DOI: 10.1534/genetics.108.090852

A Collection of *Ds* Insertional Mutants Associated With Defects in Male Gametophyte Development and Function in *Arabidopsis thaliana*

Leonor C. Boavida,* Bin Shuai,*,[1] Hee-Ju Yu,†,[2] Gabriela C. Pagnussat,† Venkatesan Sundaresan† and Sheila McCormick*,[3]

**Plant Gene Expression Center and Plant and Microbial Biology, U.S. Department of Agriculture/Agricultural Research Service and University of California, Albany, California 94710 and †Section of Plant Biology and Department of Plant Sciences, University of California, Davis, California 95616*

Manuscript received May 1, 2008
Accepted for publication February 13, 2009

ABSTRACT

Functional analyses of the Arabidopsis genome require analysis of the gametophytic generation, since ~10% of the genes are expressed in the male gametophyte and ~9% in the female gametophyte. Here we describe the genetic and molecular characterization of 67 *Ds* insertion lines that show reduced transmission through the male gametophyte. About half of these mutations are male gametophytic-specific mutations, while the others also affect female transmission. Genomic sequences flanking both sides of the *Ds* element were recovered for 39 lines; for 16 the *Ds* elements were inserted in or close to coding regions, while 7 were located in intergenic/unannotated regions of the genome. For the remaining 16 lines, chromosomal rearrangements such as translocations or deletions, ranging between 30 and 500 kb, were associated with the transposition event. The mutants were classified into five groups according to the developmental processes affected; these ranged from defects in early stages of gametogenesis to later defects affecting pollen germination, pollen tube growth, polarity or guidance, or pollen tube–embryo sac interactions or fertilization. The isolated mutants carry *Ds* insertions in genes with diverse biological functions and potentially specify new functions for several unannotated or unknown proteins.

PLANTS alternate growth of the diploid sporophyte with a highly reduced haploid gametophyte. Specialized cells within sporophytic structures of the flower undergo meiosis and subsequent mitoses to form the male (pollen) and female (embryo sac) gametophytes. Upon maturation, the embryo sac consists of seven cells, classified into four cell types with specialized functions (Drews and Yadegari 2002; Dresselhaus 2006). The mature male gametophyte (pollen grain), composed of a generative cell (or two sperm cells) and a vegetative cell, is an autonomous organism. Upon hydration on the stigma the vegetative cell produces a tip-growing extension, the pollen tube, which transports and discharges the sperm cells into the embryo sac, where double fertilization occurs (Boavida *et al.* 2005).

Gametogenesis depends on coordination of sporophytic and gametophytic gene expression (McCormick 2004; Yadegari and Drews 2004). Expression profiles of female (Yu *et al.* 2005; Steffen *et al.* 2007) and male gametophytes (Becker *et al.* 2003; Honys and Twell 2003, 2004; Pina *et al.* 2005) have provided lists of genes that may be under gametophytic selection. Microscopic-based screens for semisterility or for alterations in pollen morphology or cell division patterns have been used to identify genes required in the male (Chen and McCormick 1996; Park *et al.* 1998; Johnson and McCormick 2001; Lalanne and Twell 2002) or female (Christensen *et al.* 1998; Shimizu and Okada 2000; Siddiqi *et al.* 2000; Huck *et al.* 2003) gametophytes. Functional analysis of gametophytic-expressed genes is still incomplete, especially for genes required for pollen tube growth or for interaction with female tissues. Since gametophytic mutations affect haploid-expressed functions, they are not transmitted through the defective gamete and can be recovered only in a heterozygous state. Therefore, genetic screens for these traits are challenging to perform, unless a pollen reporter marker is used (Johnson *et al.* 2004). For this reason, transposon and T-DNA insertional mutagenesis emerged as an attractive alternative, since identification of gametophytic mutants is based on altered segregation ratios of a linked antibiotic resistance marker (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998; Johnson *et al.* 2004; Lalanne *et al.* 2004; Pagnussat *et al.* 2005).

Here we describe the genetic and molecular characterization of a collection of *Ds* insertion lines (Sundaresan *et al.* 1995) that show transmission defects through the

[1]*Present address:* Department of Biological Sciences, Wichita State University, Wichita, KS 67260.

[2]*Present address:* National Horticultural Research Institute, RDA, I-Mok dong 475, Jang-An Gu, Suwon, Gyeonggi-Do, 440-706, Republic of Korea.

[3]*Corresponding author:* Plant Gene Expression Center and Plant and Microbial Biology, USDA/ARS and University of California, 800 Buchanan St., Albany, CA 94710. E-mail: sheilamc@nature.berkeley.edu

male gametophyte. A genetic screen performed with this collection by Pagnussat *et al.* (2005) led to the identification of 130 mutants affected in female gametophyte development and function. We characterized the phenotypes of 67 male gametophytic mutants and grouped them into five categories, according to their developmental defects. For 28 lines, only one flanking sequence was recovered, suggesting that deletions or chromosomal rearrangements were linked with the transmission defects. Genomic sequences flanking both sides of the insertion site were recovered for 39 lines, and of these, 16 were associated with annotated genes, while another 16 were associated with translocations and deletions. In 7 of the 39 lines the *Ds* elements were inserted in intergenic or unannotated genomic regions, suggesting that unannotated proteins or other mechanisms (antisense, microRNA, or small RNA) might be involved in regulating expression of genes essential for male gametophyte development or function.

MATERIALS AND METHODS

Plant material and growth conditions: *Ds* insertion lines were generated and selected as described in Sundaresan *et al.* (1995). We analyzed plants grown from F_4 seeds. Seeds were surface sterilized with 20% sodium hypochlorite solution for 10 min, washed three times with sterile water, and plated on basal MS medium (Murashige and Skoog 1962) supplemented with vitamins (1 mg/liter thiamine, 0.5 mg/liter pyridoxine, 0.5 mg/liter nicotinic acid, 0.1 mg/liter myo-inositol), 0.5 g/liter 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma, St. Louis), 1% (w/v) sucrose (Fisher Scientific, Pittsburgh), 0.8% agar (Fisher Scientific), and 50 mg/liter Kanamycin (Kan) (Sigma), adjusted to pH 5.7. Seeds were stratified for 3 days at 4° in the dark and then in a growth chamber with 16 hr light at 21° for 14 days. Resistant seedlings were transferred to soil and grown in a greenhouse with 16 hr light, day/night temperatures of 21°/18°, and ~50% relative humidity.

