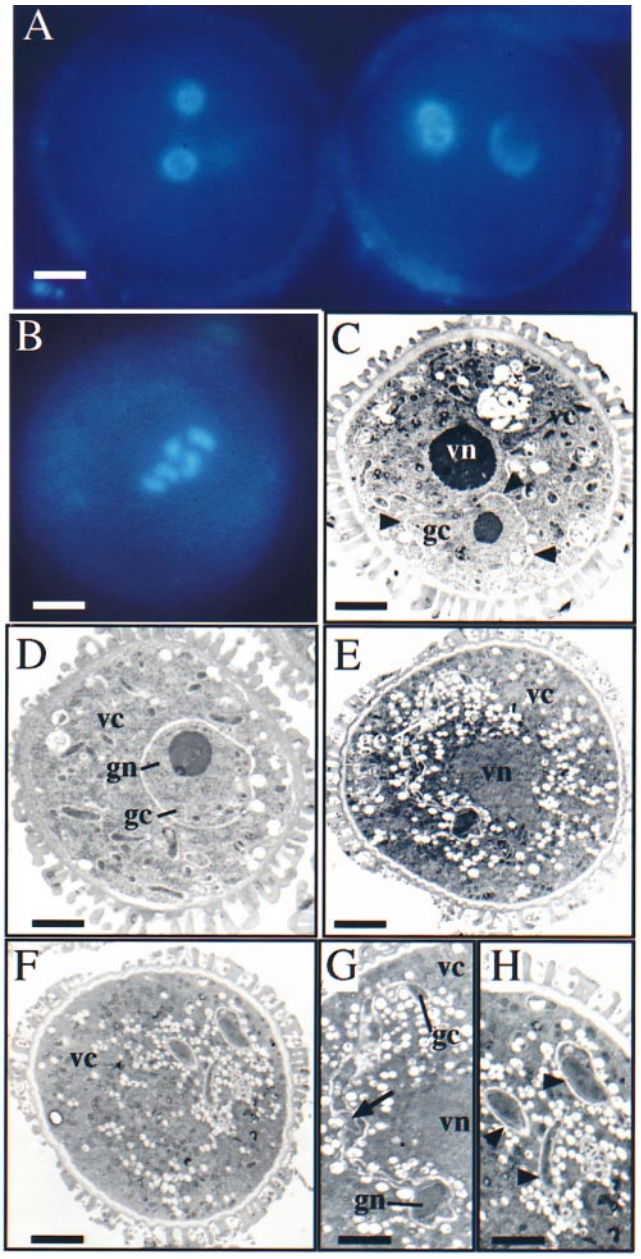

Figure 9.—Phenotype of *bod3* pollen. (A–B) Fluorescent micrographs of DAPI-stained whole-mount pollen. (C–H) Transmission electron micrographs of sectioned anthers. Bar, 3 μm if not indicated otherwise. (A–B) Pollen from mature anthers. (A) Comparison of wild-type (left) and mutant pollen (right). The vegetative nucleus of the wild-type pollen is not in the focal plane. The mutant pollen has one enlarged generative nucleus. (B) Instead of the generative nucleus five distinct spots can be recognized. The vegetative nucleus is out of the focal plane. (C) Bicellular I stage. The generative cell extends far into the vegetative cell but is still connected to the pollen wall. Arrowheads mark cell wall of the generative cell. (D) Bicellular II stage. The generative nucleus appears to be enlarged and no small vacuoles accumulate around the generative cell. (E–H) Tricellular stage. (E) Elongated generative cell. (F) Pollen with three generative cell-like compartments. (G) Magnification of E. Arrow indicates additional constriction. Bar, 1 μm. (H) Magnification of F. Arrowheads indicate three generative cell-like compartments. Bar, 1 μm. gn, generative nucleus; vn, vegetative nucleus; vc, vegetative cell; gc, generative cell.

gests that many sporophytic genes are involved in controlling postmeiotic microspore development. Evidence for gametophytically acting genes affecting male gametogenesis comes from genetic analysis of deficiencies in maize (Kindiger *et al.* 1991). Arrest in various developmental stages of microsporogenesis was correlated with the loss of specific chromosome arms. Because the phenotypes are most likely caused by the loss of several loci only the phenotype of the earliest-acting gene can be recognized.

More recently, a few male gametophytic mutants have been described. Particularly interesting are two mutants, *side-car pollen* (*scp*), and *gemini pollen 1* (*gem1*) that affect the asymmetric division and cell-fate decision during mitosis I (Chen and McCormick 1996; Twell and Howden 1998; Twell *et al.* 1998). Another T-DNA tagged mutant, *limped pollen* (*lpd*), in which mitotic divisions appear to be normal but the two sperm cells remain attached to the pollen wall has been described (Twell and Howden 1998). The *solo pollen* mutant exhibits a very early defect. In *solo pollen* mutants a high number of pollen contain only one vegetative nucleus, suggesting a defect in nuclear and/or cell division (Twell and Howden 1998).

