Selection of T-DNA-Tagged Male and Female Gametophytic Mutants by Segregation Distortion in Arabidopsis

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ABSTRACT

As a strategy for the identification of T-DNA-tagged gametophytic mutants, we have used T-DNA insertional mutagenesis based on screening for distorted segregation ratios by antibiotic selection. Screening of ~1000 transgenic Arabidopsis families led to the isolation of eight lines showing reproducible segregation ratios of ~1:1, suggesting that these lines are putative gametophytic mutants caused by T-DNA insertion at a single locus. Genetic analysis of T-DNA transmission through reciprocal backcrosses with wild type showed severe reductions in genetic transmission of the T-DNA through the male and/or female gametes. Direct evidence for mutant phenotypes in these lines was investigated by DAPI staining of mature pollen grains and by the analysis of seed set and embryo sac morphology in cleared ovules. One line, termed *limpet pollen*, showed a novel pollen phenotype in that the generative cell failed to migrate inward after pollen mitosis I, such that the generative or sperm cells remained against the pollen wall. Two other lines, *andarta* and *tistrya*, were defective in female transmission and showed an early arrest of embryo sac development with the viable megaspore not initiating the nuclear division cycles. These data demonstrate the efficacy of a segregation ratio distortion strategy for the identification of T-DNA-tagged gametophytic mutants in Arabidopsis.

POLLEN development and function is one of the most accessible developmental systems that presents an opportunity to study cell polarity, cell differentiation, and cell-cell signaling events in flowering plants. Pollen development from sporogenous cells to mature pollen in angiosperm plants requires complex and coordinate gene expression programs in the haploid gametophytic cells and in the sporophytic tissues. In particular, male gametophyte development involves a large number of genes (~20,000) whose expression is tightly regulated. To date, considerable effort has been focused on the isolation and regulation of pollen-expressed genes, which has led to the identification of shared and unique *cis*-regulatory sequences that control expression from the haploid genome (for recent review, see Twell 1994). Although similar studies of the developing female gametophyte are likely to yield equally significant data concerning specific gene expression and regulation, such studies have been hampered by the inaccessibility of the embryo sac that is located deep within the sporophytic tissues of the ovule.

An alternative to the direct molecular isolation of important haploid-expressed genes is the use of a genetic or mutational approach. *Arabidopsis thaliana* has many advantages in this regard, and significant progress has been made in studying plant reproduction using this model species (for recent review, see Preuss 1995). Mutations that interfere with the production of male and female gametophytes are common and have been described in more than 100 species. The vast majority of these mutations show a sporophytic requirement and are nuclear recessive, with only very few showing a gametophytic requirement (for recent review, see Grossniklaus and Schneitz 1998). Sporophytic mutants that disrupt pollen and ovule development have been identified in Arabidopsis by screening for reduced fertility. Recessive sporophytically acting mutations disrupting reproduction result in self-sterility when homozygous but are fertile when heterozygous. Gametophytic lethal mutations that are fully penetrant in one sex, but are transmitted at least partially through the other, can only be recovered as heterozygotes. Plants heterozygous for a female gametophyte lethal mutation are semisterile because half of the ovules carry defective megagametophytes and do not develop into seeds. In contrast, although plants heterozygous for a male gametophyte lethal mutation produce 50% nonfunctional pollen, they are fully self-fertile due to a large excess of wild-type pollen relative to the number of ovules available for fertilization.

Deficiency analysis and transmission studies in maize and Arabidopsis suggest that a large number of genes

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are required during the haploid gametophytic phase (Patterson 1978; Buckner and Reeves 1994; Vizir *et al.* 1994; Vollbrecht and Hake 1995). Nevertheless, very few mutants affecting gametophyte development have been studied. In Arabidopsis, Redei (1964, 1965) identified the first mutations that were not transmitted normally through the male or female gametophytes, respectively. In addition, a gametophytic/sporophytic overlap of gene activity was identified for two embryo lethal mutants in Arabidopsis that showed reduced gametophytic transmission (Meinke 1982).

To date, only three mutations affecting the development of the megagametophyte have been described, all of which affect early steps of megagametogenesis: *Female gametophyte factor* (*Gf*) is not transmitted through the female and shows reduced transmission through the male (the mutant allele is transmitted with 32% efficiency of the wild-type allele; Redei 1965). Recently, using confocal laser scanning microscopy Christensen *et al.* (1997) showed that viable megaspores carrying the *Gf* mutation do not divide. *hadad* (*hdd*), a gametophyte lethal mutation with very low transmission through the female (<2% as compared to the wild-type allele) and variable efficiency through the male shows an arrest after one or sometimes two nuclear divisions (Moore *et al.* 1997). Using gene trap-mutagenesis Springer *et al.* (1995) identified the first female-specific gametophytic mutation, *prolifera* (*prl*), which results in reduced megagametophyte viability (50% female transmission efficiency) but does not affect transmission through the male. Embryo sacs mutant for *prl* variably arrest during the mitotic division cycles (U. Grossniklaus, P. Springer, and R. Martienssen, unpublished results), although many complete development and are fertilized. The *PRL* gene is related to the *MCM2-3-5* genes of yeast that are required for the initiation of DNA replication, suggesting an essential function of *PRL* in all dividing cells with the exception of the haploid pollen cell lineage (Springer *et al.* 1995).

In addition to the *pollen abortion factor* (Redei 1964), a second male gametophyte-specific mutant termed *sidecar pollen* (*scp*) has been identified in Arabidopsis (Chen and McCormick 1996). *scp* plants produce mature pollen grains with two cells with vegetative cell identity, rather than one, and only one of these cells undergoes asymmetric cell division to produce the two sperm cells. Recently, three novel gametophytic mutant phenotypic classes affecting pollen cell division were observed in a genetic screen for mutations affecting polarity and cell division: *solo* mutants with unicellular pollen failing to divide at pollen mitosis I (PMI), *duo* mutants with bicellular pollen failing to divide at pollen mitosis II (PMII), and *gemini* mutants affecting the symmetry of cell division during PMI (Twell and Howden 1998). In the *gemini* mutants symmetrical and partial divisions occur frequently, suggesting a lesion in components of the polarity determination and/or expression systems. Despite such studies, evidence for gametophytic mutations in Arabidopsis and information concerning haploid-expressed genes that specifically control gametophyte development is very limited. To address this issue we have adopted a T-DNA-based gene tagging strategy, in particular because T-DNA insertional mutagenesis is a powerful and well-proven approach to isolate genes in Arabidopsis.

