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Arabidopsis *hapless* Mutations Define Essential Gametophytic Functions

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ABSTRACT

In flowering plants, the egg develops within a haploid embryo sac (female gametophyte) that is encased within the pistil. The haploid pollen grain (male gametophyte) extends a pollen tube that carries two sperm cells within its cytoplasm to the embryo sac. This feat requires rapid, precisely guided, and highly polarized growth through, between, and on the surface of the cells of the stigma, style, and ovary. Pollen tube migration depends on a series of long-range signals from diploid female cells as well as a short-range attractant emitted by the embryo sac that guides the final stage of tube growth. We developed a genetic screen in *Arabidopsis thaliana* that tags mutant pollen with a cell-autonomous marker carried on an insertion element. We found 32 haploid-disrupting (*hapless*) mutations that define genes required for pollen grain development, pollen tube growth in the stigma and style, or pollen tube growth and guidance in the ovary. We also identified genomic DNA flanking the insertion element for eleven *hap* mutants and showed that *hap1* disrupts *AtMago*, a gene whose ortholog is important for Drosophila cell polarity.

IN flowering plants, haploid and diploid cells with distinct gene expression programs interact to produce a network of signals that guide pollen tube growth toward eggs (Figure 1). Mutations that eliminate the functions of the pollen [male gametophyte (MG)] or of the embryo sac [female gametophyte (FG)] cannot be transmitted through the defective gametes; consequently, these mutants can be carried only as heterozygotes. Here, we employed a novel strategy to identify heterozygous *hapless* (*hap*) mutants with alterations that impair the development or function of haploid gametophytes. This screen tagged mutant pollen tubes with an autonomous marker, yielding new mutant phenotypes that define key signaling events.

Gametophytes are derived from the diploid sporophytic generation, the dominant stage of the angiosperm life cycle. The MG comprises three cells, two immobile sperm cells contained within a larger vegetative cell (reviewed in Twell 1994); the FG has seven cells, the egg, two synergids, the central cell, and three antipodals (Christensen *et al.* 1997). Upon pollination, MGs are partially desiccated and metabolically inactive; contact with a receptive stigma triggers hydration and germination of a pollen tube. The tip of this highly polarized cell travels across cell boundaries and through intracellular spaces and is guided by multiple discrete signals from the FG and the surrounding diploid cells (reviewed in Johnson and Preuss 2002). The pollen tube penetrates the stigma, grows through the transmitting tissue of the style and ovary, migrates along the septum to the funiculus of an ovule, and grows into the micropyle in response to signals emitted from the synergid cells and the ovule (Elleman *et al.* 1992; Hulskamp *et al.* 1995b; Ray *et al.* 1997; Higashiyama *et al.* 2001; Palanivelu *et al.* 2003). After entering the micropyle, the tube bursts to release two sperm, one of which fuses with the egg to produce a zygote, and the other merges with the central cell to generate the endosperm (reviewed in Faure and Dumas 2001).

Genetic screens for recessive loss-of-function mutations have identified several sporophytically expressed factors critical for gametogenesis (Schiefthaler *et al.* 1999; Hauser *et al.* 2000; Skinner *et al.* 2001; Wilson *et al.* 2001; Sorensen *et al.* 2003) and pollen-pistil interactions (Preuss *et al.* 1993; Hulskamp *et al.* 1995a; Wilhelmi and Preuss 1996; Palanivelu *et al.* 2003). Because these mutations affect genes expressed in diploid cells, plants heterozygous for a recessive sporophytic mutation can transmit the mutant allele efficiently through male and female gametophytes, forming homozygous progeny. In contrast, mutations that disrupt essential haploid-expressed functions show non-Mende-

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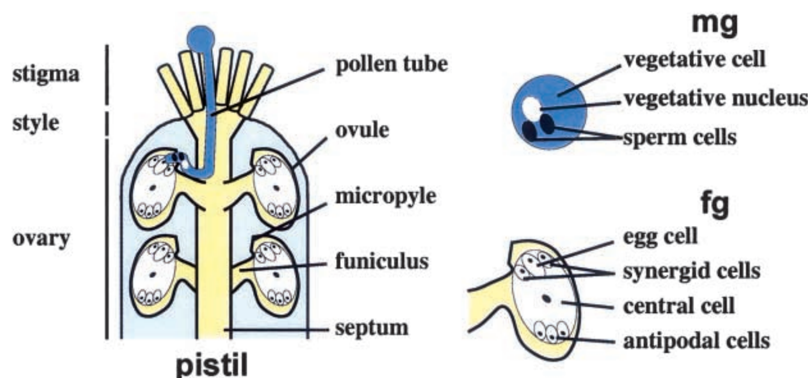

Figure 1.—Arabidopsis reproductive structures. Key features of the pollen tube (MG) growth pathway through the stigma and style to the ovary are shown. At the septum, pollen tubes migrate into the ovary and approach an ovule containing an FG. The MG and FG comprise three and seven haploid cells, respectively.

lian inheritance: Because fully penetrant, unconditional mutations cannot be transmitted through the affected gamete, homozygotes cannot be obtained (reviewed in Drews and Yadegari 2002; Johnson and Preuss 2002).

Several groups have screened for gametophytic mutants by searching for aberrant transmission of an antibiotic resistance gene associated with a T-DNA or transposon insertion (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Christensen *et al.* 1998; Howden *et al.* 1998; Procissi *et al.* 2001; Huck *et al.* 2003; Oh *et al.* 2003; Lalanne *et al.* 2004). Coupling these screens with a visual assessment of seed formation has yielded mutants defective in FG development (Christensen *et al.* 1998, 2002) or in the ability of pollen tubes to release their sperm after entering the micropyle (Huck *et al.* 2003; Rotman *et al.* 2003). Distorted segregation screens have also identified mutations that affect pollen grain development and pollen tube growth (Howden *et al.* 1998; Procissi *et al.* 2001; Oh *et al.* 2003; Lalanne *et al.* 2004). Direct phenotypic screens have also been used to identify gametophytic mutations resulting in abnormal pollen grain development (Chen and McCormick 1996; Park *et al.* 1998; Johnson and McCormick 2001; Lalanne and Twell 2002). The large number of genetic resources available for reverse genetic studies in Arabidopsis are also leading to the discovery of new genes that are essential for pollen development (Gupta *et al.* 2002; Kang *et al.* 2003) and pollen tube growth (Sanderfoot *et al.* 2001; Mouline *et al.* 2002; Golovkin and Reddy 2003; Steinebrunner *et al.* 2003; Hicks *et al.* 2004). A limitation of screens for gametophytic mutants has been the difficulty in analyzing the phenotypes of mutations that disrupt late aspects of pollen tube growth or guidance. Gametophytic mutants must often be maintained as heterozygotes and the challenges of distinguishing mutant pollen tubes from wild type make it difficult to determine the role of genes involved in pollen tube guidance.