A common observation associated with all gametophytic mutations described so far is a high variability combined with a range of different mutant phenotypes. Also all gametophytic mutants described in this study showed a pleiotropic phenotype. This was particularly the case for gametophytic mutants affecting both sexes. This suggests that this mutant class represents factors involved in more general cellular processes, as might be expected from genes required for both male and female gametophytic development. The three male-specific gametophytic mutants *mad1*, *mad2*, and *mad3* exhibited more specific phenotypes. *mad1* mutants do not enter a second mitotic division and occasionally undergo cell divisions. Thus, similar to *gem1* mutants, cytokinesis takes place in the absence of karyokinesis. *mad2* mutants appear to be defective in the differentiation of the generative cell after mitosis I. The generative cell usually lacks the generative-like characteristics and in some cases displays a phenotype reminiscent of the vegetative cell. This phenotype is very similar to *gem1*. The primary cause of this phenotype, however, appears to be different. While in *gem1* mutants the vegetative cell fate appears to be a consequence of an equal instead of an unequal division, the phenotypic spectrum of *mad2* mutants suggests a later function during the differentiation of the generative cell. *mad3* mutants appear to be affected at a slightly later stage in cell division after replication has taken place. Although the phenotypes of the three male gametophytic mutants hint at specific gene functions, we emphasize that the pleiotropic effects make it difficult to distinguish between primary and secondary defects.

Female gametogenesis is affected with a low fre-

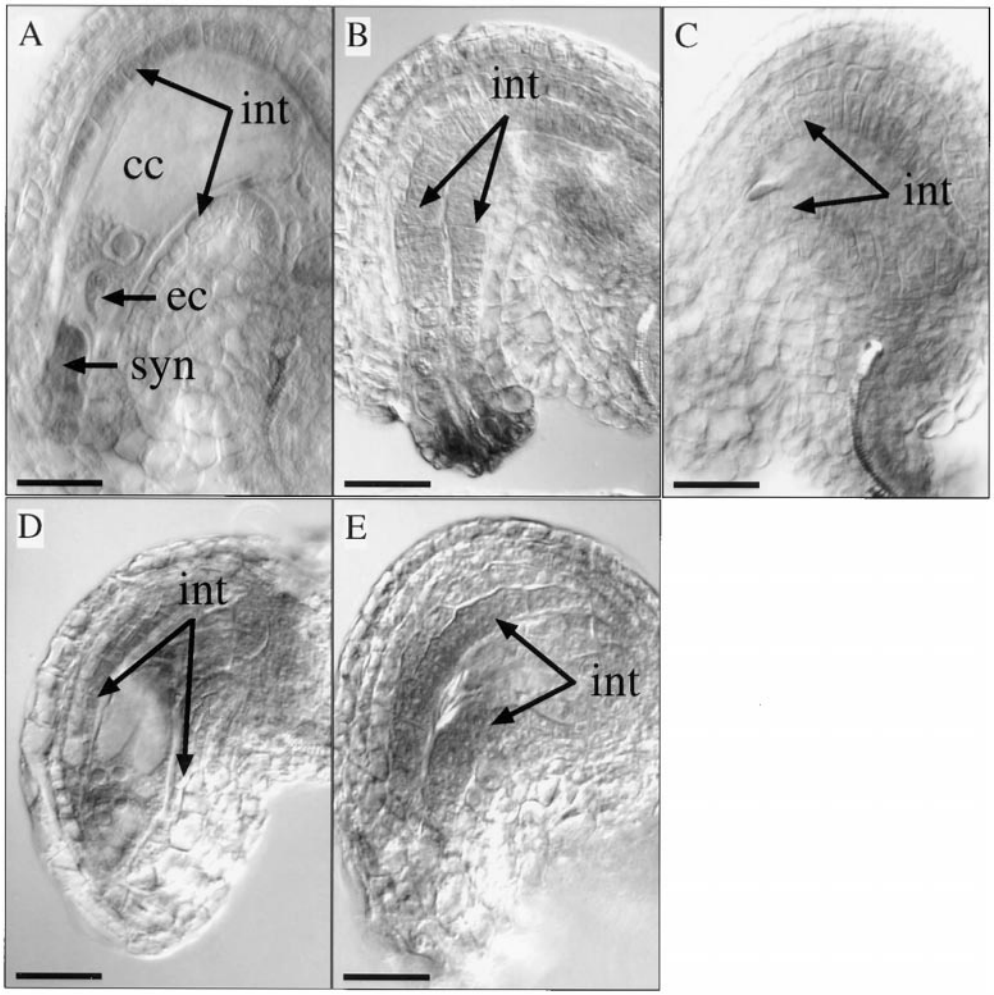

Figure 10.—Embryo sac phenotype of *bod1*, *bod2*, and *bod3*. Micrographs of optical sections of cleared whole-mount ovules stained with Mayer's hemalum. (A) Mature wild-type embryo sac. (B) *bod1*, no embryo sac. (C) *bod2*, embryo sac appears to be collapsed. (D) *bod2*, embryo sac smaller than in wild type. (E) *bod3*, embryo sac appears to be missing. Bar, 20 μm. syn, synergid cells; ec, egg cell; cc, central cell; int, integuments.

