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Analysis of Transposon Insertion Mutants Highlights the Diversity of Mechanisms Underlying Male Progamic Development in Arabidopsis

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ABSTRACT

To identify genes with essential roles in male gametophytic development, including postpollination (progamic) events, we have undertaken a genetic screen based on segregation ratio distortion of a transposon-borne kanamycin-resistance marker. In a population of 3359 Arabidopsis *Ds* transposon insertion lines, we identified 20 mutants with stably reduced segregation ratios arising from reduced gametophytic transmission. All 20 mutants showed strict cosegregation of *Ds* and the reduced gametophytic transmission phenotype. Among these, 10 mutants affected both male and female transmission and 10 mutants showed male-specific transmission defects. Four male and female (*ungud*) mutants and 1 male-specific mutant showed cellular defects in microspores and/or in developing pollen. The 6 remaining *ungud* mutants and 9 male-specific (*seth*) mutants affected pollen functions during progamic development. *In vitro* and *in vivo* analyses are reported for 5 *seth* mutants. *seth6* completely blocked pollen germination, while *seth7* strongly reduced pollen germination efficiency and tube growth. In contrast, *seth8*, *seth9*, or *seth10* pollen showed reduced competitive ability that was linked to slower rates of pollen tube growth. Gene sequences disrupted in *seth* insertions suggest essential functions for putative SETH proteins in diverse processes including protein anchoring, cell wall biosynthesis, signaling, and metabolism.

THE life cycle of flowering plants alternates between a highly reduced haploid gametophytic phase and the familiar diploid sporophytic phase. Male (pollen) and female (embryo sac) gametophytes develop within the sexual organs of the flower. Each multicellular embryo sac is intimately associated with sporophytic cells forming the ovule. In contrast, the unicellular microspores and two- or three-celled pollen grains develop as a population within the anther locule and at maturity are capable of rapid growth by tip extension. Pollen ontogeny involves two distinct developmental phases: microgametogenesis, which involves the specification of vegetative and gametic cells through two consecutive haploid mitoses (reviewed in Twell *et al.* 1998), and postpollination (progamic) development, which involves rapid directional pollen tube growth within the female reproductive tissues (reviewed in Pruitt 1999; Johnson and Preuss 2002; Lord 2003). Although postpollination events have been extensively described at the cellular level, the identification and characterization of mutants affecting male progamic phase functions remains a challenge because of two major constraints: First, female sporophytic tissues constitute a physical barrier for morphological observation of growing pollen tubes; second, mutant pollen grains strictly affecting progamic development are by definition morphologically indistinguishable from their wild-type counterparts.

Numerous pollen-expressed genes with potential progamic function have been identified in a range of species by classical molecular cloning approaches (recently reviewed in Twell 2002) and, more recently, on a genomic scale in Arabidopsis (reviewed in Da Costa-Nunes and Grossniklaus 2003), using gene arrays (Becker *et al.* 2003; Honys and Twell 2003) or serial analysis of gene expression (Lee and Lee 2003). Despite such advances, progress toward demonstrating their functional roles is still limited. Reverse genetic approaches have shown that pollen germination and tube growth depend upon the gametophytic synthesis of cellular components involved in various signaling networks (Muschietti *et al.* 1994; Kost *et al.* 1999; Li *et al.* 1999; Tang *et al.* 2002), including calcium-mediated signaling (reviewed in Hepler *et al.* 2001; Golovkin and Reddy 2003), vesicle trafficking (Sanderfoot *et al.* 2001), potassium transport (Mouline *et al.* 2002), metabolism (Steinebrunner *et al.* 2003), and cell wall biosynthesis (Goubet *et al.* 2003).

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Mutational analysis involving forward genetic screens is a proven approach to dissect the mechanisms that regulate development and is especially useful in identifying novel components that are unique to plants. Although morphological screening of mature pollen from mutagenized populations has proven valuable for the identification of gametophytic loci playing vital roles during microgametogenesis (Chen and McCormick 1996; Park *et al.* 1998; Johnson and McCormick 2001; Lalanne and Twell 2002), this approach does not allow the identification of loci with essential roles during progamic development. Visual screens for reduced fertility or semisterility have identified gametophytic mutations affecting mainly the female control of late progamic phase events, such as pollen tube guidance and gamete release (Wilhelmi and Preuss 1996; Moore *et al.* 1997; Christensen *et al.* 1998; Shimizu and Okada 2000; Huck *et al.* 2003; Palanivelu *et al.* 2003; Rotman *et al.* 2003). In contrast, direct visual screens for progamic phase defects *in planta* would be particularly challenging and have not been reported to date. As an inclusive and direct strategy for the functional identification of essential male gametophytic genes, including those with a role in postpollination events, we and others have used insertional mutagenesis based on screening for distorted segregation ratios of antibiotic resistance or classical markers (Howden *et al.* 1998; Bonhomme *et al.* 1998; Grini *et al.* 1999). Segregation ratio distortion is a good indicator of the gametophytic transmission and the importance of the mutated sequence. We have applied this strategy to identify transposon-tagged genes that have gametophytic roles (see also Moore *et al.* 1997; Page and Grossniklaus 2002) and, in particular, to identify genes that are required for postpollination development in Arabidopsis.

In a screen of 3359 *Ds* transposon insertion lines, we isolated 20 independent lines showing stably reduced segregation ratios arising from reduced genetic gametophytic transmission. We describe 10 "*ungud*" mutants affecting both male and female gametogenesis and 9 "*seth*" mutants that specifically affect pollen function during progamic development. One additional male-specific mutant arising from this screen was termed *halfman*. It specifically affects pollen maturation after pollen mitosis I and has been described previously (Oh *et al.* 2003). To assess the effects of progamic phase mutations, we developed routine genetic and cytological approaches that we have applied here to the analysis of 5 *seth* mutations. Genomic sequences disrupted in 4 *ungud* and all 9 *seth* mutations are reported. The functions of putative SETH and UNGUD proteins are discussed in relation to the observed developmental failures.

MATERIALS AND METHODS

Mutant lines and growth conditions: *Ds* lines were generated by U. Grossniklaus and co-workers at Cold Spring Harbor Laboratory as described in Sundaresan *et al.* (1995) and Moore *et al.* (1997). Mutant lines have the following Cold Spring Harbor Laboratory designations: *ung1* (GT323), *ung2* (GT2177), *ung3* (GT952), *ung4* (ET734), *ung5* (GT415), *ung6* (ET366), *ung7* (GT3210), *ung8* (ET4959), *ung9* (ET648), *ung10* (GT1005), *seth1* (GT508), *seth3* (ET3502), *seth4* (GT294), *seth5* (GT6098), *seth6* (GT691), *seth7* (GT602), *seth8* (GT722), *seth9* (ET401), and *seth10* (ET4877). *seth5-2* (SAIL_357A09) was identified in the Syngenta *Arabidopsis* insertion line (SAIL) collection (Sessions *et al.* 2002). F_4 seeds (~100) were sterilized and plated onto kanamycin (50 μg/ml) supplemented medium as described in Lalanne *et al.* (2004). Kanamycin-resistant seedlings were transferred to 3-cm-square pots containing a 3:1 compost:sand mix and grown under greenhouse conditions.