Here we describe an approach for identifying Arabidopsis gametophytic mutants that enables precise analysis of pollen mutant phenotypes. We mutagenized plants with a T-DNA that carried β-glucuronidase (GUS) under the control of the postmeiotic pollen-specific promoter, LAT52 (Twell *et al.* 1989); this cell-autonomous, MG-specific reporter is detectable in pollen grains and pollen tubes, making it possible to track mutant MGs throughout pollination. Phenotypes can be followed separately from the transmission of the selectable marker carried by the T-DNA, allowing cosegregation tests to unambiguously associate the mutant phenotype with a single T-DNA insertion. We also employed the *quartet* (*qrt1*) mutation, which causes pollen grains to be released as intact meiotic tetrads (Preuss *et al.* 1994) and makes the consequences of chromosomal rearrangements readily apparent. With *qrt1* and the cell-autonomous GUS reporter, it is possible to classify plants as either homozygous or hemizygous for the T-DNA insertion by staining their pollen grains. We identified 30 MG mutants that can be grouped into three phenotypic classes: (1) pollen grain development, (2) pollen tube germination or growth within the stigma/style, and (3) pollen tube growth or guidance in the ovary. We identified T-DNA insertion sites for 11 *hap* mutations and showed that *hap1*, a mutation causing aberrant pollen tube growth, can be rescued with the tagged gene, Arabidopsis *Mago nashi*, the ortholog of a Drosophila gene required for oocyte polarity.

MATERIALS AND METHODS

Genetic screening: *Arabidopsis thaliana qrt1* lines (Columbia ecotype) carrying T-DNA insertions were generated by Agrobacterium-mediated transformation with the pCSA110 T-DNA (McElver *et al.* 2001). pCSA110 encodes GUS under the control of the postmeiotic, pollen-specific LAT52 promoter (Twell *et al.* 1989), as well as resistance to the herbicide Basta ($Basta^R$). Individual $Basta^R$ transgenic plants (primary transformants, T_1) were self-fertilized to yield T_2 seed stocks. T_2 stocks were plated on Murashige and Skoog (MS) medium [MS salts (4.33 g/liter; Carolina Biological Supply), 10% sucrose, pH 5.7 (KOH), 7% Bacto Agar] containing 50 mg/liter Basta (glufosinate ammonium; Crescent Chemical) and the percentage of $Basta^R$ seedlings was determined. One stage 14 flower (Smyth *et al.* 1990) from each $Basta^R$ plant retained was fixed (80% acetone, 30 min, 22°) and stained in X-Gluc (5 mm potassium ferrocyanide, 5 mm potassium ferricyanide, 50 mm $NaPO_4$, pH 7, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid) overnight at 37°.

Pollen tetrads were assayed for meiotic segregation of the

LAT52:GUS gene using an inverted Zeiss Telaval 31 microscope. Transmission of the T-DNA through the MG was tested by pollinating stage 14 (Smyth *et al.* 1990) *ms1* flowers (*m*ale-*s*terile, Landsberg *erecta* ecotype) with mature pollen from $hap^{+/-}$ plants; transmission through the FG was monitored by emasculating stage 12 $hap^{+/-}$ flowers and pollinating them 24–40 hr later with *qrt1* (Columbia ecotype) pollen. F_1 seed was plated on Basta-containing MS media and seedlings were scored for resistance or sensitivity. A frequency of ≤30% $Basta^R$ progeny in the F_1 of either cross differs significantly from the expected frequency of 50% (χ^2, $P = 0.01$) when ≥50 progeny are scored.

Phenotypic analysis: Pollen behavior was examined after crossing three plants from each *hap* line to three or more *ms1* pistils and allowing 12 hr for pollen tube growth. Pistils were excised and mounted on double-sided tape; ovary walls were then removed under a dissecting scope using a 27.5-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Pistils were immediately placed in a microtiter dish containing 100 μl 80% acetone for 30 min to fix cells and remove chlorophyll. Pistils were then incubated overnight at 37° in X-Gluc at high humidity. Pistils were mounted on microscope slides in 50% glycerol and imaged using DIC optics on a Zeiss Axioskop. For closer examination of pollen tube behavior on the ovule, pistils were stained in 0.1% Congo red following incubation in X-Gluc. Congo red is a fluorescent dye that stains pollen tubes along with other cells, allowing analysis by confocal laser scanning microscopy (Palanivelu *et al.* 2003; Zeiss LSM 510 microscope).

Identification of *HAP* genes: Genomic sequences flanking the right and left T-DNA borders were amplified with thermal asymmetric interlaced (TAIL) PCR (McElver *et al.* 2001). The *HAP1* gene was amplified by PCR (primer sequences 5′-TGCACAAACACAAGCCAGTCC-3′ and 5′-GCGAAATTCAACAGCCCTCCTTAC-3′), sequenced, cloned into pCAMBIA2200 (GenBank no. AF234313), and introduced into Agrobacterium (GV3101); *hap1* plants were transformed with the floral dip method (Clough and Bent 1998).

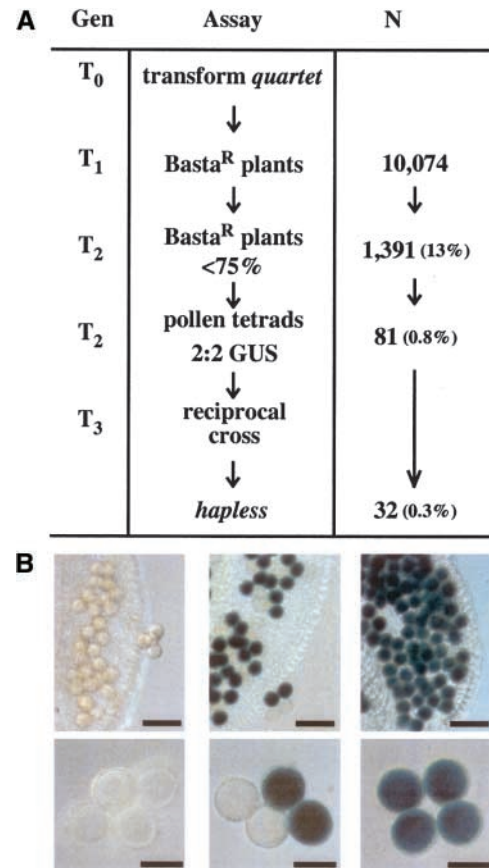

Figure 2.—The *hapless* screen. (A) 32 *hap* mutant lines were identified by screening >10,000 T_1 plants for distorted segregation of $Basta^R$ and LAT52:GUS. (B) Segregation of LAT52:GUS in pollen tetrads was used to assign genotypes to the descendants of transformed plants; homozygous wild type (0 GUS^+:4 GUS^-, left), hemizygotes (2 GUS^+:2 GUS^-, middle), and homozygotes carrying a GUS transgene (4 GUS^+:0 GUS^-, right) are shown. A control line exhibited Mendelian (1:2:1) segregation of wild type, $GUS^{+/-}$, and $GUS^{+/+}$ plants. An anther locule (top; bar, 50 μm) and individual pollen tetrads (bottom; bar, 25 μm) are shown.