The segregation of T-DNA-borne antibiotic resistance in self-progeny of hemizygous transformants may have particular significance according to the ratios observed. For example, in recessive T-DNA-tagged embryonic lethal mutants, homozygous mutant individuals are not recovered because they fail to complete embryogenesis. Embryo lethal mutants therefore often produce a 2:1 (resistant:sensitive) ratio. With this approach in mind, we developed a strategy to identify T-DNA-tagged gametophytic mutants in Arabidopsis using segregation ratio distortion. Because insertional inactivation of many important genes controlling gametophytic development is likely to lead to reduced transmission through the male or female gametes, it is possible to screen for the reduced transmission of a T-DNA-borne resistance marker. For example, if T-DNA insertion inactivates a postmeiotically expressed gene that is essential for male gametophytic development, then the ratio of resistant:sensitive seedlings in self-progeny would be 1:1 rather than the expected 3:1, due to the loss of the T-DNA containing homozygote class and half of the heterozygote class (Figure 1). If this gene were also partially required for female gametophytic development, the ratio would be <1:1. Similarly, if T-DNA resulted in a mutation that conferred fully penetrant gametophytic lethality for both gametes, T-DNA would not be transmitted to the progeny at all and could not be recovered in such a screen. According to this rationale, ~1000 independent Arabidopsis transformants were screened by determination of the segregation of the seedling hygromycin resistance phenotype conferred by the T-DNA. Here we describe the isolation, genetic transmission, and phenotypic characterization of eight mutant lines. Southern blot analysis revealed that five possessed a single copy of T-DNA and confirmed that a novel pollen mutant phenotype identified in one of the lines cosegregated with hygromycin resistance and the T-DNA. Two other lines defective in female transmission showed an arrest of development of the viable megaspore. These results demonstrate that a screening strategy based on T-DNA segregation ratio distortion is an effective approach for the identification of genes that play critical roles in either, or both, male and female gametophytic development.

MATERIALS AND METHODS

Plant material: The T-DNA lines used in this research were obtained from MOGEN (Leiden, The Netherlands). Approxi-

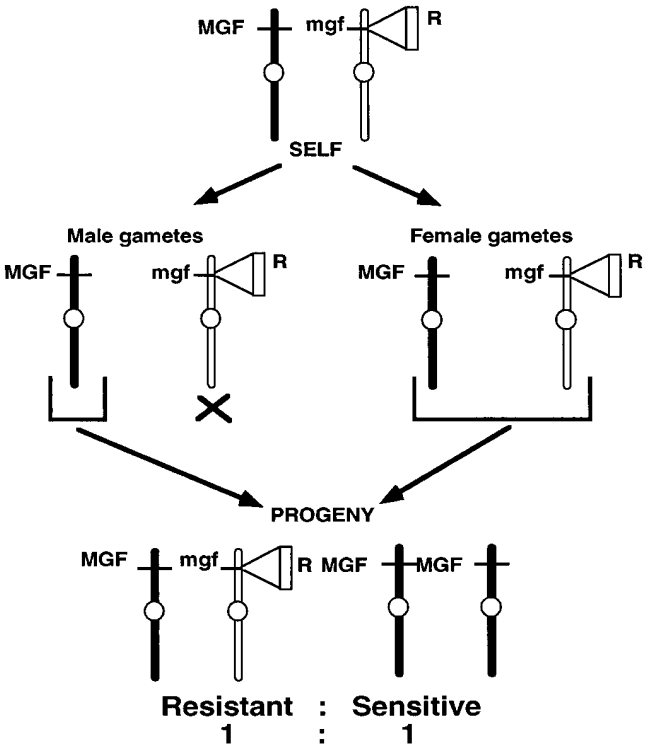


Figure 1.—Screening strategy for T-DNA-tagged gametophytic lethal mutants based on segregation ratio distortion. MGF, male gametophytic factor essential for pollen development; R, T-DNA harboring a drug resistance marker.

mately 1000 independent transformant lines of Arabidopsis were generated by the root explant transformation of Arabidopsis ecotype C24, according to the method of Valvekens *et al.* (1988). The T-DNA construct used was pMOG553 in which T-DNA carries the hygromycin phosphotransferase (*hpt*) gene and a promoterless β-glucuronidase (*gus*) gene (Goddjin *et al.* 1993).

Selection of candidate-transformed plants: Independent mutant seed lines of Arabidopsis were selected on the basis of their segregation ratios for hygromycin resistance. Surface sterilized seed (~200) was plated onto hygromycin (10 μg/liter)-supplemented medium (half-strength Murashige and Skoog salts, 1% sucrose, 0.8% agar, pH 5.8). After 2 days at 4°, the seed was grown under continuous light at 20°. The hygromycin phenotype (resistant or sensitive) was scored after 2 wk. Plants from lines that appeared to be segregating 1:1 (resistant:sensitive) were grown and seed collected from T_2 individuals. Hygromycin-resistant seedlings were transferred to 10-cm-square pots containing a 3:1 compost:sand mix. Plants were grown under greenhouse conditions with supplementary lighting (16 hr light, 22°).

Genetic transmission through male and female gametes: To determine gametophytic transmission of the T-DNA, reciprocal test crosses were performed between the wild type and each transgenic line. Harvested seed from individual siliques was sown on hygromycin-containing plates and the resistance phenotype scored. The transmission efficiency (TE) of the T-DNA through each gamete (TE_{male} and TE_{female}) describes the fraction of gametes successfully transmitting T-DNA to the progeny relative to the number of gametes expected to carry the T-DNA. The expected number of gametes carrying the trait is taken to equal the number of gametes lacking it, assuming random segregation during meiosis and the absence of post-meiotic selection; therefore, TE (%) = (observed Hyg^R/observed Hyg^S) × 100. In earlier publications, the expected number of gametes carrying the trait has been calculated as half the total number of progeny scored (Redei 1965; Feldmann *et al.* 1997; Moore *et al.* 1997). However, this formula for the calculation of the expected gametes carrying the trait leads to an overestimation of the TE because a fraction or all of the gametophytes carrying the trait do not produce scorable progeny.

The recombination frequency of lines showing reversion to 3:1 was calculated as follows: recombination (%) = (no. of lines showing 3:1/total tested lines) × 100. The approximate genetic map distance was estimated as described in Koornneef and Stam (1992) using the mapping function of Kosambi (Kosambi 1944).