Identification of male gametophytic mutants and genetic analysis: All *Ds* insertion lines were crossed as females with the *qrt1* homozygous mutant in the Landsberg *erecta* (Ler) ecotype. F_1 seedlings were selected on Kan and the resistant plants were allowed to self-fertilize. F_2 plants that were *qrt1/qrt1* and harboring the *Ds* insertion were used for further analysis. For segregation ratio analyses ~300–400 seeds from self crosses and 150–400 seeds from reciprocal backcrosses were plated on medium supplemented with Kan and seedlings were scored for resistance:sensitivity (Kan^R:Kan^S) ratios. Transmission efficiency (TE) was defined as the ratio between Kan^R and Kan^S plants in the progeny of self or reciprocal crosses. Stage 12 flowers (Smyth *et al.* 1990) of wild-type and insertion mutants were emasculated and cross-pollinations were performed 24 hr later.

Mapping Ds insertion loci: DNA was isolated from young rosette leaves as described in Edwards *et al.* (1991) or using the hexadecyletrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). To isolate sequences flanking the *Ds* insertion, thermal asymmetric interlaced (TAIL)–PCR (Liu *et al.* 1995) or adapter ligation-mediated PCR (Siebert *et al.* 1995) was used. DNA restriction and ligation steps were performed simultaneously: 3 μg of genomic DNA were digested with 1 unit of *Dra*I and 1 unit *Eco*RV and ligated in the presence of 3 units of T4 DNA ligase in a 40-μl reaction. The reaction mixture contained 25 μM of the adapters. The reaction was conducted overnight at room temperature (22°–24°). The adapter was a mixture of two oligonucleotides at equimolar concentrations: ADA1, 5′-CTAATACGACTCAC TATAGGGCTCGAGCGGCCGCCGGGGAGGT-3′; and ADA2, 5′-P-ACCTCCCC-N3′. The adapter was generated by heating ADA1 and ADA2 to 65° for 5 min and then cooled to room temperature for annealing. Digested-ligated genomic DNA was purified and PCR amplification was conducted in a 20-μl reaction containing 2.5 μl DNA, 1× PCR buffer [10 mM Tris (pH 8.8), 50 mM KCl, 0.08% Nonidet P40], 1.5 mM $MgCl_2$, 0.1 mg/ml BSA and 0.5 μl Taq DNA polymerase, 200 μM of dNTPs, 5 pmol of each primer (AP1 and the *Ds*-specific primers *Ds*5-1a and *Ds*3-1a), and 1 unit of Taq DNA polymerase. PCR conditions were as follows: 5 min at 94°; 35 cycles of 30 sec at 94°, 45 sec at 67°, and then 2 min at 72°; followed by a final elongation step at 72° for 5 min. The first PCR was diluted 50-fold before use as template for the second amplification with the second set of nested primers (AP2 and *Ds*5-2a and *Ds*3-2a), using the same conditions as the first PCR. The sequences of the *Ds*-specific primers *Ds*5-1a, *Ds*5-2a, *Ds*3-1a, *Ds*3-2a are 5′-ACGGGATCCCGGTGAAACGGT-3′, 5′-TCCGTTCCGTTTT CGTTTTTTAC-3′, 5′-CTTCTTATGTTAGCCAAGAGC-3′, and 5′-CCGGATCGTATCGGTTTTCG-3′, respectively. The sequences of the adapter-specific primers AP1 and AP2 are 5′-GTAATACGACTCACTATAGGGC-3′ and 5′-ATAGGGCACGC GTGGTCGA-3′, respectively. The PCR products were directly sequenced using the *Ds*3-7 and *Ds*5-4 primers 5′-GTTACC GACCGTTTTCATCC-3′ and 5′-CTGGCCATATTGCAGT CATC-3′, respectively. Sequencing was performed using an ABIPRISM 3130xl Sequencer (Applied Biosystems, Foster City, CA). Sequences were searched against the Arabidopsis genome sequence using BLASTN, to identify genomic regions flanking the *Ds* insertion. Gene-specific primers were used in combination with *Ds*-insertion primers to confirm the sequences obtained.

Phenotypic analysis: Pollen morphology was scored microscopically by spreading pollen from one or two flowers onto double-sided tape attached to a microscope slide. To score for abnormalities in morphology or cellular constitution, pollen from five to eight flowers was collected in liquid pollen germination medium as described (Becker *et al.* 2003). To evaluate pollen viability, pollen was incubated in a 17% sucrose solution with 5 mM fluorescein diacetate (FDA) (Heslop Harrison *et al.* 1984). To determine at which developmental stage the mutant arrested, inflorescences containing buds at different developmental stages were fixed in ethanol:acetic acid (3:1; v/v) and stored at 4°. Buds were dissected on a microscope slide and microspores or pollen were stained in 1 μg/ml 4-6-diamidino-2 phenylindole (DAPI). Pollen germination assays were performed on solid medium as described in Boavida and McCormick (2007) and were repeated at least three times. To monitor pollen tube growth inside the pistil and to determine if pollen tubes were correctly targeted to ovules, at least 10 pistils were dissected, with the help of a needle, to expose ovules; the pistils were fixed in 3.5% formaldehyde, 5% acetic acid, and 50% ethanol (FAA) solution, incubated in 4 M NaOH overnight, washed twice in water, and then stained with decolorized aniline blue (DAB) overnight (Martin 1959). Seed set was scored in at least five siliques of each plant, by counting abnormal (aborted or undeveloped ovules) and normal seeds. For ovule clearing, 2- to 4-day-old siliques were dissected by performing cuts on both sides of the replum, using a 30-gauge syringe needle. Ovules were fixed for 20 min in FAA, washed in water for 5 min, and cleared overnight in Hoyer's solution (chloral hydrate:water: glycerol, 8:3:1, w/v/v) (Liu and Meinke 1998). Images were acquired with a Zeiss Axioskop 2 (Carl Zeiss, Jena, Germany)

microscope using fluorescence or DIC optics, with 2.5× (0.075 NA), 10× (0.25 NA), or 20× (0.50 NA) objectives. Images were captured with Axiovision 4.3 software using an AxioCamMR camera and processed using Paintshop Pro.

RT–PCR expression analysis: Total RNA was extracted from developing inflorescences (containing both open flowers and closed flower buds), roots, seedlings, rosette leaves, siliques, unpollinated pistils, unfertilized ovules, and pollen of wild-type Col plants using the RNeasy Plant mini kit (QIAGEN, Valencia, CA). DNase digestion was performed on columns and prior to cDNA synthesis (Invitrogen, Carlsbad, CA) to remove genomic DNA. Two micrograms of total RNA were reverse transcribed with the SuperScript II system (Invitrogen), according to the manufacturer's instructions, using oligo(dT) primers in a 20-μl reaction. PCR reactions were performed using 32 cycles if not otherwise indicated. The primers were designed to span intron sequences. Primer sequences are listed in supplemental Table 2.