RESULTS

A novel screen for mutations that distort Mendelian inheritance: Our screening procedure utilized two features that facilitated the identification of gametophytic mutants by distorted segregation. First, in addition to an herbicide resistance marker (Basta), the T-DNA element used for mutagenesis, pCSA110 (McElver *et al.* 2001), contained a LAT52:GUS pollen-specific reporter gene, providing a cell-autonomous tag for pollen grains that carry an insertion. Second, the screen was carried out in the *qrt1* mutant, which sheds pollen tetrads at dehiscence, allowing rapid monitoring of transgene inheritance in each strain. We screened 10,074 families derived from the self-fertilization of primary transformants and identified 32 *hap* mutants.

The screen was conducted in three stages (Figure 2):

1. Between 50 and 100 seeds descended from each T_1 plant were plated on media containing Basta. Mendelian inheritance predicts 75% of the seeds derived from self-fertilization of a hemizygous T_1 plant will be $Basta^R$. However, if the T-DNA insertion impairs the development or function of either gametophyte, the percentage of $Basta^R$ plants will be reduced because the mutant allele is not transmitted as frequently as the wild-type allele. We retained lines whose T_1 progeny yielded <70% $Basta^R$ as candidate *hap* mutants (1391 lines).
2. Twelve $Basta^R$ seedlings from each candidate line were transferred to soil and flowers were stained to reveal GUS expression. Observing GUS segregation in *qrt1* pollen tetrads makes it possible to distinguish homozygous (4 GUS^+:0 GUS^-) from hemizygous (2 GUS^+:2 GUS^-) plants. An insertion with no effect on the gametophytes is expected to segregate one homozygous plant (4 GUS^+:0 GUS^-) for every two hemizygotes (2 GUS^+:2 GUS^-), when only $Basta^R$ plants are analyzed. We retained lines as candidate *hap* mutants if all 12 $Basta^R$ plants were hemizygotes (81 lines). Some plants produced pollen tetrads with

a mixture of patterns (2:2, 3:1, and 4:0) or lacked GUS expression entirely. Lines with these expression patterns were discarded as they could have resulted from multiple insertions, incomplete insertions, or silencing of the T-DNA.

3. The 81 hemizygous lines that were retained after the first two assays were reciprocally crossed to wild type, and the percentage of Basta^R in the F_1 progeny from this cross was determined. If mutant and wild-type gametophytes are equally functional, this cross is expected to yield 50% hemizygous (Basta resistant) and 50% wild-type (Basta sensitive) progeny. Lines that showed ≤30% Basta^R F_1 progeny, whether the *hap* plant was crossed as a male or female, were considered to have a gametophytic mutation; 32 *hap* mutants met these criteria. These reciprocal crosses also allowed us to eliminate recessive embryo-lethal mutations, which were expected to pass the first two stages of the screen. Although these mutations yield 66% Basta^R T_2 progeny and no homozygous mutant plants, they show no transmission defect when hemizygotes are crossed to wild type.

Further tests of Basta^R segregation confirmed reduced transmission of *hap* mutations (Table 1). A *hap* mutant with one completely nonfunctional gametophyte is expected to yield 50% Basta^R offspring when self-fertilized; ~21 lines had this phenotype. Nine lines, however, produced significantly <50% Basta^R progeny, indicating both male and female gametophytes are likely affected. Two others showed >50% Basta^R (but <75%), indicating milder gametophytic defects.

To determine the extent to which a given *hap* mutation affected the MG, FG, or both gametophytes, we collected large sets of F_1 progeny from reciprocal crosses of $hap^{+/-}$ to $HAP^{+/+}$ (Table 1). The number of Basta^R F_1 progeny was monitored, allowing a direct comparison of the reproductive success of each *hap* gametophyte relative to *HAP* counterparts produced by the same meiosis. When a hemizygous control line was used as either the male or the female parent in crosses to wild type, 50% Basta^R progeny were obtained, indicating that expression of LAT52:GUS does not impair transmission (Table 1). Sixteen mutants showed reduced transmission through the MG but did not appreciably affect the FG; 14 *hap* defects were general, decreasing, but not eliminating, the function of both gametophytes, and two *hap* mutations disrupted only the FG (Table 1). Three of the male-specific mutants (*hap6*, *hap13*, and *hap16*) completely disrupted male functions, siring no Basta^R progeny when crossed to wild type, and 6 had a strong, but not complete impact on pollen function (≤5% Basta^R F_1). Of the two female-specific *hap* mutations, *hap29* was nearly completely penetrant (4.2% Basta^R F_1), and *hap19* had a milder impact (22.4% Basta^R F_1). Two other mutations completely eliminated the function of the FG while having a mild impact on MG function (*hap27* and *hap30*). Last, of the mutations that affected both sexes, some were severe in both males and females (*hap7* and *hap23*), whereas others severely

TABLE 1

***hap* mutations display distorted segregation, affecting the male, female, or both gametophytes**

	Self-fertilized		Male		Female	
	% Basta^R	*n*	% Basta^R	*n*	% Basta^R	*n*
Male only[a]						
hap1	43.2	1308	1.8	328	54.0	174
hap2	52.8	1581	0.7	403	47.1	467
hap3	50.0	1987	1.3	151	52.3	155
hap6	48.4	153	0.0	411	39.0	118
hap9	46.6	1518	8.7	585	42.9	196
hap10	51.1	2051	28.9	166	48.5	233
hap11	71.5[b]	899	18.0	133	53.5	198
hap13	52.4	1356	0.0	135	61.1	113
hap14	61.5	1582	14.7	109	45.8	144
hap15	55.2	1343	0.9	109	50.1	363
hap16	49.4	875	0.0	115	54.0	139
hap21	48.1	828	5.1	551	46.5	258
hap24	54.9	886	20.1	413	48.2	110
hap26	55.8	265	15.9	453	50.1	407
hap28	55.2	1915	4.4	1196	50.0	252
hap31	57.1	303	18.0	523	48.1	135
Female only[c]						
hap19	55.2	647	40.4	109	22.4	147
hap29	55.3	485	48.1	341	4.2	72
Affect both[d]						
hap4	42.7	1814	16.9	498	40.7	236
hap5	50.4	1275	19.3	316	29.7	148
hap7	10.6	1222	0.0	124	5.9	271
hap8	52.2	1037	5.2	461	32.0	172
hap12	41.6	1282	0.0	126	25.2	206
hap17	35.9	395	1.2	244	32.1	156
hap18	38.8	201	8.9	621	37.0	319
hap20	24.1	826	0.5	622	32.0	275
hap22	50.5	105	14.7	693	28.2	117
hap23	13.7	497	4.8	832	9.1	187
hap25	36.6	123	27.6	174	31.0	174
hap27	38.9	610	20.2	391	0.0	70
hap30	40.4	178	18.9	386	0.0	146
hap32	29.1	2245	1.3	237	35.5	169
Control[e]	75.9[b]	686	53.4	706	49.5	188

Self fertilized: $hap^{+/-} \times hap^{+/-}$, data are shown for the T_3 or T_4 generation. Male: *ms1* × $hap^{+/-}$, *ms1* females were hand pollinated using $hap^{+/-}$ anthers. Female: $hap^{+/-}$ × *qrt1*, $hap^{+/-}$ females were emasculated and hand pollinated using *qrt1* anthers. % Basta^R, the percentage of Basta^R F_1 progeny from the indicated cross.