Preparation of genomic DNA for Southern blot analysis: Plant genomic DNA was extracted by a method slightly modified from that described by Dellaporta *et al.* (1983). Approximately 2 cm^2 of leaf tissue frozen in liquid nitrogen was ground to a fine powder using an electric grinder. Seven hundred fifty microliters of extraction buffer (50 mm Tris-HCl, pH 8.0; 10 mm EDTA, pH 8.0; 100 mm NaCl; 1% SDS; 10 mm β-mercaptoethanol) was immediately added and incubated at 65° for 10 min. One hundred fifty microliters of ice-cold 5 m potassium acetate was added. After a 20-min incubation on ice, the sample was centrifuged in a microfuge for 10 min. Seven hundred fifty microliters of the supernatant was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. The DNA pellet was washed in 80% ethanol and redissolved in 30 μl TE buffer.

Genomic DNA from two separate plants for each line was digested with *Eco*RI, which cuts once within the pMOG553 T-DNA, and separated on a 1.0% agarose gel. The DNA was transferred to Zeta-Probe GT membrane (Bio-Rad, Hemel Hempstead, Herts, UK) by capillary blotting. DNA was labeled with [^{32}P]dCTP according to the instruction manual of Prime-It II kit (Stratagene, La Jolla, CA). *GUS* gene DNA was isolated from plasmid pMKC4 as a *Nco*I/*Bam*HI fragment and *hpt* gene DNA from plasmid pMOG22 as a *Eco*RI/*Pst*I fragment. Membranes were (pre-) hybridized and washed according to the Zeta-probe GT protocol. Membranes were either exposed to X-ray film at −80° or analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Histochemical analysis of pollen: Sufficient pollen for analysis was obtained by placing 3–4 fully open flowers in a microfuge tube with 300 μl of buffer (50 mm $NaPO_4$, pH 7.0; 1 mm EDTA; and 0.1% Triton) containing DAPI (0.5 μg/ml) for 5 min. After vortexing, the tubes were briefly centrifuged in a microfuge. The pollen pellet (4 μl) was transferred to a microscope slide and viewed by light and by UV epi-illumination using a Nikon (Melville, NY) Optiphot microscope.

Intact silique analysis and whole-mount preparation of ovules: For the clearing of intact siliques, fresh material (the longest mature green siliques) was frozen for 1 hr at −20° and thawed in distilled water for 30 min at room temperature. Material was cleared in lactophenol (equal volumes of lactic acid, phenol, glycerol, and water) at 70° for 1.5–3.5 hr and stained in cottonblue-lactophenol/lactophenol (1:10 v/v) at 70° for 1 hr. Specimens were cleared and destained in lactophenol at room temperature for 1–3 hr. After treating with increasing concentrations of lactic acid in lactophenol over a period of 48 hr, specimens were transferred to pure lactic acid. Elongated siliques were dissected by removing one ovary wall to reveal the developing seed. Material was viewed with a dissecting microscope (Zeiss, Thornwood, NY). Images were captured using a 3-CCD Color Video Camera (KY-F55B; JVC) and ImageGrabber PCI 1.1 software.

For whole mounted specimens, the carpel valves of siliques

TABLE 1

Segregation of hygromycin resistance and T-DNA copy number for eight putative T-DNA-tagged gametophytic mutants

Line	Selfed T_3		Selfed T_4		Number of T_4 lines[b]				T-DNA copy
	Hyg^R:Hyg^S	%R[a]	Hyg^R:Hyg^S	%R[a]	1:1	3:1	All Hyg^R	Total	Number[c]
1	134:147	47.7	3197:2988	51.7	35	—	—	35	2
2	119:90	56.9	5224:4629	53.0	45	1	1	47	1
3	132:151	46.6	4731:4385	51.9	46	—	—	46	1
4	148:124	54.4	3671:3457	51.5	36	—	5	41	2
5	121:142	46.0	1747:1711	50.0	25	—	3	28	1
6	148:113	56.7	2337:2313	50.3	34	—	—	34	1
8	140:141	49.8	1048:1007	51.0	22	—	—	22	1
9	89:141	38.7	1773:2468	41.8	37	—	—	37	3–5

[a] % R, percentage of Hyg^R seedlings.
[b] The number of lines show segregation ratios of 1:1, 3:1, and 100% Hyg^R progeny.
[c] T-DNA copy number was determined by Southern blot analysis of *Eco*RI-digested genomic DNA with *gus* and *hpt* probes.

at various stages of development were removed using hypodermic needles (1 cc insulin syringes U-100 28G1/2; Becton Dickinson Franklin Lakes, NY). Dissected carpels were fixed in formaldehyde acetic acid [10:7:2:1 of ethanol (95%):dH_2O:formaldehyde (37%):acetic acid] for 2 hr and were transferred either directly or progressively into a drop of Herr's solution (Herr 1971) on a slide. After further dissection they were allowed to clear at least overnight. Specimens were viewed on a Leica DMR microscope with differen tial interference contrast (DIC) optics. Photographs were taken on Techpan film (Kodak, KJP, Nottingham, Berks, UK). Negatives were scanned on a 35 Sprintscan slide scanner (Polaroid, KJP, Nottingham, Berks, UK) and processed for publication using Adobe Photoshop 3.0.

RESULTS

Selection of putative T-DNA-tagged gametophytic mutants by segregation distortion: T_2 seed from 1150 transgenic lines, harboring at least one T-DNA insert from plasmid pMOG553 (Goddjin *et al.* 1993), which carries a promoterless *gus* gene and a hygromycin resistance marker, were screened for segregation ratio distortion away from 3:1. In total, 37 lines were initially selected, based on plating small numbers of T_2 seeds, which showed distorted segregation ratios of between 2:1 to 1:1. Further screening of T_3 generation seeds led to the identification of eight independent lines that reproducibly exhibited distorted segregation ratios approaching 1:1.