RESULTS

Genetic analysis of male gametophytic mutants: An insertional mutagenesis system (*Ac/Ds*) was used to generate transposon-based gene trap insertions in *Arabidopsis thaliana* (Sundaresan *et al.* 1995). Gene trap lines were originally designed to monitor expression of individual genes upon insertion within a transcription unit, but can also be used in reverse genetic approaches to analyze gene function, since these insertions can disrupt essential genes that might result in mutant phenotypes (Springer 2000). The *Ds* element contains the *NPTII* gene, which confers resistance to Kan, so plants carrying the *Ds*-transposed element can be identified. If a *Ds* insertion inactivates a haploid-expressed gene that is essential for development or function of one of the gametophytes, the expected ratio of Kan^R:Kan^S in self progeny is 1:1, rather than 3:1. If the gene is required for development or function of both gametophytes, the ratio expected would be <1:1 or, if the mutation is fully penetrant, no Kan^R progeny will be recovered. The initial screen, carried out on self progeny of 24,000 independent transformants, indicated that 1.38% (333) of the transformants were potential gametophytic mutants, with segregation ratios for Kan^R <2:1 (Pagnussat *et al.* 2005). The female and male transmission efficiency was determined by reciprocal crosses; those affecting female transmission were characterized by Pagnussat *et al.* (2005).

We had 109 lines showing predominant defects in male gametophyte transmission. To facilitate phenotypic analysis of pollen, each insertion line was crossed as female with *quartet* (*qrt1*) homozygous plants (Johnson-Brousseau and McCormick 2004); F_2 plants that were homozygous for *qrt1* and harbored the *Ds* insertion were used for further analysis. Reciprocal crosses and segregation analyses were performed to confirm the male transmission efficiency and the penetrance of each mutation. Lines that showed Kan^R:Kan^S segregation ratios of 1:1 or <1:1 after selfing, and reduced transmission through the male gametophyte (TE < 0.6), were analyzed further. Table 1 summarizes the data for these 67 lines, including transmission efficiencies for each line in self and reciprocal crosses, a female gametophytic defect if characterized, the genomic location and orientation of the *Ds* insertion, and, when both flanking sequences were recovered, the annotation of the targeted gene or ORF, if known. The remaining 42 lines were excluded from further analysis because they exhibited inconsistent distortion ratios, multiple insertions, a predominantly female gametophytic defect, or embryo lethality (see supplemental Table 1 for details). The progeny of Kan-resistant siblings from the 67 selected lines showed complete cosegregation of the phenotype with the antibiotic resistance marker and consistent segregation ratios, suggesting that *Ds* was tightly linked to or responsible for the reduced genetic transmission and behaved as a single-locus insertion.

Transmission analysis showed that 29 of the 67 lines had reduced transmission efficiency only when crossed as males (TE < 0.6), while the remaining 38 lines had reduced transmission through both sexes (Table 1). Among lines affecting both gametophytes, some were described previously (Pagnussat *et al.* 2005) and had defects in embryo sac development (so-called EDA mutants), in fertilization (so-called UNE mutants), or in embryogenesis (so-called MEE mutants) (Table 1). The transmission efficiency of male gametophytic mutations varied from very low or no transmission (37 lines) (TE = 0) to some with a mild transmission defect (18 lines) (TE > 0.2), nine of which also had reduced female gametophyte transmission (TE < 0.6). We failed to isolate homozygous plants for most mutations with low penetrance through the male, suggesting either that the female gametophyte defect contributed to the reduced *Ds* transmission or that homozygous progeny were not viable. Heterozygous plants for all but one line (*tgd5*) showed no obvious growth or morphological defects, as expected for diploids carrying recessive gametophytic mutations. In *tgd5*, the flowers of the terminal inflorescence had fewer petals and sepals.

Phenotypic characterization of male gametophytic mutants: We scored the 67 lines for pollen development defects by examining pollen morphology and cellular constitution. If no defects were observed in pollen development, pollen tube growth was assayed *in vitro* and if no defects were noted there, *in vivo* pollen tube growth was examined in self-pollinated pistils or after reciprocal crosses with wild type. This analysis allowed us to place each mutation in one of five phenotypic classes: (1) pollen development defective (PDD), showing defects before pollen maturation; (2) pollen germination defective (PGD), showing defects in pollen germination; (3) tube growth defective (TGD), showing defects in pollen tube growth or guidance; (4) pollen tube–embryo sac interaction (PEI) defective, showing defects in pollen tube–embryo sac interaction or fertil-