Cloning of sequences flanking *Ds* insertions: Thermal asymmetric interlaced (TAIL)-PCR reactions were performed according to Liu *et al.* (1995) with modifications described in Grossniklaus *et al.* (1998). The 3′ *Ds*-flanking sequences were amplified using three *GUS*-specific nested primers (Lalanne *et al.* 2004) combined with the AD1, AD2, or AD3 degenerate primers (Liu *et al.* 1995). The 5′ *Ds*-flanking DNA junction was confirmed by direct PCR amplification using *Ds5-1* (Grossniklaus *et al.* 1998) and gene-specific primers (*SETH1* and *SETH2*, as described in Lalanne *et al.* (2004); *SETH3*, 5′-ATTGTTTCCGGTCCAATTG-3′; *SETH4*, 5′-TCAAAGCCA AAACGGAAAAG-3′; *SETH5*, 5′-CTCCAGCAGAGATTCTCC ACC-3′; *SETH6*, 5′-CGCAATGAAGGACGAGTTAT-3′; *SETH7*, 5′-GCTTTTGGTGGGTTTGTGAT-3′; *SETH8*, 5′-CGGAGGC TGGTTCGGCAGTT-3′; *SETH9*, 5′-CCACAAAGTCTCGCAC CCTCTGC-3′; and *SETH10*, 5′-CCGCAAACAGATTTGTCCG TCTTAGC-3′). All 5′ and 3′ *Ds*-flanking DNA junctions were directly sequenced.

Genetic transmission analyses: Transmission of mutations through the male and female gametes was determined by carrying out reciprocal testcrosses of hemizygous mutants and wild-type (L*er*) plants. Male and female transmission efficiencies (TEmale and TEfemale) were calculated according to Howden *et al.* (1998). Limited pollinations were performed on stigmas of male-sterile *ms1-1* plants to avoid contamination through self-pollination. Up to 25 pollen grains were deposited onto *ms1-1* stigmas using a dissecting needle and a dissecting microscope (Zeiss Stemi SV-6).

Cytological analysis of pollen phenotype: For mature pollen analysis, mutant plants were crossed with *qrt1* (Preuss *et al.* 1994). Heterozygotes *seth*/+ or *ung*/+ plants homozygous for the *qrt1* mutation produce tetrads containing two wild-type and two mutant pollen grains, allowing direct morphological comparison of pollen phenotype. Mature pollen grains were incubated in 4′,6-diamidino-2-phenylindole (DAPI) staining solution and observed by use of light and epifluorescence microscopy as described in Park *et al.* (1998). Alexander and aniline blue staining of mature pollen grains was performed as described in Alexander (1969) and Park and Twell (2001), respectively. Phenotypic analyses of developing microspores and pollen using DAPI staining were performed as described in Park *et al.* (1998). *In vitro* germination assays were performed using conditions modified from Derksen *et al.* (2002) as follows: 10 open flowers were collected in microtiter plates (Corning, 24-well cell culture cluster) containing 200 μl of liquid germination medium [0.01% w/v H_3BO_3, 0.07% $CaCl_2 \cdot 2H_2O$, 3% w/v polyethylene glycol (8000), 20% w/v sucrose] per well. Plates were sealed and incubated for 4 hr at 22° under continuous light with gentle rocking at 30 rpm. Pollen germination was scored by direct observation of plates using a Zeiss Axiovert 100 inverted microscope. The Openlab 3.1.5 software (Improvision, Coventry, UK) was used to measure pollen tube lengths from images captured with an Orca ER

CCD camera (Hamamatsu Photonics, Japan). *In vivo* pollen germination assays were performed as described in Lalanne *et al.* (2004).

RESULTS

Selection of 20 putative gametophytic mutants: We screened the self-progeny of 3359 independent *Ds* transposon insertion lines containing single enhancer trap (*DsE*) or gene-trap (*DsG*) elements marked with kanamycin resistance (Sundaresan *et al.* 1995; Moore *et al.* 1997) for segregation ratios of less than 2:1 resistant:sensitive seedlings. Thirty-eight percent of the lines produced progeny fully or highly resistant to kanamycin and 56.9% showed ratios of approximately 3:1, whereas 3.4% showed slightly reduced segregation ratios (between 2:1 and 2.5:1). Fifty-seven lines, representing 1.7% of the total lines screened, gave reduced segregation ratios in the range 0.14:1 to 2:1. A segregation ratio of approximately 2:1 could result from a recessive embryo-lethal mutation, a 50% reduction of transmission through one sex, or partially reduced transmission through both male and female gametes. To select for more highly penetrant gametophytic mutations, affecting >50% of a specific gamete population, we reexamined a group of 33 lines showing segregation ratios between 0.15:1 and 1.5:1. Progeny testing of 50 kanamycin-resistant siblings for each line revealed complete cosegregation of antibiotic resistance and segregation ratio distortion in 20 lines, indicating that all of these insertions were tightly linked to or responsible for the reduced genetic transmission. Moreover, for all of these 20 lines, distorted segregation ratios remained stable among backcross progeny and for at least three generations in several lines analyzed in more detail (data not shown). In progeny of the 13 remaining lines, plants were observed that segregated 3:1 for kanamycin resistance, indicating that the corresponding gametophytic mutations were closely linked to the transposon insertion, but not physically tagged. Therefore, ~0.6% of the *DsE/DsG* insertion lines analyzed by segregation distortion appear to represent transposon-tagged gametophytic mutations.

Genetic analysis of male and female defective mutations: We identified 10 *Ds* insertion lines that showed segregation ratios significantly less than 1:1, ranging from 0.1:1 to 0.6:1 (Table 1), suggesting defective transmission through both sexes. Male and female defective lines were named *ungud* (*ung*) after a hermaphrodite Aboriginal snake deity. We failed to isolate homozygous *ung* mutants by segregation analysis of seed from self-progeny (n >100), suggesting complete failure of transmission through one or the other sex or zygotic lethality. Genetic transmission analysis revealed reduced transmission through both sexes, but all 10 *ung* mutations showed stronger effects on male transmission. Female transmission was moderately reduced in 3 *ung* mutants (TEfemale = 50–67%) and more severely reduced (TE-