[a] Significantly different from 1:1 in male cross but not in female cross (χ^2, $P < 0.01$).

[b] Not significantly different from 3:1 (χ^2, $P > 0.01$).

[c] Significantly different from 1:1 in female cross but not in male cross (χ^2, $P < 0.01$).

[d] Significantly different from 1:1 in both the male and female crosses (χ^2, $P < 0.01$).

[e] Not significantly different from 1:1 in male or female crosses (χ^2, $P > 0.01$).

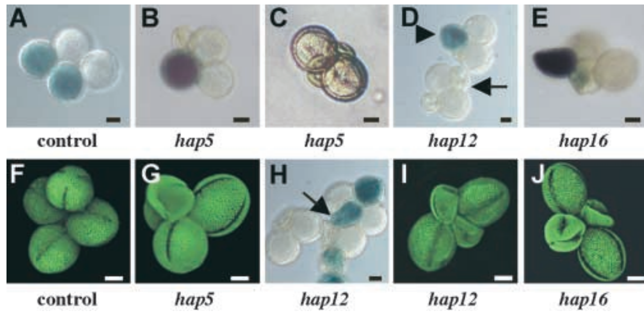

FIGURE 3.—Class 1 *hap* mutations disrupt pollen grain development. (A and F) A control line hemizygous for a LAT52: GUS insertion produced tetrads with two morphologically normal GUS$^+$ grains. Lines carrying *hap5* (B, C, and G) and *hap12* (D, H, and I) mutations produced tetrads that had two normal GUS$^-$ pollen grains and two grains with varying degrees of abnormality; development of these tetrads was sometimes arrested, inhibiting their ability to express GUS (D, arrow), while in other cases GUS expression was seen in apparently normal (D, arrowhead) or misshapen (H, arrow) grains. Lines carrying *hap16* (E and J) produced two aberrant GUS$^+$ pollen grains in each tetrad. Light micrographs (A–E and H, differential interference contrast optics) and fluorescent micrographs (F, G, I, and J, auramine-O stained to highlight pollen surface, confocal microscopy) are shown; bar, 10 μm.

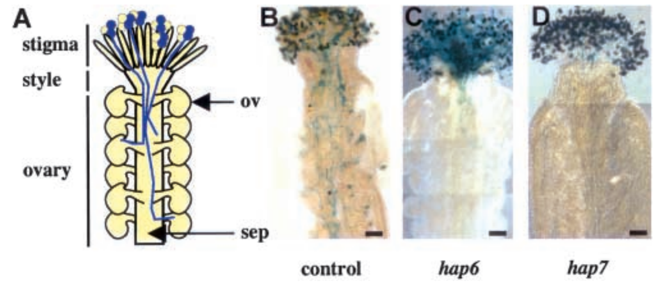

FIGURE 4.—Class 2 *hap* mutations alter early stages of pollen tube growth. (A and B) Pollinating wild type with a LAT52:GUS control revealed GUS$^+$ pollen tubes that germinated, penetrated the stigmatic papillae, grew through the style, entered the ovary via the transmitting tract, and targeted ovules for fertilization. Only GUS$^+$ pollen tubes are visible. (C) Pollen grains carrying a *hap6* defect germinated, but failed to exit the style. (D) *hap7* pollen tubes were less likely to germinate and rarely entered the style. Bars, 100 μm.

affected one gametophyte while only modestly disrupting the other (*hap12*, *hap27*, and *hap30*).

Assaying pollen phenotypes: We used the LAT52:GUS reporter to track the behavior of pollen grains and tubes carrying the T-DNA insertion. Tetrads from hemizygous controls produced two GUS$^+$ pollen grains and two GUS$^-$ pollen grains (Figure 3A), all of which germinated efficiently. Assays for later stages of growth were performed 12 hr after pollination; in control lines, GUS$^+$ pollen tubes germinated, penetrated the stigmatic papillae, grew through the style, entered the ovary through the transmitting tract, and migrated to the ovule (Figures 4, A and B, and 5, A and B). After entering the micropyle, GUS$^+$ pollen tubes burst, releasing an aggregate of GUS activity (Figure 5B, arrowhead) that marks the final stage of pollen tube function. We pollinated wild type with a hemizygous GUS$^+$ control and after 12 hr of pollen tube growth found 45% ($n = 422$) of the ovules showed GUS staining; the slight decrease from the expected rate of 50% suggests that 12 hr of pollen growth was too brief for the fertilization of every ovule.

We monitored pollen development and pollen tube growth behavior for each *hap* mutant, examining self-pollinated flowers and crosses of *hap* pollen onto wild type. This analysis placed each *hap* mutant into one of four major phenotypic classes (Table 2), having alterations in pollen grain development (class 1; 3 mutants), defective pollen tube growth through the style (class 2; 12 mutants), alterations in pollen tube growth or guidance in the ovary (class 3; 14 mutants), or no obvious defect (class 4; 3 mutants). In instances where some mutant pollen arrested at an earlier stage and other pollen progressed to a later stage, the mutant was assigned to the class representing the majority of mutant pollen.

Class 1, alterations in pollen grain development: Three *hap* mutants (*hap5*, *hap12*, and *hap16*) affected pollen grain development (Table 2, Figure 3). Neither *hap12* nor *hap16* pollen grains sired progeny, while *hap5* was less extreme, showing a 2.6-fold decrease from wild-type transmission levels (Table 1). The morphology of *hap16* pollen grains was consistently aberrant, with tetrads typically forming two almond-shaped GUS$^+$ (*hap16*) grains that were slightly collapsed and two normal GUS$^-$ (*HAP16*) pollen grains. In some cases *hap16* pollen did not express GUS (Figure 3, E and J), and mutant grains never formed pollen tubes; these phenotypes are consistent with an essential function expressed early in pollen development.

The morphological defects of *hap5* and *hap12* were less severe and showed variable expressivity. By monitoring GUS staining and pollen grain morphology, we scored tetrads as follows: (i) four grains with normal morphology (2 GUS$^+$:2 GUS$^-$); (ii) three normal and one morphologically aberrant grain (2GUS$^-$:1 GUS$^+$:1 aberrant); and (iii) two normal and two morphologically aberrant grains (2 GUS$^-$:2 aberrant). For *hap 5*, categories i, ii, and iii contained, respectively, 12, 75, and 13% of the tetrads ($n = 187$; Figure 3, A–C, F, and G); and for *hap 12*, 0, 47, and 53% of the tetrads ($n = 127$; Figure 3H, arrow; 3D, arrowhead; 3D, arrow; and 3I). Despite these morphological defects, both *hap5* and *hap12* produced some pollen tubes that germinated; those from *hap12* were arrested in the style (as in class 2 below), while *hap5* pollen grains that developed normally were able to fertilize FGs.