TABLE 1

Male gametophytic mutants grouped by phenotypic category

SGT name	Line name	AGI ID	Annotation and/or female gametophytic defect	Insertion site[a] and *Ds* orientation[b]	TE (Kan^R/Kan^S)		
					Self	Male	Female
			Pollen development defective (PDD)				
SGT 08397	*pdd1*	At3g10490[c]	Arabidopsis NAC domain protein, ANAC051, ANAC052	141 bp upstream	1.1	0.0	0.9
SGT 07406	*pdd2*	At2g01820[c]	LRR kinase	12 bp upstream	0.7	0.0	0.8
SGT 09930	*pdd3*	At5g63890	Histidinol dehydrogenase (At HDH)	145 bp upstream; 5′–3′	0.7	0.0	0.6
SGT 14109	*pdd4*	At4g13890[c]	Serine hydroxymethyltransferase 5 (SHM5), EDA37	Exon 2	0.2	0.0	0.4
SGT 01712	*pdd5*	At2g34880[c]	Transcription factor jumonji (jmj), MEE27	381 bp upstream	0.2	0.0	0.3
SGT 03118	*pdd6*	At2g35210	ARF-GAP-like (At AGD10), MEE28	Exon 3; 5′–3′	0.1	0.0	0.3
SGT 05238	*pdd7*	At2g34830/35[c]		Intergenic, 2.6 kb upstream of At2g34835 and 2.5 kb upstream of At2g34830	0.2	0.0	0.3
SGT 01706	*pdd8*	Unannotated	Upstream small ORF, 53 aa (ath_mu_ch2_12997 bottom)	Intergenic, 1.2 kb upstream of At2g34850 and 1.3 kb upstream of At2g34860; 5′–3′	0.2	0.0	0.3
SGT 02837	*pdd9*	At2g34920[c]	Protein binding/zinc ion binding, EDA18	Exon 4	0.3	0.0	0.3
SGT 04915	*pdd10*	At2g37690[c]	Phosphoribosylaminoimidazole carboxylase	33 bp upstream	0.1	0.0	0.3
SGT 04025	*pdd11*	At2g35060/70[c]		Intergenic, 2.8-kb At2g35060 and 2.5 kb upstream of At2g35070	0.6	0.0	0.4
SGT 02824	*pdd12*	At2g34220	Unknown protein, MEE20	Exon 3; 5′–3′	0.2	0.0	0.3
SGT 00458	*pdd13*	At4g04040–At4g04870	MEE51	525 kb deletion; 5′–3′	0.3	0.0	0.1
SGT 01526	*pdd14*	At3g10000/At2g34850/60		Translocation	0.2	0.0	0.2
SGT 14667	*pdd15*	At5g44700–At5g44760	EDA23	26-kb deletion; 5′–3′	0.2	0.0	0.1
SGT 08841	*pdd16*	At2g01200[c]	AUX/IAA family protein, IAA32, MEE10	Exon 1	0.6	0.1	0.9
SGT 03904	*pdd17*	At4g14790	RNA helicase (At SUV3)	108 bp upstream; 3′–5′	0.5	0.1	0.4
SGT 09368	*pdd18*	At1g60870[c]	Expressed protein, MEE 9	44 bp downstream	0.5	0.4	0.4
SGT 06608	*pdd19*	At1g79860–At1g80740	MEE64	300-kb deletion; 5′–3′	0.5	0.4	0.5
SGT 06991	*pdd20*	At5g58230	MSI1, MEE70	Exon 1; 5′–3′	0.9	0.4	0.0
SGT 13017	*pdd21*	At3g10405[c]	Unknown protein	190 bp upstream	0.5	0.0	0.5
SGT 08584	*pdd22*	At4g00700–At4g00910		100-kb deletion; 3′–5′	0.6	0.0	0.8
SGT 06569	*pdd23*	At3g56200[c]	Polyamine transporter	Exon 1	1.5	0.0	1.1
SGT 02152	*pdd24*	At3g10960/70[c]		Intergenic, 2.2 kb downstream of At3g10970 and 3.9 kb upstream of At3g10960	0.6	0.0	0.5
SGT 02381	*pdd25*	At4g00330	Calmodulin-binding receptor-like kinase (CRCK2)	Exon 1; 5′–3′	1.0	0.0	0.8
SGT 03906	*pdd26*	At4g14790	RNA helicase (AtSUV3)	130 bp upstream; 3′–5′	0.6	0.0	0.7
SGT 13701	*pdd27*	At4g01530/At2g47470		Translocation	1.0	0.1	1.0
SGT 03135	*pdd28*	At2g34850/60[c]		Intergenic, 1.1 kb upstream of At2g34860 and 1.2 kb upstream of At2g34850	1.0	0.2	0.7
SGT 11685	*pdd29*	At4g36170/At2g01420		Translocation	1.0	0.3	1.0
SGT 04228	*pdd30*	Unannotated	Upstream small ORF, 106 aa; GenBank EST EG515492	Intergenic, 2 kb upstream of At1g26810 and 1.3 kb downstream of At1g26820; 3′–5′	1.5	0.3	0.8
SGT 04087	*pdd31*	At2g34825/At2g34830	AtWRKY35	476 bp downstream of At2g34830 and 1.8 kb downstream of At2g34825; 3′–5′	0.2	0.0	0.4
SGT 12162	*pdd32*	At3g63290[c]	Unknown protein	Exon 1	1.0	0.2	0.3
SGT 14110	*pdd33*	At4g13890/At2g01200	EDA36	Translocation	0.4	0.0	0.6

(*continued*)

TABLE 1

(Continued)

SGT name	Line name	AGI ID	Annotation and/or female gametophytic defect	Insertion site[a] and *Ds* orientation[b]	TE (Kan^R/Kan^S)		
					Self	Male	Female
SGT 09926	*pdd34*	At5g54740[c]	Lipid transfer protein (LTP)	330 bp upstream	1.3	0.3	1.0
			Pollen germination defective (PGD)				
SGT 08289	*pgd2*	At3g10620–At3g11270		200-kb deletion; 5′–3′	0.9	0.0	1.0
SGT 07358	*pgd3*	At3g10560[c]	Cytochrome P450 (CYP77A7), UNE9	Exon	0.4	0.0	0.6
SGT 03671	*pgd4*	At2g35070	Unknown protein	Intron 1; 5′–3′	0.1	0.0	0.3
SGT 03669	*pgd5*	At2g34090[c]	Unknown protein, MEE18	71 bp upstream	0.2	0.0	0.3
SGT 01084	*pgd6*	At4g05450	Adrenodoxin-like ferredoxin 2	315 bp upstream; 5′–3′	0.3	0.1	0.1
SGT 03063	*pgd7*	At2g35300	LEA group 1 domain protein	70 bp downstream; 3′–5′	1.2	0.0	0.9
SGT 01001	*pgd8*	At3g05770	Unknown protein	Intron 1; 5′–3′	0.7	0.1	1.0
SGT 11239	*pgd9*	At3g10405–At3g10116		110-kb deletion; 3′–5′	0.7	0.0	0.7
			Pollen tube growth defective (TGD)				
SGT 01850	*tgd1*	At3g03810[c]	Unknown protein, EDA30	141 bp downstream	0.3	0.0	0.2
SGT 04586	*tgd2*	At2g34850[c]		Intergenic, 426 bp downstream of At2g34850	0.7	0.4	0.4
SGT 07405	*tgd3*	At3g07330[c]	Cellulose synthase-like C6 (ATCSLC6)	Exon 1	1.5	0.5	0.7
SGT 03295	*tgd4*	At2g12945/At5G54745		Translocation	1.1	0.6	0.4
SGT 05752	*tgd5*	At1g77131	Pseudogene of PGSIP, glycogenin glycosyltransferase	754 bp upstream of At1g77130; 3′–5′	1.0	0.0	1.0
SGT 13609	*tgd6*	Unannotated	Downstream small ORF, 53 aa	Intergenic, 1.8 kb downstream of At4g11550 and 2.3 kb upstream of At4g11540; 3′–5′	1.2	0.4	0.9
SGT 11205	*tgd7*	At3g06530/At3g06540		Intergenic, 771 bp downstream of At3g06540 and 750 bp downstream; 3′–5′	1.3	0.1	1.0
SGT 02510	*tgd8*	At3g05000	Transport protein particle (TRAPP) component, Bet3 family protein	Exon 1; 5′–3′	1.0	0.3	1.0
SGT 09403	*tgd9*	At3g59760[c]	*O*-acetylserine (thiol) lyase isoform C (OASC)	Exon 1	1.2	0.6	0.9
SGT 11437	*tgd10*	At3g06430	Pentatricopeptide repeat protein, EMB2750	Exon 1; 5′–3′	1.1	0.6	0.4
SGT 01267	*tgd11*	At3g03650[c]	Exostosin family protein, EDA5	Exon 1	0.2	0.0	0.6
SGT 00656	*tgd12*	At3g03690[c]	Glycosyl transferase family 14, UNE7	Intron 1	0.2	0.0	0.2
SGT 15318	*tgd13*	At3g10280–At3g10915		235-kb deletion; 3′–5′	0.3	0.0	0.2
SGT 08149	*tgd14*	At1g25240/At1g25250		Intergenic, 1.5 kb upstream of At1g25250 and 1.2 kb downstream; 5′–3′	1.2	0.2	1.0
SGT 04229	*tgd15*	At2g34590[c]	Transketolase family protein; similar to Pyruvate dehydrogenase E1 beta (PDH-E1 BETA)	66 bp upstream	1.4	0.3	0.8
SGT 15447	*tgd16*	At2g47510	Fumarase (FUM1)	Exon 1; 3′–5′	1.0	0.5	0.8
SGT 07020	*tgd17*	At1g25450[c]	Very-long-chain fatty acid condensing enzyme (CUT1)	Exon 1	0.6	0.2	0.2
SGT 07170	*tgd18*	At2g47470	UNE5	400 bp upstream of At2g47470; 5′–3′	1.2	0.4	0.6