TABLE 1

Genetic transmission analysis of *ung Ds* insertions

	+/*ung* × +/*ung* (self)		+/*ung* × +/+ (female transmission)		+/+ × +/*ung* (male transmission)		Pollen phenotype	Pollen viability (%)	*Ds* insertion site		Predicted protein
Mutant	kan^R:kan^S	Ratio	kan^R:kan^S	TEfemale (%)	kan^R:kan^S	TEmale (%)			Locus	Position	
ung1	63:409	0.14	44:268	16.4	0:409	0	48.7% abnormal	46	ND		—
ung2	131:920	0.14	67:224	29.9	0:253	0	50% abnormal	47	ND		—
ung3	211:866	0.24	24:131	18.3	8:318	2.5	46.9% abnormal	51	At2g34550	+1633	Gibberellin 2-oxidase
ung4	234:577	0.41	87:129	67.4	0:411	0	48.7% abnormal	47	ND		—
ung5	41:86	0.48	78:236	33.1	78:195	40.0	9% abnormal	87	ND		—
ung6	195:492	0.40	108:260	41.5	37:332	11.1	Normal	93	At4g04710	+2457	Calcium-dependent protein kinase
ung7	233:581	0.40	74:217	34.1	16:201	8.0	Normal	97	ND		—
ung8	450:1047	0.43	58:141	41.1	14:178	7.9	Normal	93	ND		—
ung9	288:570	0.51	68:123	55.3	8:190	4.2	Normal	93	At2g34680	+6175	Auxin-induced protein (AIR9)
ung10	379:549	0.69	105:209	50.2	36:353	10.2	Normal	96	At3g54090	+1920	Fructokinase-like protein

Numbers of self-progeny producing wild-type and mutant pollen are shown together with the calculated transmission efficiency (TE) of each mutant allele (TE = number of mutant/number of wild-type progeny × 100) through male (TEmale) and female (TEfemale) gametes. The pollen phenotype and percentage pollen viability (FDA staining) are shown. The viability for the wild-type control was 96%. Where known, the locus, *Ds* insertion position (relative to the start codon), and the annotation of the predicted proteins are shown. ND, not determined.

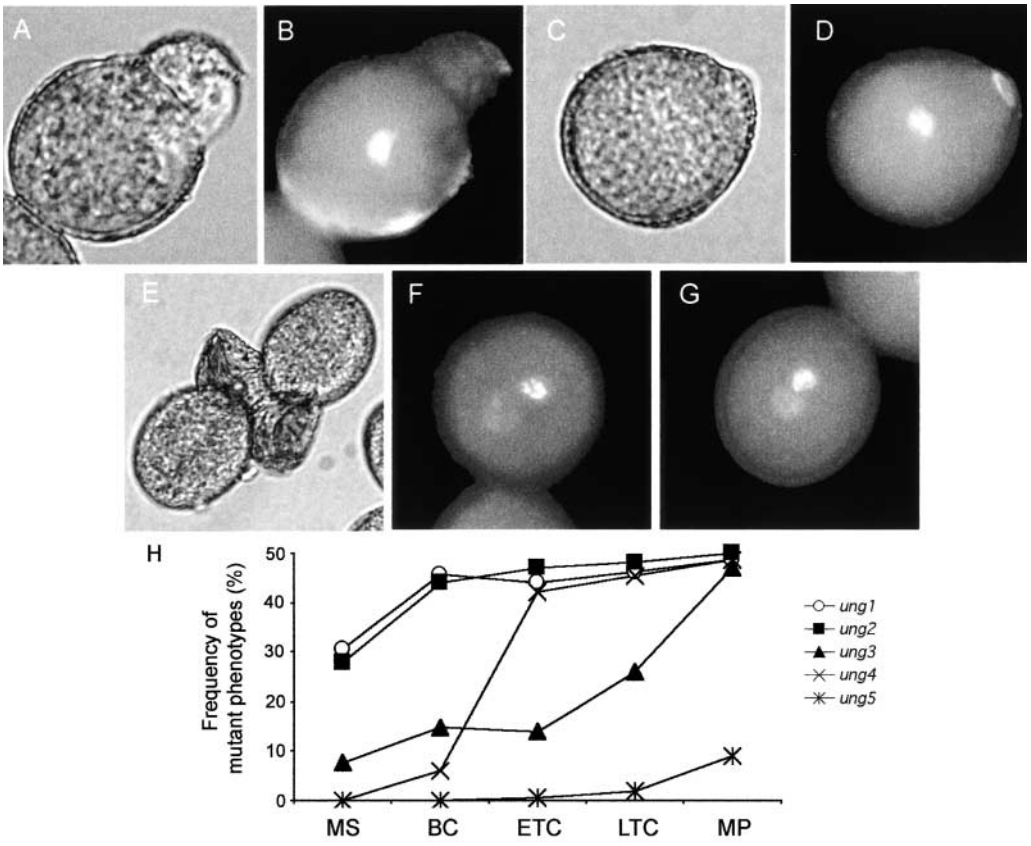

FIGURE 1.—Pollen morphology and developmental analysis for *ung1–ung5* hemizygotes. (A–D) Mutant *ung1* pollen stained with DAPI viewed by light (A and C) and epifluorescence (B and D). (E) +/*ung2* tetrad showing two intact and two collapsed members. (F and G) Mutant *ung4* pollen arrested at pollen mitosis II stained with DAPI. (H) Frequency of wild-type and mutant phenotypes in hemizygous *ung1*, *ung2*, *ung3*, *ung4*, and *ung5* plants at microspore (MS), bicellular (BC), early tricellular (ETC), late tricellular (LTC), and mature pollen (MP) stages. More than 200 spores were counted at each stage.

female = 18–41%) in the remaining 7 *ung* mutants. In contrast, male transmission in *ung1-4* and *ung9* was completely blocked or residual (TEmale = 0–4.2%) and strongly reduced (TEmale = 7.8–11%) in *ung5-8* and *ung10* (Table 1). Hemizygous *ung* plants showed no obvious growth or morphological defects, except for *ung3*, which showed a dominant semidwarf phenotype that was associated with dark green leaves and delayed flowering (not shown). Progeny testing ($n > 100$) showed that this phenotype strictly cosegregated with the *ung3* insertion.

Four *ung* mutants affect male and female gametogenesis: Plants hemizygous for the *ung1–ung4* insertions showed no or residual male transmission. Analysis of mature pollen using DAPI staining revealed that hemizygous *ung1* plants produced 27.3% aborted pollen, with no visible nuclei and 21.4% divided pollen, similar to phenotypes observed in the *gemini pollen1* mutant (PARK *et al.* 1998), with pollen containing one (Figure 1, A and B) or two nuclei (Figure 1, C and D). Mature *ung2* flowers produced 41% aborted pollen (Figure 1E) and 9% divided pollen, while hemizygous *ung3* plants produced 45.3% aborted pollen. Mature pollen from *ung4* hemizygotes also showed highly penetrant defects, with 36% aborted pollen and 12.8% smaller pollen with a single condensed generative-like nucleus or with a generative nucleus arrested in mitosis (Figure 1, F and G). Progeny analysis in *ung1*, *ung2*, *ung3*, and *ung4* revealed complete cosegregation of the aberrant pollen phenotype with antibiotic resistance, indicating that all of these insertions were either responsible for the observed pollen phenotype or tightly linked to a mutation inducing such phenotype.

Vital staining using fluorescein diacetate (FDA) revealed ~50% pollen viability in plants hemizygous for *ung1*, *ung2*, *ung3*, or *ung4*, consistent with no or residual (*ung3*) male transmission (Table 1). We conclude that the *ung1*, *ung2*, *ung3*, and *ung4* mutations result in cellular defects during male gametogenesis, leading to developmental arrest and loss of viability.