The percentages of hygromycin-resistant seedlings in progeny of selfed T_3 plants of these eight lines varied between 38.7 to 56.9%, which was significantly different from the expected 75% resulting from a normal 3:1 segregation ratio. Therefore, the T-DNA was not being transmitted normally (Table 1). We also investigated the segregation ratios of T_4 progeny lines for all eight lines and determined the frequency of lines showing ratios not significantly different from 1:1 and 3:1 using the χ^2 test (Table 1). As expected for the progeny descended from a heterozygous plant with a putative male or female gametophytic lethal mutation, all of the T_4 progeny harboring T-DNA from five independent lines (1, 3, 6, 8, and 9) appeared to be hemizygous for the T-DNA and showed segregation ratios of 1:1. Lines 2, 4, and 5 also gave rise to exceptional segregants that produced 100% Hyg^R progeny, presumably representing cases in which T-DNA became homozygous as a result of transmission through both male and female gametes (Table 1). Furthermore, line 2 also gave rise to a single progeny plant with a 3:1 segregation ratio out of 47 tested. The reversion of progeny to a 3:1 segregation ratio could indicate that the T-DNA is only closely linked but not the direct cause of the mutation. For example, in the parent plant of a 3:1 revertant line, a recombination event may have occurred between T-DNA and the gametophytic mutation. Based upon recombination values (2.1%), we estimated that the map distance between the T-DNA and mutation was 2.1 cM in line 2. In all lines showing 1:1 ratios there was no major difference in the frequencies of hygromycin-resistant progeny derived from T_3 lines and their T_4 progeny plants, as shown in Table 1. These results strongly suggest that six out of the eight lines are gametophytic mutants caused by T-DNA insertion at a single locus.

Southern blot analysis of restricted genomic DNA and examination of the fragments hybridizing to the *gus* and *hpt* probes was used to determine T-DNA copy number for each line (Table 1). Based on the segregation data, the expectation was that T-DNA was present at a single locus. With both probes, five lines (2, 3, 5, 6, and 8) showed a single hybridizing band consistent with the presence of a single intact T-DNA, whereas two lines (1 and 4) showed two bands indicating the presence of two complete T-DNA copies. Based upon the observed

TABLE 2

Genetic analysis of T-DNA transmission

	Male > C24[a]			Female < C24[b]			$\%R_{self}$	
Line	Hyg^R:Hyg^S	$\%R^c$	$TE_{male}{}^d$	Hyg^R:Hyg^S	$\%R^c$	$TE_{female}{}^d$	Expected[e]	Observed[f]
1	140:210	40.0	66.7	146:376	28.0	38.8	56.8	51.7
2	5:183	2.7	2.7	129:168	43.4	76.8	45.1	53.0
3	244:282	46.4	86.5*	60:154	28.0	39.0	61.1	51.9
4	102:174	37.0	58.6	205:356	36.5	57.6	60.0	51.5
5	79:96	46.2	82.3*	88:338	20.7	26.0	56.4	50.5
6	112:163	40.7	68.7	69:142	32.7	48.6	60.3	50.3
8	26:142	15.5	18.3	174:284	38.0	61.3	47.3	51.0
9	36:152	19.1	23.7	77:156	33.0	49.4	45.7	41.8

[a] Transgenic lines were used as the pollen donor.
[b] C24 was the pollen donor.
[c] %R = percentage Hyg^R seedlings in backcross progeny.
[d] TE_{male} and TE_{female} = the transmission efficiency (%) of the T-DNA.
[e] Expected percentage Hyg^R seedlings in self-progeny based on observed male and female transmission was calculated as $\%R_{self} = \%R_{female} + \%S_{female} \times (\%R_{male}/100)$.
[f] Observed $\%R_{self}$ was from selfed T4 progeny seedlings.
* The data showed no significant difference from 100% or a 1:1 segregation ratio.

segregation ratios, the two T-DNAs present in lines 1 and 4 are behaving as a single locus. This may result from the presence of two T-DNA copies at a single locus not directly linked in tandem or from two unlinked T-DNAs, only one of which is functional (*i.e.*, expressing the hygromycin resistance gene). Line 9 showed a more complex pattern of hybridizing fragments. With the *gus* and *hpt* probes, four and five hybridizing fragments were observed, respectively, some with different intensities, suggesting that there may be five *gus* and six *hpt* sequences present. Southern analysis of 12 progeny plants of line 9 gave identical hybridization patterns with no observed segregation of individual T-DNA bands (data not shown). Therefore, in line 9 multiple copies of T-DNA linked in a complex array are behaving as a single locus.

Genetic transmission of gametophytic mutations: The eight lines identified as putative T-DNA-tagged gametophytic mutants were all reciprocally backcrossed with the wild type (C24) in order to determine whether T-DNA transmission was reduced through the male and/or female gametes. If the T-DNA was transmitted normally (TE = 100%), seed from reciprocal backcrosses between hemizygous and wild-type individuals should segregate Hyg^R:Hyg^S = 1:1.

A summary of the percentage Hyg^R seedlings (%R) in reciprocal backcross progeny and the TE of the T-DNA through each sex is given in Table 2. In three lines, TE was reduced predominantly through the male. Line 2 showed only 2.7% TEmale; line 8, 18.3%; and line 9, 23.7%. Lines 8 and 9 also showed a lower but significant reduction in TE through the female (TE female: line 8, 61.3 %; and line 9, 49.4%). This shows that the putative gametophytic mutation in these lines has a greater effect on the success of the male than the female gametes. Four lines showed a predominant reduction in TE through the female (TEfemale: line 1, 38.8%; line 3, 39%; line 5, 26%; and line 6, 48.6%). For lines 3 and 5 there was no significant reduction in the TEmale, such that the observed distorted segregation ratios in self-progeny were solely due to reduced female transmission. Thus, mutations resulting from T-DNA insertion in lines 3 and 5 appear to be female gametophyte-specific.

We predicted the %R in self-progeny based on the observed TEmale and TEfemale and compared these values with the observed %R obtained in self-progeny of T_4 plants (Table 2). In general there was good agreement between the expected and observed %R, but some deviation was observed. Six lines (1, 3, 4, 5, 6, 9) showed an observed %R 4–10% below that expected. One explanation for this is that the calculated (expected) %R included the homozygote class. Because the block on transmission was always only partial (*i.e.*, the gametophytic mutation was incompletely penetrant), the expectation was that T-DNA homozygotes should occur but at a reduced frequency. However, homozygotes were very rarely observed in selfed T_4 individuals, such that only five T_4 individuals (in lines 2, 4, and 5) produced 100% Hyg^R progeny (Table 1). The absence of homozygote progeny in lines 1, 3, 6, 8, and 9, together with the severely reduced frequency of homozygotes in lines 2, 4, and 5, suggests that all mutations show full or partial sporophytic lethality.