(*continued*)

TABLE 1

(Continued)

SGT name	Line name	AGI ID	Annotation and/or female gametophytic defect	Insertion site[a] and *Ds* orientation[b]	TE (Kan^R/Kan^S) Self	Male	Female
SGT 14828	*tgd19*	At3g05200–At3g05320		30-kb deletion; 5′–3′	1.0	0.0	0.9
			Pollen tube–embryo sac interaction (PEI) and fertilization defective				
SGT 05178	*pei1*	At3g58350/At2g34850		Translocation	0.7	0.1	0.6
SGT 12231	*pei2*	At3g14940/At3g14950		Intergenic, 366 bp downstream of At3g14940 and 346 bp downstream of At3g14950; 5′–3′	1.3	0.4	0.9
			No obvious phenotype				
SGT 11748		At1g04620/30/ At2g34850/60		Translocation	0.3	0.4	0.1
SGT 12347		At2g15890[c]	Unknown protein, MEE14	Exon 1	0.5	0.4	0.1
SGT 01214		At3g14920–At3g15030	MEE35	44-kb deletion; 5′–3′	0.5	0.4	0.1

The genetrap (SGT) number is indicated. The annotation is according to TAIR. Transmission efficiency (TE) (Kan^R/Kan^S) is shown from self and reciprocal crosses. The female gametophyte phenotype noted by Pagnussat *et al.* (2005) is indicated, if present. AGI ID, Arabidopsis Genome Initiative identification number.

[a] The insertion location relative to the transcribed region of the annotated gene.

[b] Orientation of the *Ds* insertion relative to the transcribed region of the annotated gene (shown only for insertions where both flanking sequences were recovered and map to the same chromosome). For lines in which the distance from the closest ORF is >1 kb upstream or 0.3 kb downstream, the genomic coordinate of the *Ds* insertion is indicated.

[c] Lines for which only one genomic flanking sequence was recovered.

ization; and (5) no obvious phenotype. Phenotypic classifications and transmission data from self and reciprocal crosses for each independent line are summarized in Table 1. In the following we describe each class separately and comment on the potential roles of the proteins encoded by the disrupted genes.

PDD mutants: We analyzed the pattern of pollen abortion and stage of developmental arrest in heterozygous mutants using DAPI (Figure 1). Heterozygous mutants with a fully penetrant gametophytic mutation in this category are expected to produce an equal number of wild-type and aberrant pollen grains. In wild type (Figure 1, A–E) after meiosis, the free microspores increase in volume and small vacuoles accumulate, which ultimately coalesce into a single vacuole that displaces most of the cytoplasm and the nucleus to the cell periphery (Figure 1B). An asymmetric division during pollen mitosis I (PMI) yields two cells with different fates, the vegetative cell and the generative cell. The generative cell is at the periphery (Figure 1C). At the early bicellular stage, the generative cell detaches from the intine wall and is engulfed by the vegetative cell cytoplasm, acquiring a central position (Figure 1D). During pollen mitosis II (PMII) the generative cell divides to form two sperm cells. In mature pollen, the assemblage of two sperm cells in association with the vegetative nucleus is termed the male germ unit (MGU) (Figure 1E).

As shown in Table 1, 34 of the 67 lines (51%) exhibited obvious defects in pollen development and maturation. In all of the PDD mutants, a collapsed pollen phenotype was seen in >20% of the pollen grains; in these there was eventually complete degeneration of the cytoplasm and there were no visible nuclei after PMI or during the transition to the late bicellular stage (Figure 1). The *ppd6* (Figure 1, F–J) and *pdd34* (not shown) mutants showed defects in PMII nuclear division. Pollen development in *pdd6* arrested at the early bicellular stage; the defects were visible during generative cell displacement, and occasionally the generative cell failed to migrate to a central position. Mutant pollen grains in the late bicellular stage failed to undergo PMII and no sperm cells were formed; however, they conserved the integrity of their cytoplasm and their nuclei stained until rather late in pollen development, when cytoplasm degeneration and pollen collapse occurred (Figure 1, F–J).

For other *pdd* mutants no significant abnormalities in positioning of the vegetative nucleus or generative cell were found. In *pdd17* (Figure 1, K–O) the first alterations in cytoplasm organization were observed during the transition from the vacuolated microspore stage to the early bicellular stage (PMI), but microspores collapsed later, at the late bicellular stage (Figure 1, K–O). In *pdd18* (Figure 1, P–T), pollen grains underwent PMI, but the first signs of cytoplasmic degeneration occurred during displacement of the generative cell to a central position and pollen grains aborted during the transition to the late bicellular stage. Another example is *pdd23* (Figure 1, U–Y), in which 30% of the pollen grains showed an initial delay in polarization and vacuolization of the microspore before PMI and pollen grains collapsed early during the bicellular stage (Figure 1, U–Y).

Most (28 of 34) of the mutants affecting pollen development were fully penetrant, abolishing (TE = 0) or strongly reducing (TE < 0.2) transmission through