To understand the origins of these pollen phenotypes, counts were made of DAPI-stained spores at several stages of development (late microspore, late bicellular, early tricellular, late tricellular, and mature pollen). At late microspore stage, a significant percentage (8–30%) of spores from *ung1*, *ung2*, and *ung3* mutants (Figure 1H) showed a more central nucleus, not clearly polarized toward the pollen wall as in the wild type. By late bicellular stage, ~50% of pollen from *ung1* and *ung2* plants showed aberrant phenotypes (Figure 1H). Therefore, *ung1* and *ung2* mutations first act prior to microspore division and subsequently affect bicellular pollen development. In contrast, the percentage of mutant spores in *ung3* plants increased progressively during pollen development (Figure 1H). This pleiotropic phenotype may be influenced by the dominant sporophytic phenotypes observed in hemizygous *ung3* plants. In contrast, abnormal pollen phenotypes in *ung4* plants were

observed only at the late bicellular stage, with a significant percentage of smaller, early bicellular pollen grains. The percentage of abnormal phenotypes increased from 6 to 42% between late bicellular and early tricellular stages (Figure 1H), suggesting that *ung4* acts throughout the period of pollen mitosis II.

Reduced female transmission observed in the *ung1–ung4* mutants indicated that these mutations also affect megagametophyte development and/or function. Analysis of seed set in siliques of hemizygous *ung1*, *ung2*, *ung3*, and *ung4* plants showed a significant percentage of infertile ovules (37, 27.5, 37.5, and 12.7%, respectively). Low percentages of aborted seeds (2.5–6%) were also observed, with 2% aborted seeds being normal for wild-type controls. We conclude that reduced female transmission in *ung1–ung4* results mainly from megagametogenesis and/or fertilization defects.

Six *ung* mutants primarily affect male progamic development: Pollen morphology and viability in the remaining six *ung* mutants, *ung5–ung10*, was analyzed by DAPI and FDA staining. Pollen from hemizygous *ung6–ung10* plants was similar to the wild type in size, appearance, and viability (Table 1). Moreover, no significant percentages of aborted seeds or failed ovules were observed, suggesting that these mutations act specifically on male progamic development at stages prior to occupancy of the micropyle. Pollen from *ung5* mutants also showed high viability (87%), with a low frequency (9%) of aborted pollen. However male transmission was more severely reduced (TEmale = 40%), suggesting that mutant *ung5* pollen also fails primarily during progamic development. A significant percentage of failed ovules (10.9%) and aborted seeds (7.1%) was also observed in hemizygous *ung5* plants, suggesting additional roles for *UNG5* in megagametogenesis and seed development.

Molecular identity of insertion sites in four *ungud* mutants: Genomic sequences flanking *Ds* insertions were isolated for four *ung* mutants by TAIL-PCR (Liu *et al.* 1995; Table 1). Both 5′ and 3′ *Ds*-genomic DNA junctions were confirmed by direct PCR amplification and sequencing. In *ung3*, *Ds* was inserted into the 3′-untranslated region (UTR) of a gene encoding a potential gibberellin 2-oxidase that shares 69% identity with its *Phaseolus coccineus* homolog (Thomas *et al.* 1999). The *ung6 Ds* insertion was located in a gene encoding a putative calcium-dependent protein kinase (CDPK) related to 12 other Arabidopsis CDPKs. The putative *UNG6* protein (At4g04710) shows 68% identity over 486 amino acids with its closest homolog in Arabidopsis (At4g21940) and 64% identity with a CDPK from tobacco (GenBank accession no. T01989). The *ung9 Ds* insertion was in the unique At2g34680 gene, which has no nucleotide neighbors and encodes a putative extracellular protein containing a short-chain dehydrogenase/reductase family signature, a leucine-rich repeat domain, and a glycosyl hydrolase family 5 signature. The putative *UNG9* gene corresponds to *AIR9*, an mRNA that accumulates during auxin-induced lateral root formation (Neuteboom *et al.* 1999). The *ung10 Ds* insertion was in the At3g54090 gene encoding a fructokinase-like protein with 41% identity with its closest homolog in Arabidopsis. The Arabidopsis genome encodes three additional fructokinases (Pego and Smeekens 2000) and seven fructokinase-like proteins containing carbohydrate kinase signatures of the pfkB (*p*hospho*f*ructo*k*inase-*B*) family.

Genetic characterization of 10 male-specific mutations: Analysis of self-progeny revealed that 10 lines segregated approximately 1:1 (Table 2). These included nine *seth* mutants, named after the brother and murderer of the Egyptian fertility god Osiris, and 1 line, *halfman*, that has been described previously (Oh *et al.* 2003) and showed developmental defects as a result of a large genomic deletion. Hemizygous *seth* insertions had no effect on sporophytic development and no homozygous mutants were identified by segregation analysis of self-progeny ($n > 100$). No or only residual male transmission (TEmale = 0–1%) was observed for six *seth* mutants, *seth1* and *seth3–seth7*. The three remaining *seth* mutants, *seth8–seth10*, showed significant, but less severely reduced male transmission (TEmale = 15–28%). In contrast, female transmission was normal in *seth1–seth4*, *seth6*, *seth8*, and *seth9* and only slightly reduced (TEfemale ~80%) in *seth5*, *seth7*, and *seth10* (Table 2).

***seth* mutations specifically affect progamic development:** Mature pollen from all nine *seth* mutants appeared tricellular and similar to the wild type (Figure 2A). More than 93% of pollen grains were viable and Alexander staining did not reveal cytoplasmic defects (Figure 2, B and C). Moreover, we did not observe increased levels of seed or ovule abortion in any *seth* mutants. Their reduced male transmission and normal pollen morphology define these as male gametophytic progamic phase mutations that act upon pollen germination, tube growth, or guidance.

***seth6* and *seth7* affect pollen germination:** *In vitro* pollen germination assays (Figure 2, D–F) showed that pollen germination was strongly reduced in *seth6* and *seth7* hemizygotes (Figure 2F). To monitor pollen germination efficiency *in planta*, we developed an *in vivo* pollination assay. This involved pollinating excised pistils from male-sterile (*ms1-1*) plants and treating pistils with Alexander stain after 4 hr, to allow pollen germination and tube growth to be scored (Figure 3, A–C). In wild-type pollinations 95% of pollen grains on the papillar cells were not strongly stained, showing that the majority had germinated and transferred cytoplasm into the pollen tube (Figure 3A). In contrast, ~48% of pollen grains from *seth6* (Figure 3B) and *seth7* hemizygotes remained strongly stained as a result of failure to germinate or establish long pollen tubes (Figure 3C).