Cytological analysis of male gametophytic phenotypes: The eight lines identified as having distorted segregation ratios were analyzed for mutant phenotypes. For the investigation of male gametophytic phenotypes, mature pollen from T-DNA-tagged plants was analyzed under the light microscope and by DAPI staining, which

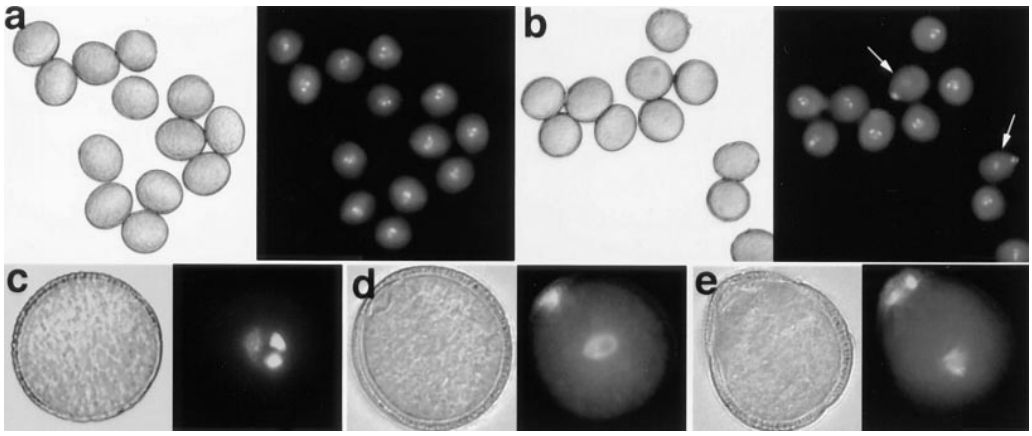


Figure 2.—Phenotype of wild-type (C24) and *limpet pollen* (*lip*). Light panels represent micrographs of mature pollen grains by light microscopy and adjacent dark panels represent the same pollen grains viewed by fluorescence microscopy after staining with DAPI. (a and c) Mature C24 pollen; all pollen grains possess two sperm nuclei and one centrally located vegetative nucleus. (b, d, and e) Mature *lip* pollen; the generative (d) or sperm cells (e) remain adjacent to the pollen wall. Arrows indicate pollen grains showing the *lip* phenotype (b).

allowed the examination of pollen cell morphology and nuclear phenotype (Figure 2a, b, c, d, and e). Mature pollen from all eight lines was examined. Although several of the lines, including lines 2 and 8 that showed a predominantly male reduction in TE, often showed some collapsed and smaller pollen compared with wild type, no consistent phenotypic differences could be detected. Pollen grains were consistently tricellular with wild-type nuclear phenotypes, two intensely staining sperm nuclei and one diffusely staining vegetative nucleus. However, line 9 showed a clear nuclear pollen phenotype by DAPI staining (Figure 2b). Compared to wild-type pollen, the most striking phenotypic difference was that the generative cell failed to migrate inward after pollen mitosis I, such that the generative or sperm cells remained against the pollen wall (Figure 2d and e). This mutant was named *limpet pollen* (*lip*). Despite the altered cell morphology in *lip* pollen grains, vegetative and sperm cell nuclear staining appeared normal with the two compact sperm cell nuclei staining intensely and the larger vegetative nucleus staining diffusely. The frequency of the pollen population showing the *lip* phenotype was ~25% (487 pollen with the *lip* phenotype out of 1979 scored).

Analysis of seed set: A straightforward assessment of the success of the female gametophyte can be made through analysis of seed set, because fully penetrant gametophytic mutations that disrupt female functions should reduce seed set by half (see also Moore *et al.* 1997). Removal of the ovary wall of elongated siliques showed that not all ovules had successfully developed into seed in lines 1 and 5, compared with wild-type siliques of similar age (Figure 3a and b). Clearing and staining of intact siliques was found to be a superior method of silique analysis because undisturbed seeds attached to the placenta via the funiculus could be observed without dissection. Funiculus staining was poor in ovules that had not been successfully fertilized, whereas the funiculus of well-developed seed stained intensely (Figure 3a and b). Seed set was analyzed quantitatively in wild type (C24) and in lines 1, 5, and 9 (*lip*) (Table 3). Wild-type plants showed 94.8% well-developed seed, whereas lines 1 and 5 both showed significantly reduced frequencies (40.9 and 46.2%, respectively) of well-developed seed. Both of these lines showed a strong reduction in T-DNA transmission through the female gametes (Table 2; TEfemale = 38 and 26%, respectively). Because the genes affected in lines 1 and 5 are required for female fertility, we named them *andarta* (*ada*) and *tistrya* (*tya*) after fertility goddesses of Gallic and Persian origin, respectively. Line 9 (*lip*) showed 81.5% well-developed seed consistent with the expectation of a reduced effect on female transmission (Table 2; TEfemale = 49%).

Ovule development in female gametophytic mutants: To determine the developmental defect in lines 1 (*ada*) and 5 (*tya*) we performed cytological analysis of cleared whole-mount ovules. In Arabidopsis, a single meiotic product, the viable megaspore (Figure 3c), survives and undergoes three synchronous nuclear divisions to form an eight-nucleated syncytium. Cellularization leads to the formation of a megagametophyte of the *Polygonum*-type (Maheshwari 1950) with the egg cell and two synergids at one pole, three antipodals at the other, and a binucleated central cell in the middle whose nuclei fuse prior to fertilization (Figure 3e). Double fertilization of egg and central cell initiates seed development. The ovules of a flower and the gametophytes within develop largely in synchrony (Schneitz *et al.* 1995; Christensen *et al.* 1997; Moore *et al.* 1997). Thus, cytological analysis of ovules from a semisterile plant heterozygous for a megagametophytic mutation allows comparison of mutant and wild-type embryo sacs within the same gynoecium.