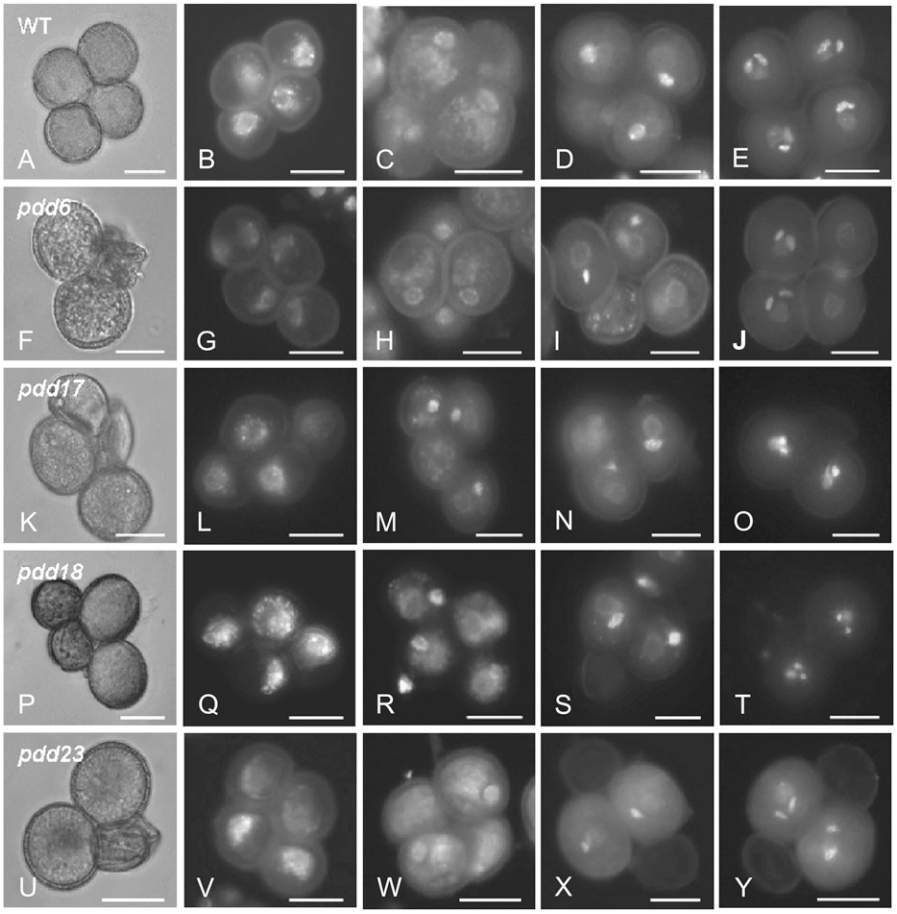

FIGURE 1.—Developmental analysis of *pdd*/+ mutants. Mature pollen and earlier developmental stages of wild type (A–E), *pdd6* (F–J), *pdd17* (K–O), *pdd18* (P–T), and *pdd23* (U–Y) are shown. Transmitted light images of mature pollen grains and DAPI stain for different pollen developmental stages are shown: (B, G, L, Q, and V) polarized and vacuolated microspore stage; (C, H, M, R, and W) early bicellular stage; (D, I, N, S, and X) late bicellular stage; and (E, J, O, T, and Y) early tricellular pollen. More than 500 spores or pollen grains were scored in the *qrt1* background. Bars: 20 μm.

the male, although six lines showed less severe effects (TE $>$ 0.2) (Table 1). For the *pdd* mutants with low penetrance, pollen abortion was often variable (25–40%) and mixed tetrad classes were often observed, indicating that some of the mutant pollen grains could still achieve maturation. Twenty of the *pdd* mutants also had reduced female transmission, but in general the defects were less severe (TE $>$ 0.2) than in the male gametophyte (Table 1).

We recovered both flanking sequences for 18 of the *pdd* lines; 9 of these target annotated genes or small unannotated ORFs. The genes associated with the *Ds* insertions include two thought to play roles in signal transduction pathways (an *ARF-GAP*, *AGD10*, and a calmodulin-binding receptor-like kinase, *CRCK2*), *MSI1*, involved in chromatin remodeling, a histidinol dehydrogenase, a gene encoding a protein of unknown function, and one encoding an RNA helicase (*At4g14790*). For the RNA helicase, we had two independent insertions in the promoter region and both lines showed the same phenotype, confirming that the insertions in this gene were responsible for the phenotype. To verify expression of the candidate genes in the male gametophyte and assess expression in different tissues, we performed RT–PCR (Figure 4). In three of the PDD lines (*pdd8*, *pdd30*, and *pdd31*) the insertions were located in intergenic regions. The transcriptome Tiling array (YAMADA *et al.* 2003) predicted the existence of two small ORFs downstream of the *pdd8* and *pdd30* insertions, and these ORFs had EST support. For *pdd8* a transcript encoding a putative 53-aa secreted protein was detected in several tissues tested but was absent from mature pollen (Figure 4), while for *pdd30* the transcript predicted to encode a putative 106-aa secreted protein was not detected in any tissues tested. For *pdd31* the insertion was located between two genes and therefore a tagged gene could not be assigned. The remaining 8 lines represent putative translocations or deletions, ranging from 26 to 525 kb.

PGD and TGD mutants: Mutants in these categories showed normal pollen morphology: the sperm cells and vegetative nucleus were correctly positioned and FDA staining indicated that >96% of the mature pollen grains were viable (data not shown). However, *in vitro* pollen germination assays showed that the *pgd2-9* mutants had reduced pollen germination. For example, in *pgd4* (Figure 2), as in other *pgd* mutants (not shown), pollen tube initiation and growth occurred in only 50% of the pollen grains (likely the wild-type pollen grains in the tetrad), even though all pollen grains were viable (Figure 2, A–C). The *pgd7* mutant has a particularly interesting phenotype, since pollen grains burst during