Pollen germination and tube growth was also assayed 2 hr after pollination by staining fixed pistils with aniline blue (Figure 3, D–F). On *ms1-1* stigmas pollinated with

TABLE 2

Genetic transmission analysis of *seth Ds* insertions

Mutant	+/*seth* × +/*seth* (self)		+/*seth* × +/+ (female transmission)		+/+ × +/*seth* (male transmission)		Pollen phenotype	*Ds* insertion site		Predicted protein
	kan^R:kan^S	Ratio	kan^R:kan^S	TEfemale (%)	kan^R:kan^S	TEmale (%)		Locus	Position	
seth1	2235:2098	1.07	151:162	93.2	0:412	0	Normal	At2g34980	+67	Phosphatidylinositol-glycan synthase C
seth3	1793:1649	1.09	479:453	105.7	0:830	0	Normal	At3g54690	+596	Arabinose-5-phosphate isomerase
seth4	2580:2817	0.92	365:350	104.3	0:194	0	Normal	At4g34940	+550	ARM repeat containing protein
seth5	383:456	0.84	205:254	80.7	4:524	0.8	Normal	At4g00800	+341	Unknown transmembrane protein
seth6	1269:1324	0.96	463:428	108.2	5:463	1.1	Normal	At2g47860	−68	RPT2/NPH3-like protein
seth7	516:601	0.85	242:317	76.3	4:543	0.7	Normal	At2g41930	+1252	Ser/Thr protein kinase
seth8	522:537	0.97	103:91	113.2	77:259	29.7	Normal	At5g13650	−367	GTP-binding typA-related protein
seth9	549:460	1.19	107:92	116.3	23:124	18.5	Normal	At5g42250	+2361	Alcohol dehydrogenase-like protein
seth10	700:813	0.86	104:130	80	48:324	14.8	Normal	At2g03070	+348	Putative protein

Numbers of progeny producing wild-type and mutant pollen are shown together with the male and female transmission for each mutant allele. The loci, *Ds* insertion positions, and annotation of the predicted proteins are shown.

wild-type pollen (Figure 3D), 97.4% developed a pollen tube and 0.3% initiated germination but arrested, indicated by the polarized deposition of callose (Figure 3F). In contrast, only 55% of pollen grains developed a pollen tube from *seth6* hemizygotes (Figure 3E), 27% had initiated germination and arrested, and 18% showed no evidence of polarized growth (Figure 3F). Pollen from *seth7* hemizygotes showed a similar germination efficiency (54%), but a greater percentage (37%) failed to initiate a pollen tube (Figure 3E). We conclude that both *seth6* and *seth7* block pollen germination and early tube growth *in planta*, with *seth7* acting earlier to block the initiation of germination.

***seth8*, *seth9*, and *seth10* pollen tubes are less competitive than their wild-type counterparts:** We also tested the *in vitro* germination efficiency of pollen from *seth8*, *seth9*, and *seth10* mutants that showed higher rates of male transmission. Pollen germination efficiencies ranged from 66 to 70%, similar to the wild type (Figure 2F). To assess the potentiality of mutant pollen grains *in planta*, limited pollinations were performed on *ms1-1* stigmas. In the presence of limited numbers of wild-type competitor pollen, the male transmission of *seth6* and *seth7* did not exceed that observed in competitive pollinations (2/261 and 0/179 Kan^R:Kan^S seedlings, respectively). In contrast, male transmission increased dramatically in *seth8*, *seth9*, and *seth10*, to 62.5, 81, and 79.2% (139/213, 157/193, and 122/154 Kan^R:Kan^S seedlings), respectively. Therefore, *seth8*, *seth9*, and *seth10* pollen grains are functional, but appear less competitive than their wild-type counterparts.

To determine if the *seth8*, *seth9*, and *seth10* mutations affect pollen tube elongation rate, we measured the lengths of pollen tubes after 4 hr of *in vitro* germination (Figure 2G). The mean pollen tube length was reduced by 17.6, 8.7, and 12.8% in hemizygous *seth8*, *seth9*, and *seth10* mutants, respectively. Pollen tubes carrying the *seth8*, *seth9*, and *seth10* mutations therefore appear to grow more slowly than their wild-type counterparts *in vitro*.

Molecular identity of insertion sites in nine *seth* mutants: Genomic sequences flanking *Ds* insertions in all nine *seth* lines were isolated by TAIL-PCR and sequenced (Table 2). *SETH1* and *SETH2* encode components of the phosphatidylinositol-glycan synthase complex and are described in Lalanne *et al.* (2004). The characterization of *SETH3*, *SETH4*, and *SETH5* will be detailed elsewhere, but brief descriptions are given here. In *seth3* the *DsE* element is inserted into the first exon of At3g-54690, a single copy gene in Arabidopsis. SETH3 shows 42% identity (61% similarity) over 324 amino acids with a *Chlamydia pneumoniae* sugar phosphate isomerase (KpsF). KpsF was shown recently to be an arabinose-5-phosphate isomerase involved in the synthesis of the 3-deoxy-d-manno-octulosonic acid (Kdo) biosynthetic pathway (Tzeng *et al.* 2002). In *seth4*, the *DsG* element is inserted into the unique exon of the At4g34940 gene.

