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 (Muscheretti 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).
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 prolagmic development. Visual screens for reduced fertility or semisterility have identified gametophytic mutations affecting mainly the female control of late prolagmic phase events, such as pollen tube guidance and gamete release (Wilhelm 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 prolagmic 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 prolagmic 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 prolagmic 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 (GT5020), seth4 (GT294), seth5 (GT6098), seth6 (GT6911), seth7 (GT6024), seth8 (GT7224), seth9 (GT401), and seth10 (ET4877). seth5-2 (SAIL_357A09) was identified in the Syngenta Arabidopsis insertion line (SAIL) collection (Sessions et al. 2002). F1 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’ (Grossniklaus et al. 1998) and gene-specific primers (SETH1 and SETH2, as described in Lalanne et al. (2004); SETH3, 5’-ATTGTTCGGCTCAGATTG-3’, SETH4, 5’-TCAAAGGCAAAAGGAAAG-3’, SETH5, 5’-CTTCGACAGATTCTGCACC-3’, SETH6, 5’-CCGAGAAGCGAGATTTGAT-3’, SETH7, 5’-CTTTTGTTGGGTGTTTGTGAT-3’, SETH8, 5’-CCGAGGCTGTTTCCCAGTTGC-3’, SETH9, 5’-CCCAAAGGCCACCTTGGTC-3’, and SETH10, 5’-CCGAAAAAAGTTTGTCCGTCTTGGC-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 (Lei) 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). Heterozygous 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 microsperms 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 microtitre plates (Corning, 24-well cell culture cluster) containing 200 μl of liquid germination medium [0.01% w/v K2BO3, 0.07% CaCl2·2H2O, 3% w/v polyethylene glycol (8000), 20% w/v sucrose] per well. Plates were sealed and incubated for 4 hr at 22°C 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.
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 embryonic 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, $\sim 0.6\%$ 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 ($TE_{female} = 50–67\%$) and more severely reduced ($TE_{female} = 30–62\%$) in the remaining 7 $ung$ insertion lines. Our laboratory has not determined why only 10 mutants were isolated and further efforts are required to determine the number of all self-progeny producing wild-type and mutant pollen are shown together with the calculated transmission efficiency ($TE$) of each mutant allele. 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.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>+/+ × +/+</th>
<th>+/+ × +/−</th>
<th>−/− × +/+</th>
<th>Pollen phenotype (%)</th>
<th>Pollen viability (%)</th>
<th>Locus</th>
<th>Position</th>
<th>Predicted protein</th>
</tr>
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<tbody>
<tr>
<td>$ung1$</td>
<td>634:909</td>
<td>0.14</td>
<td>44:298</td>
<td>16.4</td>
<td>44:298</td>
<td>50%</td>
<td>Normal</td>
<td>96  At1g05154</td>
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<tr>
<td>$ung2$</td>
<td>1314:920</td>
<td>0.14</td>
<td>21:1866</td>
<td>28.9</td>
<td>21:1866</td>
<td>50%</td>
<td>Normal</td>
<td>96  At1g01576</td>
</tr>
<tr>
<td>$ung3$</td>
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<td>0.14</td>
<td>24:1311</td>
<td>18.3</td>
<td>24:1311</td>
<td>50%</td>
<td>Normal</td>
<td>96  At1g05178</td>
</tr>
<tr>
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<td>2345:777</td>
<td>0.14</td>
<td>87:2196</td>
<td>41.5</td>
<td>87:2196</td>
<td>41.5</td>
<td>Normal</td>
<td>96  At1g05180</td>
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<td>0.14</td>
<td>76:2832</td>
<td>48.7</td>
<td>76:2832</td>
<td>48.7</td>
<td>Normal</td>
<td>96  At1g05175</td>
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<tr>
<td>$ung6$</td>
<td>1954:921</td>
<td>0.14</td>
<td>105:2206</td>
<td>50%</td>
<td>105:2206</td>
<td>50%</td>
<td>Normal</td>
<td>96  At1g05176</td>
</tr>
<tr>
<td>$ung7$</td>
<td>1954:921</td>
<td>0.14</td>
<td>105:2206</td>
<td>50%</td>
<td>105:2206</td>
<td>50%</td>
<td>Normal</td>
<td>96  At1g05176</td>
</tr>
<tr>
<td>$ung8$</td>
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<td>34:1147</td>
<td>24.1</td>
<td>34:1147</td>
<td>24.1</td>
<td>Normal</td>
<td>96  At1g05176</td>
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<tr>
<td>$ung9$</td>
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<td>68:1926</td>
<td>34.1</td>
<td>68:1926</td>
<td>34.1</td>
<td>Normal</td>
<td>96  At1g05176</td>
</tr>
<tr>
<td>$ung10$</td>
<td>5795:494</td>
<td>0.14</td>
<td>30:1028</td>
<td>20.0</td>
<td>30:1028</td>
<td>20.0</td>
<td>Normal</td>
<td>96  At1g05176</td>
</tr>
</tbody>
</table>
female = 18–41%) in the remaining 7 ung mutants. In contrast, male transmission in ung1-4 and ung9 was completely blocked or residual (TMale = 0–4.2%) and strongly reduced (TMale = 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 megalametophyte 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 megalametogenesis and/or fertilization defects.