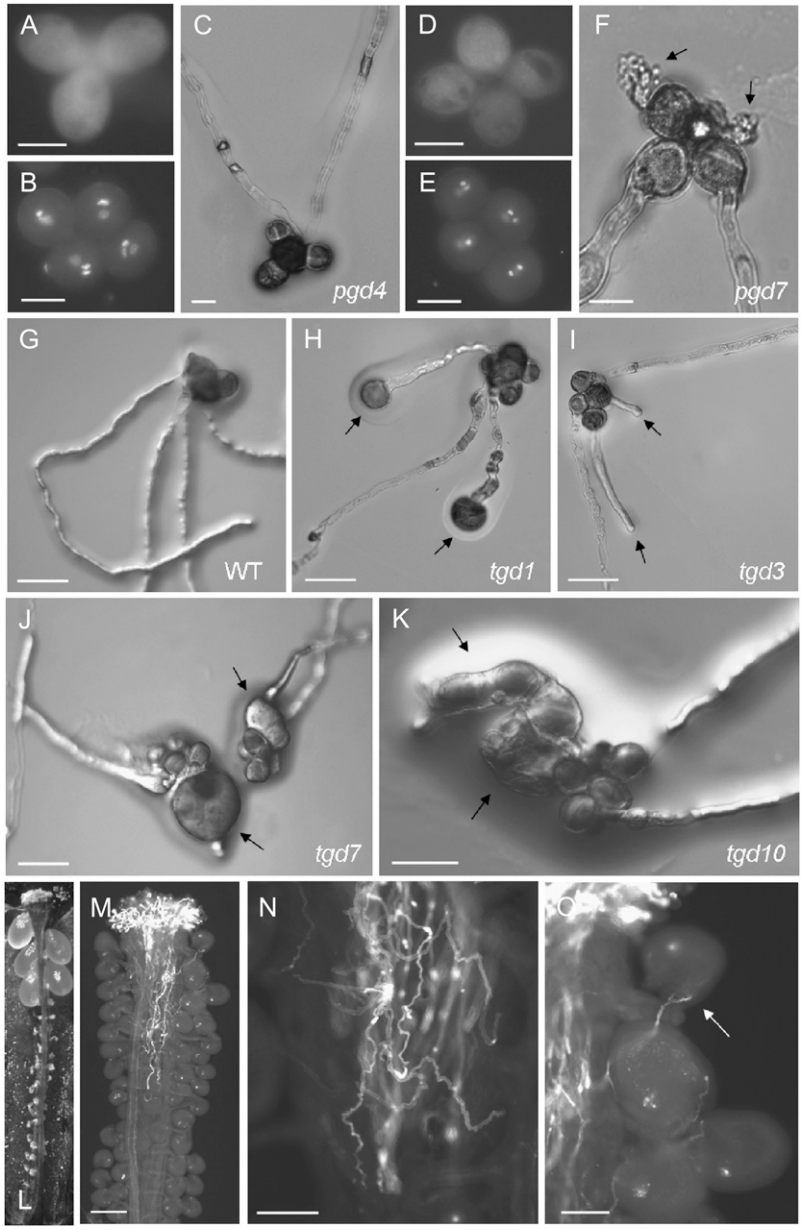

FIGURE 2.—Phenotypic characterization of *pgd*/+ and *tgd*/+ mutants. (A–F) Pollen germination defective (PGD) mutants, *pgd4* (A–C) and *pgd7* (D–F). Mature *pgd*/+ tetrads stained with FDA (A and D) and DAPI (B and E) and after *in vitro* pollen germination (C and F) are shown. (G–K) *In vitro* pollen germination assays for pollen tube growth in wild type (WT) (G) and (TGD) mutants, *tgd1* (H), *tgd3* (I), *tgd7* (J), and *tgd10* (K). Solid arrows indicate defective pollen tubes. (L–O) Phenotypic analysis of a *tgd19* homozygous line: (L) silique from a self cross showing reduced seed set (all seeds are at the top of the silique); (M–O) representative images of 3 DAP pistils stained with DAB; (M) DAB-stained pistil showing pollen tube growth in the upper third of the pistil, (N) aspect of abnormal and nondirectional pollen tube growth in transmitting tissue, and (O) detail of the upper third of the pistil showing the first ovules targeted by pollen tubes (open arrow indicates undeveloped ovule). Bars: A–F, 25 μm; G–K, 50 μm; N and O, 100 μm; M, 250 μm.

hydration (Figure 2, D–F). Consistent with the phenotypes observed, all *pgd* mutants were fully penetrant (TE < 0.1). Four of these (*pgd2*, *pgd7*, *pgd8*, and *pgd9*) are male gametophytic-specific, and four (*pgd3*, *pgd4*, *pgd5*, and *pgd6*) also have a female transmission defect (Table 1).

Genes tagged in PGD mutants include those encoding an adrenodoxin-like ferrodoxin (At4g05450), a protein with homology to a late embryogenesis abundant (LEA) domain protein (At2g35300), and two unknown proteins (Table 1). For three of the PGD lines we were able to detect the corresponding transcript in mature pollen (Figure 4), suggesting that these proteins may perform important functions during pollen hydration and germination. For four lines a tagged gene could not be assigned: two had large deletions (200 and 110 kb) and in two others only one of the *Ds* flanking sequences was recovered.

In the TGD lines (*tgd1–19*) all pollen grains in a tetrad formed a pollen tube (Figure 2). However, these mutants showed severe defects in pollen tube growth performance, *i.e.*, in the ability to initiate a tip-growing tube or to maintain polarized growth, or in pollen tube elongation or guidance (Figure 2, H–K). Some showed disturbed pollen tube shapes, likely caused by a combination of defects in polarity, orientation, and growth. As expected the associated phenotype was present in two of the pollen tubes in a tetrad, while the other two had morphologies and growth rates similar to those of wild-type pollen tubes. Mutant pollen tubes were shorter in *tgd3* (Figure 2I) and *tgd1*, *tgd2*, *tgd4*, *tgd5*, *tgd8*, *tgd9*, *tgd11*, *tgd12*, *tgd13*, *tgd14*, *tgd15*, *tgd16*, and *tgd18* (not shown).

The *tgd1*, *tgd7*, and *tgd14* (not shown) mutations affected pollen tube polarity. In *tgd1* the mutant pollen

tubes were shorter than wild type and sometimes showing bulged regions along the tube. More often pollen tube tips were swollen or formed small balloons due to isotropic growth after an initial phase of tube elongation (Figure 2H), ending in premature growth arrest. In contrast, the *tgd7* mutant failed to produce a polarized tube during initial germination. Consequently, large balloons were observed emerging directly from the pollen grains (Figure 2J). Interestingly, some of these tubes recovered polarity after the initial phase of isotropic growth, and tip-growing tubes were observed emerging from the balloons, although these pollen tubes were still compromised in their ability to grow. In *tgd10* (Figure 2K) and *tgd6* (not shown) mutant pollen tubes showed striking defects in shape, tube polarity, and growth. In *tgd10* the pollen tubes were wider and shorter than in wild type, occasionally presenting multiple tips (Figure 2K). This mutation affected the ability of pollen tubes to elongate and these tubes arrested prematurely, consistent with the strong reduction in male transmission (TE < 0.2). Seven of these mutations are male gametophytic specific (Table 1).

Despite the phenotypes observed in *in vitro* germination, reciprocal crosses showed that pollen tube elongation *in vivo* was severely compromised in only seven of these mutants (*tgd1*, *tgd5*, *tgd7*, *tgd11*, *tgd12*, *tgd1*, and *tgd14*), where male transmission was strongly reduced (TE ≤ 0.1), while all other mutants in this class could still fertilize ovules (TE ≥ 0.2), suggesting that the defect reduced competitive ability but was not sufficient to block transmission. To test this hypothesis, we separately collected seeds from the top and bottom halves of siliques of self crosses for some of the *tgd* mutants and analyzed Kan transmission. If mutant pollen tubes had impaired growth *in vivo* but were still able to fertilize some ovules, we would expect a higher proportion of Kan^R seedlings from the top half of the siliques. This hypothesis was confirmed for *tgd3*, *tgd4*, *tgd6*, *tgd7*, *tgd8*, *tgd9*, *tgd14*, *tgd15*, *tgd16*, *tgd17*, *tgd18*, and *tgd19* (Table 2).