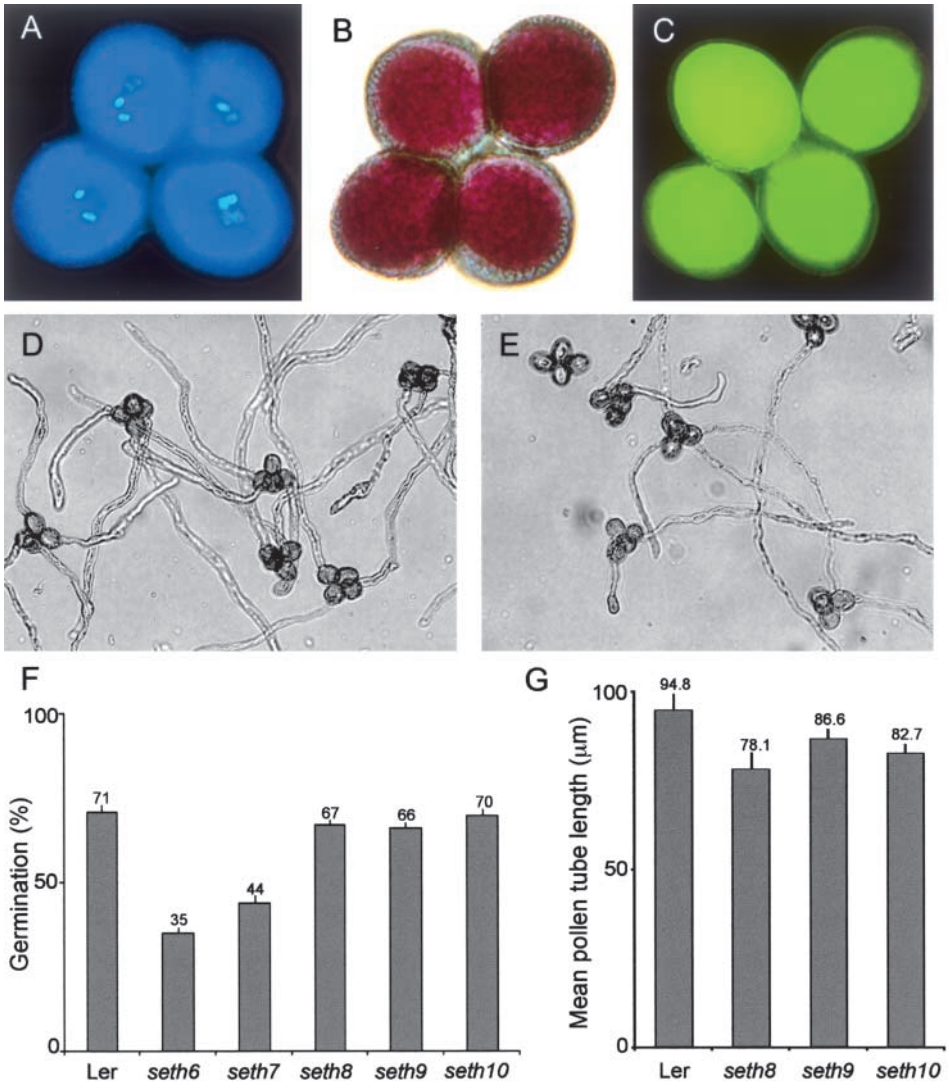

FIGURE 2.—Pollen morphology and *in vitro* germination efficiency for wild type and *seth* hemizygotes. (A–C) Mature +/*seth6* tetrads stained with DAPI (A), Alexander stain (B), and fluorescein diacetate (C). (D–G) *In vitro* germination of wild-type (D) and +/*seth6* (E) tetrads. (F) Histogram showing the percentage *in vitro* germination of pollen from wild-type (L*er*) and hemizygous *seth6*, *seth7*, *seth8*, *seth9*, and *seth10* plants. The standard error for 12 independent experiments is shown (n = 1200 pollen). (G) Pollen tube lengths of pollen from wild-type (L*er*) and heterozygous *seth8*, *seth9*, and *seth10* plants (n = 200 pollen). The standard error of 4 independent experiments is shown.

SETH4 encodes a potentially nuclear localized protein of 664 amino acids predicted to contain six armadillo repeats. In *seth5* the *DsG* insertion is within the unique At4g00800 gene predicted to encode an unknown, putative plasma membrane protein with six predicted transmembrane domains, a potential protein-protein interaction domain, and a single WD-40 motif.

In *seth6* the *DsG* element is inserted between the putative TATA box and the start codon of the At2g47860 gene. The putative SETH6 protein shows 36% identity (56% similarity) over 595 amino acids with RPT2 (*r*oot *p*hototropism 2), a signal transducer of the phototropic response (SAKAI *et al.* 2000). Two potential protein-protein interaction domains, BTB and coiled-coil domains, are present in the putative SETH6 protein, RPT2 and NPH3 (*n*on*p*hototropic *h*ypocotyl 3). In addition, putative SETH6 possesses a potential elongation factor (EF)-hand calcium-binding domain.

In *seth7* the *DsG* element is inserted into the 3′-UTR of the At2g41930 gene, 200 bp downstream of the stop codon. The putative *SETH7* gene encodes a putative Ser/Thr protein kinase of 351 amino acids containing a potential nuclear localization signal. Two genes (At2g41920 and At2g41910) showing the closest similarities with At2g41930 are present on the same bacterial artificial chromosome contiguous to At2g41930. At2g41920 and At2g41910 show respectively 54 and 47% identity over 355 amino acids with the putative SETH7 protein. These three proteins form a distinct nine-member subfamily of nontransmembrane MAP3K-related, Ser/Thr protein kinases that currently have no assigned function (http://plantsp.sdsc.edu/cgi-bin/detail.cgi?at_number=At2g41930).

In *seth8*, the *DsG* element is inserted 360 bp upstream of the initiator ATG of the At5g13650 gene. No other genes related to the putative *SETH8* were found in the Arabidopsis database. *SETH8* encodes a potential GTP-binding protein typA (tyrosine phosphorylated protein A) whose closest homologs are *Clostridium acetobutylicum* TYPA/BIPA GTPase (51% identity, 67% similarity over

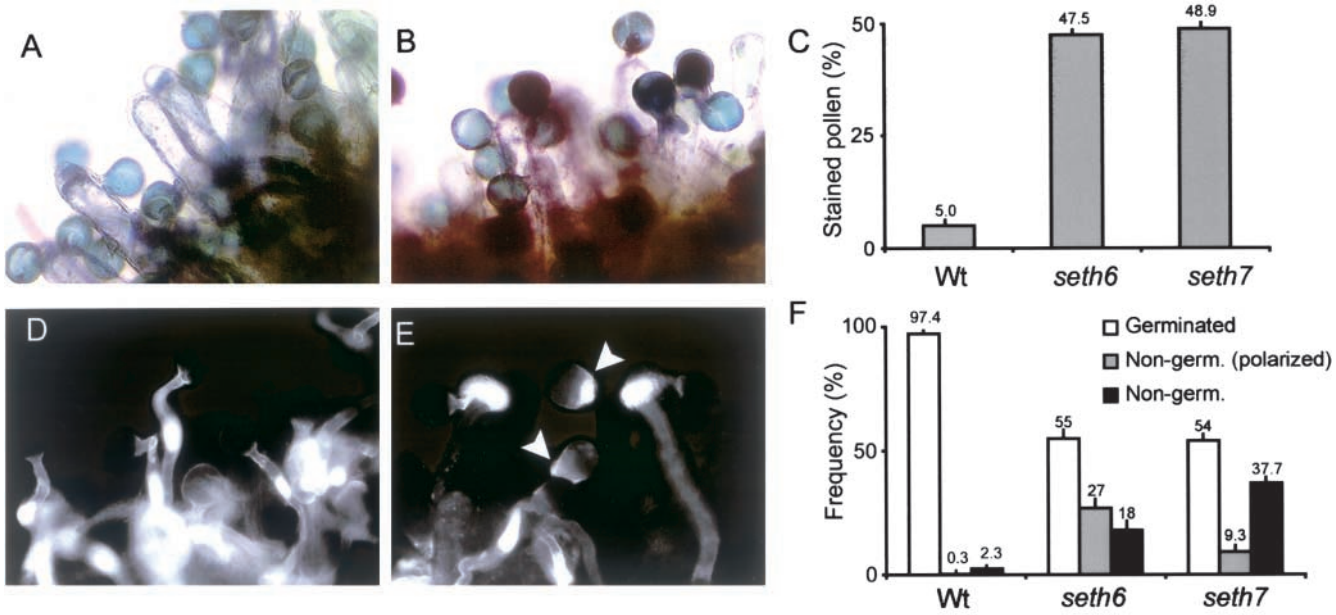

FIGURE 3.—*In vivo* germination of *seth6* and *seth7* pollen grains. The morphology and frequency of *in vivo* germination were monitored using Alexander (A–C) and aniline blue (D–F) staining after pollination of *ms1-1* stigmas. (A and B) Pollen from wild type (A) and *seth6* hemizygotes (B) 4 hr after pollination. Stained pollen grains are indicated by arrowheads. (C) Histogram showing the percentage of Alexander-stained pollen grains from wild-type and *seth6* and *seth7* hemizygous plants, 4 hr after pollination ($n > 1000$ pollen). The standard error for three independent experiments is shown. (D and E) Fluorescence micrograph of aniline blue-stained pollen from wild type (D) and *seth6* hemizygotes (E) showing germinated and polarized (arrowheads) pollen 2 hr after pollination. (F) Histogram showing the frequency of germinated, polarized, and nonpolarized pollen grains 2 hr after pollination with pollen from wild-type and *seth6* and *seth7* hemizygous plants ($n = 400$ pollen). The standard error for three independent experiments is shown.