Class 2, defective pollen tube growth at the stigma or style: After pollen tubes emerge from the grain, they establish polarized growth, grow within the cell wall of the stigmatic papillae cells, and then enter the style

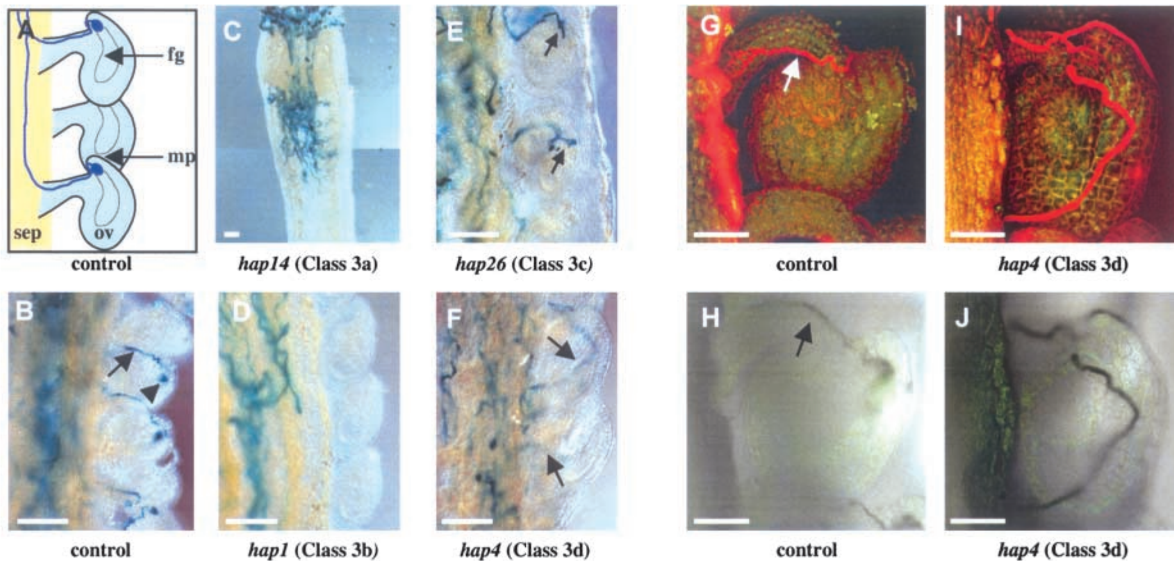

FIGURE 5.—Class 3 *hap* mutations alter late stages of pollen tube growth or guidance. (A) Diagram of wild-type pollen tube paths (blue); sep, septum; ov, ovule; mp, micropyle; fg, female gametophyte. (B–F) LAT52:GUS staining of pollen tubes growing in wild-type pistils (pollen tube, arrow; GUS activity in ovule, arrowhead). (B) *HAP* pollen tubes carrying LAT52:GUS grow up the funiculus, penetrate the micropyle, and deposit an aggregate of GUS activity in the FG. (C) *hap14* mutants (class 3a) grow short pollen tubes that target the ovules they reach. (D) *hap1* pollen tubes (class 3b) fail to exit the septum. (E) *hap26* pollen tubes (class 3c) grow up the funiculus, but fail to enter the micropyle. (F) *hap4* pollen tubes (class 3d) exhibit random growth on the surface of ovules. Confocal laser scanning micrographs of control (G) and *hap4* (I) pollen tubes stained with Congo red show that *hap4* pollen tubes grow past the micropyle without entering. Transmitted light micrographs of the same samples (H and J) show GUS activity in pollen tubes. The ovule shown in G and H has two pollen tubes growing on it: a GUS− pollen tube (arrow) is visible in G, while a GUS+ pollen tube (arrow) is visible in H. Collecting both images allows GUS− and GUS+ pollen tubes to be compared. The pollen tube visible in I is GUS+ (J) and therefore carries the *hap4* mutation. Bars: A–F, 100 μm; G–J, 50 μm.

where they grow through the nutrient-rich extracellular matrix of the transmitting tract. We identified 12 *hap* mutations that were defective in these stages of pollen tube growth (Figure 4, Table 2). These mutations affected pollen tube germination and growth and when they were reciprocally crossed to wild type, they all showed extreme defects in male reproductive success; five exhibited a 100-fold or greater decrease in transmission and the remainder showed a reduction between 6- and 50-fold (Table 1). One group was male specific (*hap3*, *-6*, *-9*, *-13*, *-15*, *-21*, and *-28*), and the other (*hap7*, *-8*, *-17*, *-20*, and *-32*) affected both sexes.

Class 3, alterations in pollen tube growth or guidance in the ovary: Despite intensive screening for sporophytic sterile mutations, few genes affecting pollen tube growth or guidance from the style to the ovule have been identified, presumably because such genes are gametophytic. In this screen we identified 14 mutants with alterations in these later stages of pollination (Table 2), two of which (*hap1* and *hap2*) are male specific and virtually 100% penetrant (Table 1). Class 3 mutants fell into four phenotypic groups that genetically define critical steps in the pollen tube growth and guidance process: (a) short tubes that target the ovules they reach (*hap10*, *-14*, *-23*, and *-31*); (b) pollen tubes that remain on the septum (*hap1*, *-18*, and *-22*); (c) pollen tubes that grow normally to the ovules, but fail to enter the micropyle (*hap11*, *-26*, and *-30*); and (d) pollen tubes that grow chaotically in the ovary (*hap2*, *4*, *24*, and *27*).

Class 3a mutants demonstrate that pollen tube growth can be genetically separated from guidance; for example, *hap14* pollen tubes never grew more than one-third of the way down the septum (Figure 5C), but nonetheless sired 14.7% of F_1 progeny when crossed to wild type (Table 1). In contrast, pollen tubes from class 3b (*hap1*) grew the length of the pistil, but did not migrate toward the ovules and sired only 1.8% of F_1 progeny when crossed to wild type (Figure 5D, Table 2); *hap18* and *hap22* had a similar phenotype. Upon close inspection of class 3b pollen tubes, we found they remained on the septum or in the transmitting tract (Figure 5D).