quency in gametophytic mutants affecting both sexes: Embryo sac development takes place inside the ovule. Genetic analysis in Arabidopsis has yielded a number of sporophytic mutants defective in ovule development, embryo sac development, or both (Robinson-Beers *et al.* 1992; Jofuku *et al.* 1994; Leon-Kloosterziel *et al.* 1994; Gaiser *et al.* 1995; Hülskamp *et al.* 1995b; Elliott *et al.* 1996; Klucher *et al.* 1996). The phenotypes of a few previously described megagametophytic mutants suggest an early requirement for haploid-expressed genes (Redei 1965; Simcox *et al.* 1986; Ohad *et al.* 1996; Christensen *et al.* 1997; Moore *et al.* 1997; Drews *et al.* 1998; Grossniklaus and Schneitz 1998; Grossniklaus *et al.* 1998; Howden *et al.* 1998). An elegant study in maize based on a systematic generation and characterization of chromosome deletions suggested three different processes that can be disrupted by mutations: progression through nuclear stages, synchronization of events, and establishment of cellular patterns (Vollbrecht and Hake 1995). In our study, we isolated

TABLE 3

Seed development in male and female gametophytic mutants and wild type

Line	Developed seed[a] (%)	Mature ES[b] undeveloped (%)	Arrested ES[b] development (%)	Total
bod1 × wt	69.0	7.6	23.4	197
bod2 × wt	70.2	12.7	17.1	362
bod3 × wt	75.7	12.1	12.1	313
mm1 / + × *mm1*	96.9	2.6	0.5	397

[a] The number of developed seeds was determined 3 days after pollination.
[b] ES, embryo sac.

TABLE 4

Pollen tube guidance in *mad4* and wild type

Line	Hydration (%)	Germination (%)	No. of pollen tubes arrived in the ovary after 24 hr (%)	Total
mad4	99	99	51	242
Wild type	99	99	98	165

three male and female gametophytic mutants that appeared to be impaired in early steps of embryo sac development. The functional requirement of *BOD1*, *BOD2*, and *BOD3* during both male and female gametophytic development, however, suggests that these three genes do not play a role in specific developmental processes but rather reflect a requirement in more general cellular processes.

Isolation of a male gametophytic mutant affected in pollen tube growth: During the fertilization process, an intimate interaction of gametophytic and sporophytic tissues is required to achieve the fusion of the gametes. While sporophytically and gametophytically active genes appear to be involved in initial pollen tube growth, pollen tube guidance and fertilization are probably governed by the function of male and female gametophytes (Heslop-Harrison 1987; Sanders and Lord 1992; Pruitt and Hülskamp 1994; Hülskamp *et al.* 1995b; Preuss 1995; Ray *et al.* 1997). Although gametophytically active genes have been implicated, no gametophytic mutations specifically affecting the fertilization process have been described previously. The only mutation known to gametophytically affect pollen tube growth, *tip1*, is also required for general non-tip growth of sporophytic cells (Schiefelbein *et al.* 1993; Ryan *et al.* 1998).

The isolation of gametophytic mutants affecting the fertilization process is particularly difficult by direct inspection for mutant phenotypes. The strength of marker-retardation-based strategies is to enable the isolation of mutants irrespective of their phenotype. We identified one male gametophytic mutation in which the fertilization process is affected. *mad4* is a male gametophytic mutant that affects pollen tube growth. The finding that mutant pollen tubes do not reach the ovary even after 24 hr suggests that pollen tube growth is either retarded relative to wild type or arrested shortly after germination. It would be interesting to see whether *MAD4* also has a role in cell morphogenesis of other sporophytic tissues similar to *TIP1*. However, although the genetic data suggest a low transmission through the male gametophyte, we have not yet identified plants homozygous for *mad4* to test this possibility.

Perspective: The gametophytic phase of the plant life cycle requires general functions, such as those involved in cell-cycle regulation or cytokinesis, as well as important developmental processes including establishment of cell polarity, differentiation, and morphogenesis of cells. It is likely that some of the latter processes are common to gametophytic and sporophytic development and hence relevant mutations might have been missed in previous screens focusing on developmental processes of the diploid plant. New strategies based on the segregation distortion of linked markers, either by insertional or conventional mutagenesis screens, will enable systematic genetic approaches for the dissection of developmental processes and the molecular isolation of relevant genes for the gametophytic phase of the life cycle in plants.

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