592 amino acids) and *Synechocystis* sp. translation elongation factor EF-G (50% identity, 65% similarity over 582 amino acids).

In *seth9* the *DsE* element is inserted into the coding sequence of the At5g42250 gene encoding an alcohol dehydrogenase-like protein. Numerous alcohol dehydrogenase-like proteins are found in the Arabidopsis database, but the closest homolog is ADH3a, a *Lycopersicon esculentum* putative alcohol dehydrogenase specifically expressed in anthers (INGERSOLL *et al.* 1994).

In *seth10 DsE* is inserted into the third intron of the At2g03070 gene that is split into 12 exons and encodes a unique glutamine and proline-rich protein of 531 amino acids. PSORT analysis suggests that the putative SETH10 protein is cytosolic and does not contain any known functional domains.

Confirmed male gametophytic functions for *SETH1*, *SETH3*, *SETH4*, and *SETH5*: To verify that the reduced transmission phenotypes were directly attributable to the disruption of the *Ds*-tagged *SETH* loci and not to closely linked mutations, we have undertaken genetic complementation and/or analysis of additional insertion alleles for selected *seth* mutants. Using these approaches, to date we have confirmed a male gametophytic function for *SETH1*, *SETH3*, *SETH4*, and *SETH5*. Characterization of *SETH1* is described together with a T-DNA knockout of the *SETH2* gene, both of which are required for glycosylphosphatidylinositol (GPI) anchor synthesis (LALANNE *et al.* 2004). The detailed characterization of *SETH3* and *SETH4* will be described in separate publications.

We present here the genetic analysis of one additional *seth5* allele, *seth5-2*, identified in the SAIL collection (SESSIONS *et al.* 2002). The hemizygous *seth5-2* insertion (located in exon 3) had no effect on sporophytic development and no homozygous mutant plants were identified in self-progeny. Similar to *seth5-1* (see Table 2), progeny of hemizygous *seth5-2* plants segregated 1:1 (Kan^R:Kan^S = 232:223) for the basta-resistance marker present within the T-DNA insertion. Moreover, progeny testing ($n = 50$) revealed strict cosegregation of basta resistance with reduced genetic transmission of the insertion in *seth5-2*. Similar to *seth5-1*, male transmission was strongly reduced for *seth5-2* (TE male = 3.8%), but female transmission was unaffected. Mature pollen grains from hemizygous *seth5-1* and *seth5-2* plants appeared >94% viable by FDA staining and were all tricellular, similar to the wild type. *In vitro* assays showed that pollen germination was significantly reduced in *seth5-1* and *seth5-2* (to 73 and 64%, respectively) compared to their respective wild-type controls (*Ler* and Col-0). We conclude that the *seth5* progamic phase phenotype is directly attributable to disruption of the *SETH5* gene encoding a unique putative plasma membrane

protein with protein interaction motifs including a WD-40 motif.

DISCUSSION

From a screen of 3359 *Ds* transposon insertion lines, we isolated 20 independent gametophytic mutants that showed strict cosegregation of the reduced transmission phenotype with *Ds* and are likely to be tagged. These included 10 male- and female-defective mutations, 9 male-specific *seth* mutations affecting progamic development, and 1 male-specific mutation, *halfman*, affecting microsporogenesis (Oh *et al.* 2003). The frequency of *Ds* insertion lines we observed showing confirmed segregation ratios of ~1:1 or less was 0.98% (33 lines of 3359), similar to the frequencies, of 0.8 and 0.88%, observed by Howden *et al.* (1998) and Bonhomme *et al.* (1998) in identical screens of T-DNA insertion lines. Characterization of these *Ds* insertion lines revealed that 9 male-specific mutations corresponded to progamic phase (*seth*) mutations, representing 0.27% of the total number of lines screened. On the basis of this frequency, at least 73 male-specific progamic phase mutants could be expected from insertions into all predicted genes in the Arabidopsis genome. However, the expression of multiple members of a large number of different gene families in mature pollen (Becker *et al.* 2003; Honys and Twell 2003; Lee and Lee 2003) implies functional redundancy that would block the identification of large numbers of genes with essential progamic functions through simple genetic screens. Such functional redundancy was recently demonstrated by combining mutations in two different pollen-expressed *apyrase* genes, revealing an essential role for nucleoside tri- and diphosphates in Arabidopsis pollen germination (Steinebrunner *et al.* 2003).

Four (*ung1–ung4*) of 10 *ung* mutations result in defects during male gametogenesis: The abnormal phenotypes in *ung1* and *ung2* increased dramatically between late microspore and early bicellular stage and resulted in the formation of significant proportions of divided, *gemini* pollen phenotypes (Park *et al.* 1998). These mutations therefore disturb aspects of division polarity and/or cytokinesis at pollen mitosis I that are known to depend upon the microtubule binding protein MOR1/GEM1 (Twell *et al.* 2002). However, the high levels of pollen abortion during bicellular stages in *ung1* and *ung2* is not characteristic of *gem1* mutant alleles (Park *et al.* 1998; Twell 2002), suggesting pleiotropic cellular defects of these *ung* mutations during the transition between microspore and bicellular pollen.

In contrast, the proportion of aberrant pollen in *ung3* plants increased throughout pollen development. The *Ds* element in *ung3* is inserted into a gene encoding a potential gibberellin 2-oxidase. Given the position of the insertion in the 3′ UTR we speculate that this could lead to mRNA stabilization and increased enzyme activity. The *ung3* phenotype that includes dwarfing and seed abortion is consistent with such overexpression leading to gibberellin deficiency (Singh *et al.* 2002; Schomburg *et al.* 2003). Our data suggest a developmental role for gibberellins during microgametogenesis, in addition to their reported role in pollen tube growth (Singh *et al.* 2002). Developmental failure in *ung4* mainly affected the transition from late bicellular to early tricellular pollen, resulting in mitotic arrest during pollen mitosis II, similar to the *mad1* mutant (Grini *et al.* 1999).

A variable proportion of embryo sacs carrying the *ung1*, *ung2*, *ung3*, and *ung4* mutations failed to develop, indicating that reduced female transmission in these lines results mainly from megagametogenesis or early postfertilization defects. The functional requirement of *UNG1*, *UNG2*, *UNG3*, and *UNG4* during both male and female gametogenesis suggests that these genes are not required for highly specific developmental events, but may be associated with more general cellular functions that operate at different stages of male and female gametophyte development.

Seven putative male-progamic development proteins with assigned functions: We identified 14 (9 *seth* and 5 *ungud*) mutants that act during male progamic development. Gene sequences disrupted by *Ds* insertions were identified for 11 mutant lines, among which 7 encode proteins with assigned functions. These proteins are involved in biological function as diverse as protein anchoring (SETH1 and SETH2), cell wall biosynthesis (SETH3), signaling (SETH6, SETH7, and UNG6), and metabolism (SETH9 and UNG10).