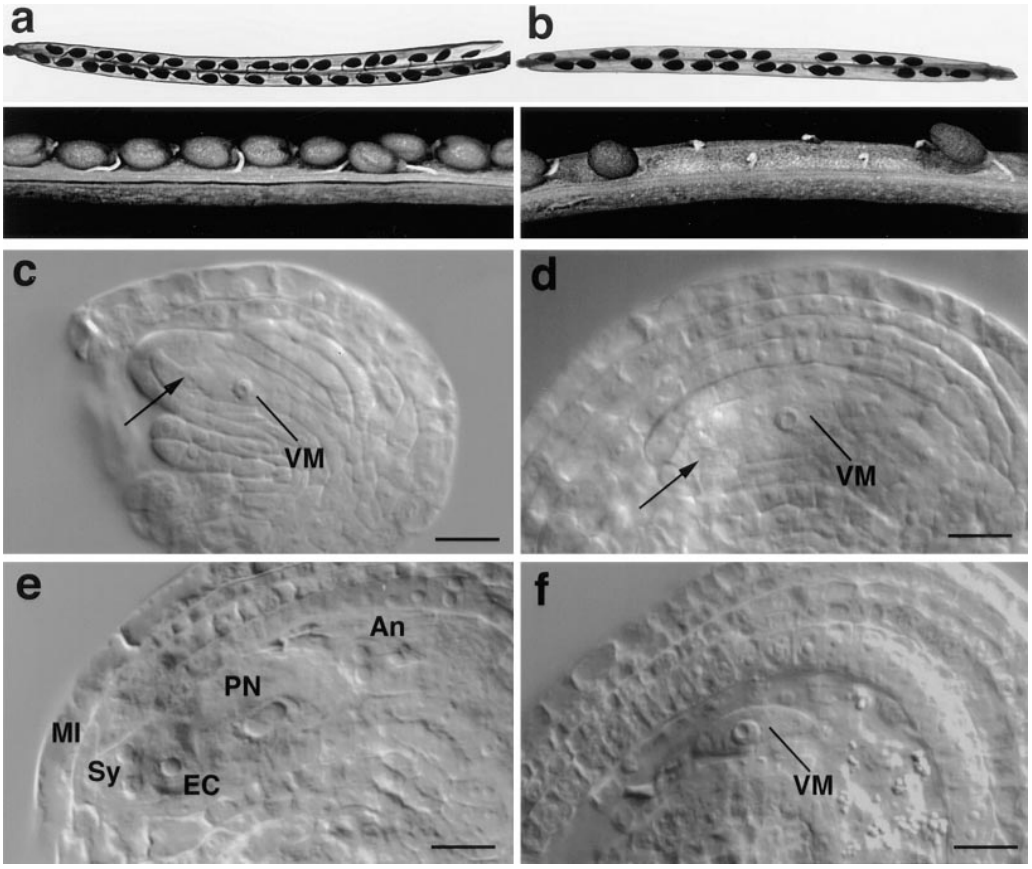


Figure 3.—Female phenotype of C24 and *andarta* (*ada*). a–b: Light panels represent intact siliques processed by clearing and cottonblue lactophenol staining; dark panels represent ovules viewed after dissection of the ovary wall. (a) Mature silique of C24. (b) Mature silique of a *ada* heterozygote showing ~50% failed ovules. (c–f) Megagametophyte development in cleared ovules of wild-type (c and e) and *ada* heterozygous plants (d and f). (c) Viable megaspore (VM) and remnants of three degenerated megaspores (arrow) at the initiation of megagametogenesis. (d) Viable megaspore nucleus (VM) and a lesser second nucleus of a persisting megaspore (arrow) in a mid-stage ovule. (e) Eight nuclear embryo sac with polar nuclei (PN) in the process of fusion. Antipodals (An) have cellularized, and the three nuclei at the micropyle (MI) cellularize to form the cells of the egg apparatus, the egg cell (EC), and the two synergids (Sy). (f) Arrested viable megaspore (VM) at the center of a late-stage ovule when sibling wild-type embryo sacs have been fertilized and initiated seed development. Bars (c–f), 10 μm.

Developmental stages from meiosis to early seed development were characterized. For *ada*, 33% of 102 ovules analyzed contained one-nuclear megagametophytes (Figure 3f) at a developmental stage when wild-type ovules were fertilized and had initiated endosperm development. A few of the defective female gametophytes had undergone one division cycle and were arrested at the two-nuclear stage (3%). The majority of the ovules (42%) contained embryo sacs where the primary endosperm nucleus had divided once or twice, and in some the zygote had initated the embryonic mitotic divisions. The remaining ovules (22%) were devoid of a normal embryo sac, and the gametophytic nuclei had apparently degenerated. In comparison, ~5% of the

TABLE 3

Analysis of seed set in mature siliques

Line	Developed seeds	Failed ovules	Total	Developed seeds (%)
C24	898	42	940	95.5
Line 1 (*ada*)	531	768	1299	40.9
Line 5 (*tya*)	438	488	926	47.3
Line 9 (*lip*)	651	148	799	81.5

The number of developed seeds and failed ovules was determined in 19 siliques from four plants of C24, 28 siliques from four plants of line 1 (*ada*), 22 siliques from four plants of line 5 (*tya*), and 17 siliques from three line 9 (*lip*) mutants. Seed set was scored following careful removal of the ovary wall.

ovules in a wild-type plant contain degenerated embryo sacs (Moore *et al.* 1997). In some ovules a second megaspore persisted and was visible in the center of the nucellus at early stages of megagametogenesis (Figure 3d). The developmental arrest of the viable megaspore was already apparent during the early mitotic division cycles, but the arrested embryo sacs persisted until after fertilization.

Embryo sacs carrying *tya* displayed a similar phenotype, but the arrested megaspore degenerated earlier than in *ada* ovules (data not shown). At early stages of development when almost 80% of the ovules in wild-type plants carry megagametophytes that have undergone at least two nuclear divisions, only 50% of 62 ovules analyzed from *tya* heterozygous plants contained megagametophytes of a corresponding stage. 24% of the embryo sacs had not undergone a single nuclear division cycle as compared to <1% in wild-type plants. The fraction of one-nucleated megagametophytes decreased in more mature ovules with a concomitant increase of degenerated embryos sacs. At a stage where about 95% of the ovules in a wild-type plant contained preglobular embryos, 53% of the ovules in *tya* heterozygotes contained degenerated megagametophytes. As occasionally observed in *ada* mutants, young ovules of *tya* plants often contained a second persisting megaspore. In addition, up to 10% of the ovules in a gynoecium carried female gametophytes with an odd number of nuclei, indicating that the nuclear division cycles in *ada* gametophytes were not synchronized. In summary, both *ada* and *tya* heterozygotes were found to carry megagametophytes arrested early during megagametogenesis, with the viable megaspore usually failing to initiate the mitotic program.