Class 3c mutants also exhibited a novel phenotype, with the growth of the pollen tubes indistinguishable from wild type except for the final step—targeting the micropyle. For example, *hap26* pollen tubes often grew up the funiculus but then stopped and failed to enter the micropyle (Figure 5E); *hap11* and *hap30* pollen tubes were similarly arrested. We did not identify any *hap* mutants with pollen tubes that entered the micropyle but continued to grow without bursting as has been observed in the FG mutants *fer* and *srn* (Huck *et al.* 2003; Rotman *et al.* 2003). The class 3c defects were all

TABLE 2

***hap* mutants fall into four pollen phenotypic classes**

Class	Description	*n*	Mutants
1	Disrupted pollen grain development	3	*hap5*, *hap12*, *hap16*
2	Short pollen tube growth—failure to exit style	12	*hap3*, *hap6*, *hap7*, *hap8*, *hap9*, *hap13*, *hap15*, *hap17*, *hap20*, *hap21*, *hap28*, *hap32*
3	Pollen tube growth in the ovary and/or guidance is disrupted		
3a	Growth in ovary is short, but pollen tubes target the ovules they reach	4	*hap10*, *hap14*, *hap23*, *hap31*
3b	Pollen tubes fail to leave the septum	3	*hap1*, *hap22*, *hap18*
3c	Pollen tube growth path appears normal, yet tubes fail to enter the micropyle	3	*hap11*, *hap26*, *hap30*
3d	Pollen tube growth is chaotic in the ovary	4	*hap2*, *hap4*, *hap24*, *hap27*
4	No obvious defect	3	*hap19*, *hap25*, *hap29*

subtle with relatively modest (two- to threefold) decreases in transmission through the MG.

Removal or impairment of the FG has been shown to result in chaotic pollen tube growth on affected ovules (Ray *et al.* 1997; Shimizu and Okada 2000), similar to that observed in the sporophytic mutant, *pop2* (Palanivelu *et al.* 2003). Here we have defined a set of pollen mutants with this phenotype (class 3d); the pollen tubes of *hap2*, *-4*, *-24*, and *-27* exit the transmitting tract, yet take unorthodox paths within the ovary and meander along the surface of the ovules (Figure 5, F, I, and J). Two of these (*hap2* and *-24*) are male specific, while one (*hap27*) also completely impairs FG function. In all of these cases, the principle defect does not appear to be one of pollen tube extension; instead, it is more likely that the gametophytes are defective in responding to signals or, alternatively, in forming appropriate surface contacts necessary for guidance.

T-DNA insertion sites: Genomic DNA was amplified from 15 of the *hap* mutants by TAIL PCR and putative insertion sites were successfully identified for 11 mutants (Liu *et al.* 1995). The junction between the T-DNA and the genome was identified on both sides of the insertion for four of these (*hap2*, *-4*, *-6*, and *-15*; Table 3). Comparison with the sequenced, annotated Arabidopsis genome localized the TAIL-PCR products to specific loci (Table 3). Other than *hap3* and *hap11*, which fell between two genes, all of the amplified insertion junctions were within a single gene or in the immediately adjacent 5′ or 3′ DNA (Table 3). With the exception of *hap2*, an expressed sequence tag or a full-length cDNA sequence supported the annotation of each of these genes (Table 3). Current annotation of the Arabidopsis genome describes the role of most of these genes as “unclassified”; the exceptions are *hap4* and *hap12*, which have been assigned roles in protein synthesis and transcription, respectively (Arabidopsis Genome Initiative 2000).

We used the predicted amino acid sequence of each gene to search for similar sequences that might provide insight into their biochemical functions and found similarity to proteins involved in gene expression (*hap1*, *hap3*, *hap4*, *hap12*, and *hap15*), secretion (*hap6* and *hap13*), molecular transport (*hap3* and *hap5*), and cellular energy production (*hap11*; Table 3). The *hap8* insertion disrupts a gene with no matches in databases and therefore appears to be unique to the Arabidopsis genome sequence; the *hap2* insertion and one of the genes potentially disrupted by *hap11* are found only in Arabidopsis and rice and may, therefore, be plant specific.

Molecular complementation of *hap1*: The *hap1* insertion was found just upstream of the Arabidopsis ortholog of *Mago nashi*, a Drosophila gene required for localization of *oskar* mRNA and, consequently, for differentiation of the oocyte posterior and germ-line formation (Boswell *et al.* 1991; Mohr *et al.* 2001). To verify the identity of *HAP1*, we introduced a wild-type copy (*HAP1tr*), including 730 bp upstream and 700 bp downstream of the open reading frame, into *hap1* mutants. A T-DNA carrying *HAP1tr* and a kanamycin resistance gene was introduced into heterozygous *hap1* plants; these plants were self-pollinated and kanamycin-resistant (Kan^R) progeny were selected. As expected, half of these plants produced pollen tetrads with 2 GUS^+ and 2 GUS^- grains and thus carried the original *hap1* insertion. These plants were self-fertilized, and the segregation of the Basta and Kan markers was analyzed by plating on selective MS medium. Lines with multiple unlinked insertions of the Kan^R gene were discarded (>90% Kan^R). A self cross of a line heterozygous for $hap1^{+/-}$ ($Basta^{R+/-}$) and $HAP1tr^{+/-}$ ($Kan^{R+/-}$) is expected to produce F_2 progeny that segregate ~67% $Basta^R$ (8/12) and ~83% Kan^R (10/12); this segregation pattern results because *hap1* MGs function only when they carry the *HAP1tr* construct. Two independent transgenic lines were thoroughly analyzed: F_2 progeny were 70% (n = 380) and 69% (n = 377) $Basta^R$ and 79% (n = 482) and 82% (n = 355)

TABLE 3

Putative insertion sites for *hapless* mutations

Mutant	Gene[a]	cDNA[b]	Description or reference gene[c]	Insertion site[f]
hap1[d]	At1g02140	FL	*Mago nashi* (*Drosophila melanogaster*, $e = 3 \times 10^{-61}$; Boswell *et al.* 1991)	93 bp upstream
hap2[e]	At4g11720	None	Unknown	Exon 12 of 14
hap3	At1g66570	EST	Sucrose transporter, *SUC1* (*A. thaliana*, $e = 0.0$; Stadler *et al.* 1999)	502 bp upstream
	At1g66580	FL	60S ribosomal protein L10	1814 bp upstream
hap4[e]	At3g52590	FL	Ubiquitin extension protein 1 (UBQ1)/60S ribosomal protein L40 (Callis *et al.* 1990)	Intron 4 of 4
hap5[d]	At1g30450	EST	Cation-chloride cotransporter (*Nicotiana tabacum*, $e = 0.0$; Harling *et al.* 1997)	Exon 13 of 13
hap6[e]	At4g21150	FL	Ribophorin II (*Homo sapiens*, $e = 8 \times 10^{-19}$; Crimaudo *et al.* 1987)	186 bp upstream[g]
hap8	At5g56250	FL	Unknown	Exon 3 of 4
hap11[d]	At5g47020	EST	Unknown	495 bp downstream
	At5g47030	FL	Mitochondrial ATP synthase δ chain (*Ipomoea batatas*, $e = 6 \times 10^{-72}$; Morikami *et al.* 1992)	89 bp upstream
hap12	At4g36900	FL	Contains AP2 domain (RAP2.10)	62 bp downstream[h]
hap13	At1g60780	FL	Clathrin adapter medium chain, *MU1B* (*Mus musculs*, $e = 10^{-154}$; Ohno *et al.* 1999)	Exon 8 of 11
hap15[e]	At1g20200	FL	26s proteasome regulatory subunit S3	Exon 3 of 9

[a] Arabidopsis gene names; two genes are listed when insertions were found between two genes.