Six ung mutants primarily affect male progametic development: Pollen morphology and viability in the remaining six ung mutants, ung3–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 progametic development at stages prior to occupancy of the micropyte. 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 progametic 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 megalametogenesis 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 (Lu 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 (phosphofructokinase-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 progametic 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 progametic 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 insertions

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Location</th>
<th>Locus</th>
<th>Position</th>
<th>Phenotype</th>
<th>Predicted protein</th>
</tr>
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<tbody>
<tr>
<td>seth3</td>
<td>67</td>
<td>Phosphatidylinositol-glycan synthase C</td>
<td>+67</td>
<td>Normal</td>
<td>At3g54690</td>
</tr>
<tr>
<td>seth5</td>
<td>550</td>
<td>ARM repeat containing protein</td>
<td>+550</td>
<td>Normal</td>
<td>At4g00800</td>
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<tr>
<td>seth6</td>
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<td>Normal</td>
<td>At2g47860</td>
</tr>
<tr>
<td>seth7</td>
<td>1252</td>
<td>Ser/Thr protein kinase</td>
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<td>Normal</td>
<td>At5g13650</td>
</tr>
<tr>
<td>seth8</td>
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<td>GTP-binding typeA-related protein</td>
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<td>At5g42250</td>
</tr>
<tr>
<td>seth9</td>
<td>2361</td>
<td>Alcohol dehydrogenase-like protein</td>
<td>+2361</td>
<td>Normal</td>
<td>At2g03070</td>
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</tbody>
</table>

Numbers of progeny producing wild-type and mutant pollen are shown together with the male and female transmission for each mutant allele. The loci, insertion sites, 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 msl1-1 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 KanR:KanS 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 KanR:KanS 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.

**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 At3g54690, 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.
**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 (root phototropism 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 (nonphototropic hypocotyl 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 typeA (tyrosine phosphorylated protein A) whose closest homologs are Clostridium acetobutylicum TYPA/BIPA GTPase (51% identity, 67% similarity over...
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 hemizygotest (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 hemizygotest (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 (Lalanne et al. 2004). 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 (KanR:KanS/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 (Le and Col-0). We conclude that the seth5 proagamic 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 proagamic 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 proagamic phase (seth) mutations, representing 0.27% of the total number of lines screened. On the basis of this frequency, at least 73 male-specific proagamic 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 proagamic functions through simple genetic screens. Such functional redundancy was recently demonstrated by combining mutations in two different pollen-expressed apyrerase genes, revealing an essential role for nucleoside tri- and diphosphates in Arabidopsis pollen germination (Steinebrunner et al. 2005).

Four (ung1–ung4) of 10 unmutations 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-proagamic development proteins with assigned functions:** We identified 14 (9 seth and 5 ungud) mutants that act during male proagamic 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-
nucleus (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 progradic 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 progradic 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 alcholic 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 progradic development proteins with unknown functions:** We identified gene sequences at insertion sites for five mutants (ung9, seth4, seth5, seth8, and seth10) that affect progradic 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 penetration 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 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 progradic 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 progradic 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 progradic 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 progradic 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 progmatic 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 progmatic development is a challenge that will require concerted systematic genetic screens and reverse genetic approaches.

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