SETH1 (this study) and *SETH2* (Lalanne *et al.* 2004) encode Arabidopsis homologs of two conserved proteins involved in the first step of the GPI biosynthetic pathway. Reduced pollen germination and tube growth is associated with abnormal callose deposition, revealing an essential role for GPI anchoring of proteins in the establishment and maintenance of polarized pollen tube growth (Lalanne *et al.* 2004).

The *SETH3* gene encodes a putative arabinose-5-phosphate isomerase involved in the synthesis of Kdo, a rare sugar present only in rhamnogalacturonan-II , characteristic of primary (extensible) plant cell walls. *seth3* could therefore affect pectin structure or synthesis during pollen tube growth. Detailed analysis of *seth3* will be reported separately.

The putative SETH6 protein belongs to large family of transducer molecules, apparently specific to plants that may function as an adapter or scaffold protein to bring together the enzymatic components of signaling pathways (Motchoulski and Liscum 1999; Sakai *et al.* 2000). The presence of potential nuclear targeting signals in putative SETH6, RPT2, and NPH3 and the plasmalemma colocalization of NPH3 suggest that these proteins could be released from the plasmalemma to activate or inhibit interacting components in the nu-

cleus (Motchoulski and Liscum 1999). In addition, SETH6 possesses a potential EF-hand calcium-binding domain, suggesting that this protein might be involved in calcium-dependent signaling. Although *seth6* pollen is largely unable to germinate both *in vivo* and *in vitro*, rare transmission of *seth6* demonstrates that mutant pollen grains that germinate can go on to fertilize the embryo sac. Therefore SETH6 may be required only at the germination step.

The putative *UNG6* protein encodes a calcium-dependent protein kinase (CDPK) that may be involved in calcium-dependent signaling, acting both in pollen during progamic development and in embryo sacs. Calcium, calmodulin, and calcium-binding proteins are known to play a crucial role during pollen germination and tube growth (Estruch *et al.* 1994; Hepler *et al.* 2001; Golovkin and Reddy 2003). Moreover, *in vitro* germination and pollen tube growth are impaired upon addition of CDPK inhibitors or antisense oligonucleotides directed against a pollen-specific CDPK mRNA from maize (Estruch *et al.* 1994).

The putative SETH7 protein belongs to a small family (nine members) of nontransmembrane Ser/Thr protein kinases that have no assigned function (http://plantsp.sdsc.edu/cgi-bin/detail.cgi?at_number=At2g41930). The observed reduced germination efficiency and short pollen tubes in *seth7* suggest a requirement for SETH7 during the initiation of pollen germination, but also to maintain pollen tube elongation. Collectively, SETH6, SETH7, and UNG6 provide an opportunity to uncover novel signaling pathways acting during male progamic development.

The putative SETH9 protein and its close relatives show a high percentage of similarity with ADH proteins. Although the Arabidopsis genome encodes only one functional ADH protein (Dolferus *et al.* 1990), ADH-like proteins are likely metabolic enzymes of alcohol fermentation, but their activity remains to be identified. Pollen tubes carrying *seth9* grow more slowly than their wild-type homologs, but in noncompetitive pollinations are able to reach and fertilize the embryo sac. As pollen tube growth requires a high rate of sugar metabolism involving alcoholic fermentation (Mellema *et al.* 2002), the expression of additional ADH-like isoforms might provide for energetic and biosynthetic demands.

The putative *UNG10* gene encodes a fructokinase-like protein containing a pfkB family carbohydrate kinase domain that could be involved in sugar metabolism. Pollen fructokinases may play a role in pollen germination and tube growth by providing fructose-6-phosphate for glycolysis or through conversion to UDP-glucose to support the biosynthesis of cell wall material (Karni and Aloni 2002).

Five putative male progamic development proteins with unknown functions: We identified gene sequences at insertion sites for five mutants (*ung9*, *seth4*, *seth5*, *seth8*, and *seth10*) that affect progamic development, whose functions are unknown. The putative *UNG9* gene encodes an unknown protein containing a short-chain dehydrogenase/reductase family signature and a glycosyl hydrolase family 5 signature. *SETH4* encodes a potentially nuclear localized protein containing six armadillo repeats and has two close homologs in Arabidopsis. The putative SETH5 protein is a potential transmembrane protein, which does not show similarity to known plant proteins. Detailed molecular and cytological characterization of these mutations will be reported separately.

The lower penetrance of *seth8* and *seth10* mutations and *in vitro* pollen tube growth assays demonstrated that pollen tubes carrying these mutations are functional, but pollen tubes grow more slowly than their wild-type counterparts. The putative SETH8 protein shares significant similarity with a family of GTP-binding proteins of unknown function that are found in prokaryotes, but not in animals. The putative SETH10 protein does not show convincing homology with other known proteins. In *seth8*, *Ds* is inserted in the promoter and therefore might not result in a complete knockout. In *seth10*, *Ds* is in inserted in coding sequence. Therefore, SETH10 does not appear to provide essential functions, but provides a competitive advantage to pollen grains.

Diversity of cellular mechanisms underlying the progamic phase: A number of male gametophytic mutants that affect pollen development and/or function in Arabidopsis have been identified by either visual (Chen and McCormick 1996; Park *et al.* 1998; Johnson and McCormick 2001; Lalanne and Twell 2002) or genetic screens (Bonhomme *et al.* 1998; Howden *et al.* 1998; Grini *et al.* 1999; Procissi *et al.* 2001; Lalanne *et al.* 2004) or through reverse genetic approaches (Sanderfoot *et al.* 2001; Gupta *et al.* 2002; Mouline *et al.* 2002; Golovkin and Reddy 2003; Goubet *et al.* 2003; Kang *et al.* 2003; Steinebrunner *et al.* 2003). Among these, six genes that act during the progamic phase have been isolated: *SYP21* and *SYP42* (Sanderfoot *et al.* 2001), *SPIK* (Mouline *et al.* 2002), *AtCSLA7* (Goubet *et al.* 2003), *NPG1* (Golovkin and Reddy 2003), and *APYRASE* (Steinebrunner *et al.* 2003).

We identified gene sequences disrupted by *Ds* in 11 mutants affecting male progamic development. Currently, we have confirmed a male gametophytic function for four of these genes (*SETH1*, *SETH3*, *SETH4*, and *SETH5*) through genetic complementation and/or the isolation of additional insertion alleles (Lalanne *et al.* 2004; E. Lalanne, C. Michaeladis and D. Twell, unpublished results). Putative proteins encoded at these loci are involved in biological functions as diverse as cell wall biosynthesis, protein anchoring (this work and Lalanne *et al.* 2004), calcium sensing, signaling, metabolism, and those that remain to be elucidated. These data reflect the variety of molecular mechanisms that operate during male progamic development. The availability of well-characterized insertion mutants will facilitate the development of new avenues of research con-

cerning the cellular functions that operate during male progamic development. Further systematic screens of insertion collections by segregation ratio distortion will reveal many more proteins with essential gametophytic functions. However, elucidation of the full complement of proteins with essential roles in male progamic development is a challenge that will require concerted systematic genetic screens and reverse genetic approaches.

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