[b] FL, annotation supported by full-length cDNA; EST, expressed sequence tag in GenBank; none, no EST or full-length cDNA in databases.

[c] Protein sequences were compared with GenBank's nonredundant database (Altschul *et al.* 1990). Genes with significant similarity for which functional data have been published are noted; *e*-value from Blast is given.

[d] In addition to TAIL-PCR, one T-DNA border was confirmed by a secondary PCR.

[e] Both T-DNA borders were recovered by PCR.

[f] Based on full-length cDNA if available, or most recent annotation; for insertions between genes, positions are relative to the translational start codon (upstream) or the translational stop codon (downstream).

[g] Insertion in 5′-untranslated region.

[h] Insertion in 3′-untranslated region.

Kan^R, respectively. Untransformed *hap1* lines showed 48% $Basta^R$ progeny (see also Table 1). These results indicate that *HAP1tr* restores function to *hap1* MGs. Further confirmation was obtained when pollen tetrads were analyzed in Kan^R F_2 plants: plants with pollen tetrads that were 4 GUS^+ to 0 GUS^- were found at expected frequencies (∼25%) among Kan^R plants (24/112 plants); these were never found in *hap1* lines that were not transformed with *HAP1tr* ($n > 200$). In addition, *HAP1tr* restored the ability of *hap1* pollen to migrate from the transmitting tract and to fertilize ovules (data not shown).

DISCUSSION

Haploid-specific genes required for pollen tube growth and guidance: Here, we describe 32 Arabidopsis mutations that affect genes expressed in haploid gametophytes. Thirty *hap* mutants altered MG functions, significantly expanding the set of previously described MG mutations. Mutant pollen cells were tagged with an autonomous marker that facilitated precise phenotypic analysis, allowing the placement of each mutant into one of three phenotypic categories: (1) altered pollen grain development, (2) failure of pollen tube growth within the stigma and/or style, or (3) failure of pollen tube growth within the ovary (Table 2).

This study identified new mutations that cause novel pollen tube growth phenotypes, as well as mutations that are phenotypically similar to previously characterized sporophytic or gametophytic mutants. The 12 class 2 *hap* mutations disrupted pollen tube germination or growth through the stigma and style similar to the previously identified *mad4*, *syp21-1*, *npg1*, *AtAPY1/AtAPY2*, *kip*, *seth1*, and *seth2* gametophytic mutations (Grini *et al.* 1999; Sanderfoot *et al.* 2001; Golovkin and Reddy 2003; Procissi *et al.* 2003; Steinebrunner *et al.* 2003; Lalanne *et al.* 2004). Four class 3a *hap* mutations arrested tube growth in the upper ovary and, like the gametophytic *tip1* defect (Ryan *et al.* 1998), did not impair the tube's ability to target ovules. Three class 3b *hap* mutations produced tubes that grew along the septum but failed to exit onto the placenta surface, reminiscent of the major defect caused by the sporophytic *pop2* mutation (Palanivelu *et al.* 2003). Three class 3c *hap* mutations yielded pollen tubes that had

normal growth to the micropyle yet failed to penetrate ovules; this phenotype has not been previously described. Finally, like the secondary *pop2* defect (Palanivelu *et al.* 2003), the four class 3d *hap* mutations generated tubes that grew toward ovules, but failed to adhere to the funicular surface or target the micropyle; no MG mutants with this defect were previously known.

Beyond defining functions required for assembling pollen grains and extending tubes, each *hap* mutant class could include MG genes that mediate responses to female signals directing pollen tube growth. Class 2 *hap* mutations might disrupt the initial interactions between pollen and stigma cells, including the pollen's ability to hydrate, establish polarity, germinate, and penetrate stigmatic papillae (Johnson and Preuss 2002). Sporophytically expressed pollen coat components, including lipids and proteins, are critical for pollen hydration (Preuss *et al.* 1993; Lush *et al.* 1998), yet MG-expressed factors likely mediate pistil interactions soon after germination. A lily *in vitro* system recently led to the identification of a complex of pectin and a small cysteine-rich protein (SCA) that forms on the stigma and transmitting tract to promote pollen tube attachment and growth (Lord 2003). Pollen tube factors that bind the pectin/SCA matrix are not known, and *hap* mutants that fail to grow in the transmitting tract may reveal components of a matrix-driven translocation system. Additionally, chemocyanin, a stigma protein that redirects pollen tube growth *in vitro*, was also recently purified from lily (Kim *et al.* 2003). Whether a similar factor directs Arabidopsis pollen tube growth remains to be determined; however, Arabidopsis does encode a protein that is 60% identical to chemocyanin (Kim *et al.* 2003). Class 2 *hap* mutants may be an important resource to identify new components of an Arabidopsis analog to this lily signaling system.

Class 3 *hap* defects could disrupt MG responses to FG signals that mediate transmitting tract exit, ovule choice, funicular growth, or micropylar targeting. Genetic ablation of the FG causes pollen tubes to bypass the affected ovules (Ray *et al.* 1997), and more subtle defects in FG development implicate signals that regulate adhesion to the funiculus and micropyle targeting (Shimizu and Okada 2000). Class 3c *hap* mutants, which arrest tube growth just before the micropyle (Figure 5E), may be defective in their response to these late guidance cues, representing male counterparts of the *maa1* and *maa3* FG mutants (Shimizu and Okada 2000). Class 3d *hap* pollen tubes resemble *pop2* pollen tubes that exit the transmitting tract, but fail to target the micropyle. In *pop2*, a 100-fold increase in gamma amino butyric acid (GABA) concentration causes aberrant tube migration; therefore, class 3d mutants may define components of a pollen-expressed GABA response pathway.

The *hap* screen complements previous screens based on distorted segregation: Many gametophytic mutants have been identified by monitoring the segregation of antibiotic resistance markers in lines generated by insertion mutagenesis (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Christensen *et al.* 1998; Howden *et al.* 1998; Procissi *et al.* 2001; Huck *et al.* 2003; Oh *et al.* 2003; Lalanne *et al.* 2004). Non-Mendelian, distorted segregation ratios identified numerous FG mutations, as well as MG mutations that alter pollen grain development, pollen tube germination, and tube growth. The inability to differentiate mutant from wild-type pollen tubes in heterozygous plants hampered characterization of MG mutants affecting the final stages of pollen tube growth (Procissi *et al.* 2001). Here, we assayed distorted segregation of an herbicide resistance marker and a pollen-specific reporter gene (LAT52:GUS) that tagged mutant pollen in *qrt1* plants, enabling a focus on single insertions that potentially affect any stage of MG growth.