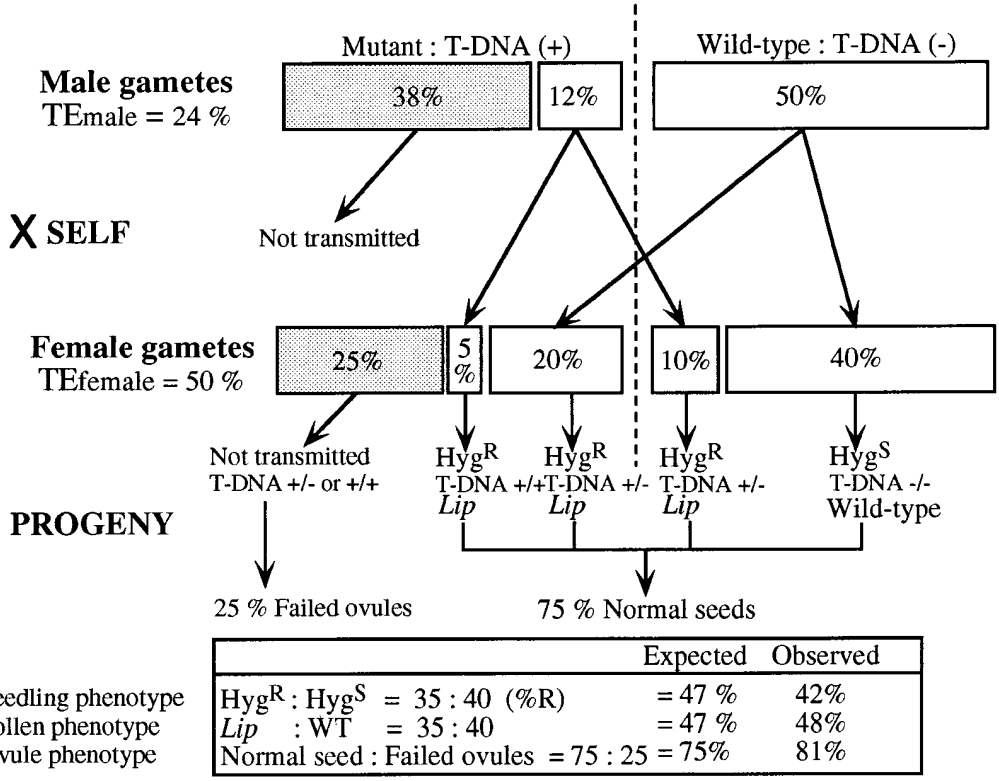
DISCUSSION

The strategy of screening for functionally important gametophytic genes through T-DNA segregation ratio distortion has led to the identification of eight independent putatively T-DNA-tagged gametophytic mutants in Arabidopsis, demonstrating the efficacy of this approach. T-DNA transformants showing segregation distortion have previously been observed in several species, including Arabidopsis (Budar *et al.* 1986; Katavic *et al.* 1994; Feldmann *et al.* 1997). Feldmann *et al.* (1997) recently described "exceptional" Arabidopsis transformants that segregated for a vast deficiency of Kan^R seedlings. These exceptional lines were detected at a frequency of 17 out of 142 (9%) lines tested and varied from those that gave rise to 100% Kan^S progeny to lines that segregated ~1:1. By comparison, our screening of ~1000 T-DNA lines identified eight lines (~1%) showing segregation distortion of ~1:1, which is similar to the frequency (3/142; ~2%) of 1:1 lines observed by Feldmann *et al.* (1997). In similar screens using transposable elements as insertional mutagens, such lines were recovered at a frequency of about 1% (Moore *et al.* 1997; R. Howden, U. Grossniklaus, and D. Twell, unpublished results).

Genetic and preliminary cytological analyses on these eight lines showed that three (2, 8, and 9) showed a predominant male gametophytic effect, four additional lines (1, 3, 5, and 6) showed a predominant female effect, and one line showed a similar effect on male and female transmission. One line (2) was essentially male-specific, whereas two lines (3 and 5) were female-specific. A similar distribution among mutant classes was observed in the study by Feldmann *et al.* (1997), although none of the mutations were found to be male- or female-specific. This may result from the different criteria used in the selection of lines for analysis. We deliberately selected lines showing ~1:1 ratios, whereas 6/7 of the lines analyzed by Feldmann *et al.* (1997) showed ratios <~1:1, preferentially selecting for more severe mutations affecting both male and female transmission. As discussed by Moore *et al.* (1997), a combination of a screen for semisterility with a segregation ratio distortion analysis allows the identification of mutations that affect the female gametophyte, either specifically or in combination with a male defect.

In all eight mutant lines analyzed here the observed reduction in TEmale and TEfemale was clearly sufficient to account for the strong reduction of T-DNA transmission in self-progeny. By way of example, the fate of wild-type and mutant gametes in self-crosses is illustrated for the *lip* mutant, which showed a strong reduction in T-DNA transmission in self (%R = 41.8) and backcross progeny (TEmale = 23.7%; TEfemale = 49.4; Figure 4). *lip* plants exhibited an obvious gametophytically transmitted phenotype and the phenotypic segregation of wild-type and *lip* progeny as 1:1. The absence or reduction in the expected frequency of the T-DNA homozygote class in all of the lines suggests that these mutations also act in the sporophyte and affect sporophytic viability to a greater or lesser extent. The high survival rate of transplanted resistant seedlings following selection further suggests that these mutations may cause embryo or seedling lethality.

Pollen mitosis I represents a highly asymmetric division of the microspore, following which the transient callose wall separating the vegetative and generative cells is degraded, and the generative cell migrates inward (Twell and Howden 1998). Degradation of the transient callose wall, presumably by β(1-3) glucanase(s), is likely to be essential for generative cell detachment and migration to proceed because it is fused at its margins to the outer (pollen) wall. The *lip* mutation results in mature pollen in which the generative or sperm cells remain closely associated with the pollen wall. In *lip* pollen, peripheral generative cells often appear to be separated from the vegetative cell by wall material. This suggests that their peripheral position may occur as a result of becoming trapped behind a



		Expected	Observed
Seedling phenotype	$Hyg^R : Hyg^S = 35 : 40$ (%R)	= 47 %	42%
Pollen phenotype	*Lip* : WT = 35 : 40	= 47 %	48%
Ovule phenotype	Normal seed : Failed ovules = 75 : 25	= 75%	81%

Figure 4.—Diagram illustrating the relationship between the expected and observed segregation of the T-DNA and *lip* phenotype in self-progeny of a heterozygous *lip* mutant. The frequencies of each phenotypic and genotypic class in self-progeny are based on the observed reduction of T-DNA transmission through the male and female gametes.

persistent dividing wall preventing inward migration. It is therefore conceivable that *LIP* encodes a β(1-3) glucanase required for degradation of the transient callose wall or a factor required for its activation or subcellular targeting. Alternatively, *LIP* may encode a factor involved in the presumed cytoplasmic (cytoskeleton) reorganization required for cell migration. It is also significant that division of the generative cell (PMII) to form two sperm cells is commonly completed in *lip*, demonstrating that PMII is not strictly dependent on complete engulfment of the generative cell by the vegetative cytoplasm. However, PMII does appear to be less efficient in *lip* because an undivided generative cell sometimes remains against the pollen wall. Although predominantly affecting male transmission, *lip* also acts in the female gametophyte. Furthermore, the absence of the homozygous mutant class suggests that *lip* also acts sporophytically and may therefore be embryo lethal. We are not aware of any other mutations known to affect generative cell migration, such that the molecular cloning of sequences at the site of T-DNA insertion to identify the *LIP* gene is of particular interest in understanding this unique case of cell migration in plants.

Two of the lines showing a predominantly female (*ada*) or female-specific (*tya*) reduction in T-DNA transmission were found to show reduced seed set consistent with failed embryo sac development. Detailed analysis showed that in both lines female gametophytes were arrested at an early stage such that the single undivided viable megaspore persisted (or subsequently degenerated) in mature ovules. The percentage of normal megagametophytes present in postfertilization ovules of *ada* and *tya* plants correlates well with the reduced seed set (*ada*: 41% fertility *vs.* 42% normal ovules; *tya*: 46% fertility *vs.* 47% normal ovules). However, this reduction in fertility cannot be fully accounted for by a megagametophytic defect. Both lines show significant transmission of the T-DNA through the female (*ada*: TEfemale = 38%; *tya*: TEfemale = 26%) such that a fertility significantly higher than 50% would be expected (66 and 60%, respectively, assuming a 5% background of unfertilized ovules). In contrast, the observed phenotypic abnormalities are in good agreement with the transmission data. In *ada* heterozygotes, 33% of the ovules are arrested at the viable megaspore stage as compared with the 30% calculated based on transmission data $[(100 - 5)/2 - \{0.38 \times (100 - 5)/2\} = 29.5\%]$. In plants carrying a mutant *tya* allele, 24% of the ovules at early stages contain one-nucleated megagametophytes and another 10% show an abnormal number of nuclei, which is close to the expected 35% as calculated from TEfemale $[(100 - 5)/2 - \{0.26 \times (100 - 5)/2\} = 35.1\%]$. What causes the additional decrease in seed set that goes beyond that expected from phenotypic and transmission analyses? A likely explanation is that the mutations cause a partially penetrant dominant sporophytic defect that leads to the development of abnormal ovules irrespective of their gametophytic genotype. This dominant effect would lead to gametophyte degeneration in an additional ~30 or ~20% of the ovules.

Indeed, a corresponding number of ovules with degenerated gametophytes was observed, such that the percentage of normal ovules just after fertilization is in good agreement with the observed seed set.

In both these lines the viable megaspore fails to initiate the mitotic phase of megagametogenesis. Therefore, they define and provide access to genes that are required for early viable megaspore development. A similar phenotype has been observed in detailed studies of the *Gf* mutation (Christensen *et al.* 1997). Mapping and determination of allelism among such mutants will be most effectively achieved through using molecular probes obtained through cloning of T-DNA flanking sequences. The mutants described here could affect the differentiation of the viable megaspore or specifically disrupt the initiation of the mitotic program. Many of the young ovules in *ada* and *tya* plants contain a second persisting megaspore (Figure 3d). Isolation of the megaspores may be important for normal megaspore selection and differentiation (Haig and Westoby 1986), and the presence of an additional megaspore may interfere with this process.

Segregation analysis of self-seed from T_4 plants corresponding to seven lines (1, 3, 4, 5, 6, 8, and 9) showed no reversion to the wild-type segregation ratio (3:1), which could result through the unlinking of the T-DNA from the mutation by a recombination event. These data strongly suggest that the T-DNA is the cause of the mutation or is very tightly linked to it. However, one T_4 individual from line 2 exhibited reversion to a 3:1 ratio, demonstrating that the T-DNA may not be the direct cause of the reduced transmission but may have inserted at a site linked to a gametophytic mutation.

In order to verify close linkage between a gametophytic mutation and a particular T-DNA locus, we performed detailed analyses of the cosegregation of T-DNA and the *lip* phenotype in line 9. The *lip* phenotype was scored in a population of 200 unselected T_4 progeny derived from T_3 *lip* parents, and seed was collected from the identified 97 *lip* mutants. All of the progeny of the 97 *lip* T_4 mutants segregated $\sim$1:1 for hygromycin resistance. Genomic DNA was isolated from 12 *lip* mutants and eight sibling plants with wild-type pollen from a segregating T_3 family. Southern blot analysis showed that the same multiple T-DNAs were present in all 12 *lip* mutants, whereas no T-DNA was present in the eight wild-type siblings (data not shown). Taken together, these data strongly indicate that the *lip* phenotype in line 9 is induced by the insertion of one or more of the multiple T-DNA copies at a single genetic locus.

The screening strategy and experimental results described in this article demonstrate that T-DNA tagging of important gametophytic genes through segregation distortion is an effective method to identify genes required for the development and/or function of male and female gametophytes. This approach initially does not allow one to distinguish between mutations affecting transmission through male or female gametes but is attractive in that: (1) it will identify important gametophytically expressed genes required for the complete developmental pathway of male and female gametophyte development, including gamete recognition and fusion; (2) genes identified in this way are very likely to be tagged or very closely linked to a molecular marker; and (3) it is simple to carry out, given the availability of large populations of transgenic lines containing a high proportion ($\sim$50%) of single locus T-DNA insertions (Feldmann 1991). For both tagged and T-DNA-linked mutations, direct access to the gene will be most efficiently achieved by the cloning of plant DNA flanking the insertion using inverse-PCR (Thomas 1996) or thermal asymmetric interlaced-PCR (Liu *et al.* 1995; Grossniklaus *et al.* 1998) procedures. If a concerted effort could be made to identify large numbers of random T-DNA or transposon-induced gametophytic mutations, a saturation approach could be taken to functionally dissect male and female gametophyte development and function.

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