Gametophytic mutations can be pleiotropic, incompletely penetrant, and can display variable expressivity (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998; Grini *et al.* 1999; Drews and Yadegari 2002). The inclusion of a cell-autonomous tag in the *hap* screen makes analysis of these characteristics more efficient. For example, the pollen grain defects in *hap16*, *hap5*, and *hap12* varied from strong (no GUS expression, completely collapsed) to weak (normal GUS expression, normal pollen grain), and this phenotypic variation was evident because the pollen tetrads were marked by LAT52:GUS (Figure 3). By tagging the mutant pollen tubes, it was clear that rare *hap12* pollen grains germinated pollen tubes that failed to leave the stigma, and that *hap3* pollen tubes occasionally grew down the length of the ovary but failed to target ovules. These variable phenotypes could result from unequal inheritance of gene products expressed in the diploid meiocyte or from variable expression of gametophytically expressed genes.

The method of Agrobacterium-mediated transformation used here targets the FG, potentially limiting our ability to recover insertions that result in FG lethality (Clough and Bent 1998; McElver *et al.* 2001; Drews and Yadegari 2002). Nevertheless, we did identify two *hap* defects that completely blocked FG function (Table 1, *hap27* and *hap30*), suggesting that these genes function at a developmental stage that occurs prior to the Agrobacterium targeting event. Consistent with previous observations (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Grini *et al.* 1999; Christensen *et al.* 2002), approximately half (14) of the *hap* mutations affected both the MG and FG to some degree, suggesting they alter basic functions required by male and female haploid cells. Interestingly, some *hap* mutants with MG and FG defects extend full-length pollen tubes with a reduced capacity to target ovules (hap4, hap18, hap22, hap27, and hap30), raising the possibility that they define factors required for signaling between male and female gametophytes. On the other hand, a greater number (16) were pollen specific, identifying a set of

genes required by the MG that have no essential role in MG development or function.

By performing the *hap* screen in the *qrt1* background, we were able to efficiently discard numerous lines with unwanted alterations that are byproducts of T-DNA mutagenesis, including multiple unlinked insertions, secondary untagged mutations, complex or incomplete insertions, and translocations (Castle *et al.* 1993; Feldmann *et al.* 1997; Nacry *et al.* 1998). We retained Basta^R plants that produced only 2 GUS$^+$ to 2 GUS$^-$ tetrads; plants that carry multiple T-DNA insertions are readily differentiated by their altered patterns of GUS segregation. By monitoring the inheritance of Basta^R, GUS, and the gametophytic phenotype, we were assured that the *hap* mutation is caused by a single insertion. Furthermore, while translocations and other genomic rearrangements have contaminated previous screens for gametophytic genes (Feldmann *et al.* 1997; Bonhomme *et al.* 1998), tetrad analysis readily identifies these events. Translocations produce two types of tetrads in equal proportion: (1) four viable pollen grains with balanced chromosomes and (2) four inviable (shriveled) pollen grains that carry duplications and deficiencies (Kindiger *et al.* 1991). In *QRT1*$^{+/+}$ plants, translocations yield 50% aborted pollen and are indistinguishable from a heterozygous gametophytic lethal mutation; however, translocations are obvious in *qrt1*, where two types of tetrads are produced as described above.

Functional genomics of the male gametophyte: A complete understanding of the ~26,000 Arabidopsis genes requires a thorough analysis of the gametophytic generation. Between 13 and 20% of Arabidopsis genes are expressed in pollen and as many as 5% are pollen specific (Becker *et al.* 2003; Honys and Twell 2003); functional analysis of only a small fraction of these genes has been performed. None of the genes identified in this study were previously identified by mutations in plants (Table 3). Screens for gametophytic mutations are efficient, rapid, and can be performed on a genome-wide scale. Approximately 180,000 T-DNA insertions are required to achieve a 95% probability of identifying at least one insertion in every Arabidopsis gene, with an average of three insertions per gene (Krysan *et al.* 1999). Here, we identified 30 MG mutants in a screen of 10,074 transgenic lines (0.3%); this number underestimates the mutant/insertion rate because we discarded ~47% of the lines because of multiple insertions (Budziszewski *et al.* 2001). Correcting for multiple inserts, we calculate that MG *hap* mutations represent 1/180 insertions. A saturation screen would consequently yield ~1000 *hap* mutations representing ~330 MG genes (assuming three alleles per gene). Traditional tests of allelism cannot be performed with gametophytic mutants because the affected cells are haploid; therefore, determining the allelic relationship between *hap* mutations requires identifying the responsible gene. The identity of *HAP1* has been confirmed by molecular complementation, and provisional assignments of several other *HAP* genes have been made by PCR. Because each *hap* strain has a single T-DNA insertion, it is likely that many of these assignments will prove correct.

Many candidate MG genes have predicted functions that could meet the unusual demands of pollen tube growth. The pollen tube extends at an astounding rate, growing >100-fold in length by absorbing metabolites from floral tissues, converting them into energy, and delivering newly synthesized membrane and cell wall components to the tube tip (Hepler *et al.* 2001). Therefore, it is not surprising that *hap* mutants with short pollen tubes implicate genes with predicted roles in sucrose transport (*hap3*; Stadler *et al.* 1999) or membrane trafficking (*hap6* and *hap13*; Crimaudo *et al.* 1987; Ohno *et al.* 1999). *hap5* acts much earlier, causing defects in pollen grain development (Figure 3, B, C, and G); this phenotype could result from alterations in a predicted cation-chloride cotransporter (Harling *et al.* 1997). Intriguingly, *hap11* acts at a very late stage, affecting pollen tube entry into the micropyle; this mutant has an insertion just upstream of a predicted mitochondrial ATP synthase δ chain (Morikami *et al.* 1992), suggesting an unexpected late-stage energy requirement. Microarray analysis of mature pollen (Becker *et al.* 2003; Honys and Twell 2003) and profiles of pollen gene expression (Mascarenhas 1990) suggest that the mature pollen grain is packed with transcripts that are translated upon tube germination. A large set of *hap* genes may regulate the expression or stability of these MG gene products (Table 3), affecting mRNA metabolism/localization (*hap1*), transcription (*hap12*), protein synthesis (*hap3* and *hap4*), and protein degradation (*hap15*). *hap1* alters late stages of tube growth, and its correspondence to *Mago nashi*, a highly conserved protein associated with mRNA processing and translocation in animals (Palacios 2002), is particularly intriguing. In Drosophila, *Mago* is predominantly nuclear, but shuttles to the cytoplasm as part of a complex that is required for proper localization of *Oskar* mRNA (Micklem *et al.* 1997; Hachet and Ephrussi 2001; Mohr *et al.* 2001); consequently, *hap1* could point to an important requirement for subcellular mRNA localization in pollen tube growth and guidance.

Considerable effort will be required to uncover all of the MG functions necessary for pollen cell maturation, growth, and communication with female cells. This work will also be valuable for understanding the functions of genes that are critical for later stages in plant development because many MG mutations identify genes that also have important sporophytic functions. Dissecting these roles will require comparing the outcomes of gametophyte screens with those of saturation screens for embryo-lethal mutations, analyzing the phenotype of rare homozygotes recovered from gametophytic mutants, or examining the sporophytic development of gametophytic mutants rescued by a gametophytically expressed trans-

gene. Such efforts will considerably advance the goal of defining the function of all Arabidopsis genes within this decade.

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