For *tgd19*, *in vitro* pollen germinations were not conclusive, as many tetrads produced four long tubes (data not shown). Analysis of Kan^R transmission from the top and bottom halves of *tgd19* heterozygous siliques suggested that the defect could be explained by reduced competitive ability of *tgd19* pollen tubes (Table 2). This hypothesis was confirmed when we recovered one homozygous plant (Figure 2, L–O). The *tgd19* homozygous plant produced just a few seeds (four to six) always located at the top of the silique (Figure 2L). To characterize this phenotype in more detail, pistils were dissected 3 days after pollination (DAP) and stained with DAB to monitor *in vivo* pollen tube growth (Figure 2, M–O). Pollen tubes grew only about one-third of the total pistil length (Figure 2M) and had irregular and nondirectional growth (Figure 2N), often failing to leave the transmitting tissue (Figure 2, M and N). Seed set near the top was probably due to the physical constriction at the ovary entrance, allowing some of the tubes to target a few ovules. However, the opportunity to fertilize might be a rare event in heterozygous plants, since there was no measurable *tgd19* male transmission (Table 1). Additionally we observed that some of the targeted ovules failed to develop (Figure 2O). Crosses performed using *tgd19* homozygous plants as female and pollinated with wild-type pollen showed no fertilization defects (data not shown), consistent with the normal transmission (TE $= 1.0$) through the female (Table 1) and suggesting that the slight reduction in seed set ($\sim$20%) in heterozygous self siliques was likely caused by the *tgd19* mutant pollen tubes. These results support the idea that *tgd19* is a male gametophytic-specific mutation with pleiotropic defects affecting pollen tube growth and guidance and pollen tube–embryo sac interactions.

Genes tagged in TGD lines (Table 1) include those encoding an annotated pseudogene of a glycogenin glycosyltransferase, a component of transport protein particle (TRAPP) from the Bet3 family, a mitochondrial fumarase hydratase (FUM1), and a pentatricopeptide repeat protein. For five of these genes the corresponding transcript was detected in mature pollen, suggesting that the genes tagged might perform functions during pollen tube growth (Figure 4). In three of the TGD lines, the insertions were located in intergenic regions. The Tiling array (Yamada *et al.* 2003) predicted the existence of a small ORF in the vicinity of the *tgd6*

TABLE 2

Analysis of *in vivo* pollen tube competitive ability in *tgd* mutants

	Kan^R/Kan^S in siliques of self crosses (*n*)	
Line	Top half	Bottom half
tgd1	0.2 (237)	0.2 (258)
tgd2	0.5 (132)	0.4 (198)
tgd3	1.4 (140)	0.6 (149)
tgd4	1.5 (163)	1.0 (131)
tgd5	0.8 (97)	0.6 (102)
tgd6	1.3 (337)	0.8 (230)
tgd7	1.4 (128)	1.0 (104)
tgd8	1.2 (118)	0.7 (157)
tgd9	1.8 (166)	1.1 (115)
tgd10	0.2 (114)	0.2 (135)
tgd11	0.6 (134)	0.6 (123)
tgd12	0.2 (339)	0.3 (396)
tgd13	0.2 (366)	0.1 (316)
tgd14	1.3 (223)	0.9 (216)
tgd15	1.6 (280)	1.2 (205)
tgd16	1.3 (154)	0.8 (159)
tgd17	1.1 (96)	0.4 (79)
tgd18	1.3 (79)	0.7 (71)
tgd19	1.3 (253)	1.0 (356)

Kan^R/Kan^S ratios were scored from seeds collected separately from top and bottom halves of siliques of self crosses of heterozygous plants. *n*, number of seeds counted.

insertion, suggesting that the phenotype might be correlated with an unannotated and pollen-specific 53-aa secreted protein (Figure 4). For *tgd7* and *tgd14* the phenotype might be associated with one of the flanking genes. In *tgd14* one of the flanking genes encodes a pollen-specific epsin N-terminal homology (ENTH) domain-containing protein. In two of the TGD lines (*tgd13* and *tgd19*) the insertions caused deletions (of 30 and 235 kb). The 30-kb deletion in *tgd19* includes 12 genes, most of which are expressed in pollen (Pina *et al.* 2005), but only one is pollen-specific and it encodes a GTP-binding protein. One *Ds* insertion line (*tgd4*) might represent a translocation and for the eight remaining lines only one of the flanking sequences was recovered; a transposition event might have caused a chromosomal rearrangement (Table 1).

PEI and fertilization-defective mutants: The PEI category includes two lines (*pei1* and *pei2*) that failed to show any of the previously described phenotypes. The PEI mutations were potential male gametophytic-specific, affecting male transmission (TE $\leq$ 0.2) (Table 1), while female gametophytic transmission was not significantly affected (TE $>$ 0.6) (Table 1). However, these two lines had undeveloped ovules in mature siliques. The ovule abortion pattern did not follow the expected 50% seed abortion phenotype typical for a female gametophytic mutation; instead seed set on a plant varied slightly from silique to silique. To test whether the seed abortion phenotype had a male gametophytic origin, additional reciprocal crosses were performed using heterozygous *pei*/+ mutants and wild-type plants, and *in vivo* pollen tube growth was analyzed in independent crosses and correlated with seed set.

The *pei1* mutant (Figure 3, A–E) shows reduced male transmission (TE = 0.1) and a slight reduction in transmission through the female gametophyte (TE = 0.6; Table 1). Consistent with these results, siliques from reciprocal crosses showed ovule abortion phenotypes in both directions, indicating that both gametophytes were affected to some extent. The defect caused by the male gametophyte was analyzed by reciprocal crosses. When *pei1*/+ was used as a pollen donor on wild type, all ovules were targeted by a pollen tube, but 33% of the ovules failed to develop into seeds (Figure 3, A–E), while when *pei1*/+ was used as female, only 25% of the ovules failed to develop (data not shown). In many of the undeveloped ovules we observed nondirectional pollen tube growth and often two pollen tubes were observed in the micropyle, either in undeveloped (Figure 3B) or in normal-appearing ovules (Figure 3C). Ovule clearings showed that most of the undeveloped ovules were unfertilized (Figure 3, D and E), although occasionally (15%) the undeveloped ovules contained an arrested embryo and endosperm.

The *pei2* mutant (Figure 3, F–J) has a particularly interesting phenotype. *pei2* had a transmission defect only through the male (TE = 0.2) and female transmission was close to normal (TE = 0.9). However, when *pei2*/+ was used as pollen donor and 3 DAP siliques were examined, 20–25% of the ovules failed to develop, even though all ovules were targeted by pollen tubes (Figure 3, F–J). Often two pollen tubes were seen in the micropyle region of the undeveloped ovules and less frequently (2%) we observed pollen tube overgrowth inside the embryo sac (Figure 3G). Ovule clearings of undeveloped ovules showed that the embryo sacs were unfertilized, but occasionally one or two extra small nuclei were seen inside the central cell (Figure 3, I and J), but not inside the egg cell. The diameters of these nuclei are similar to those of sperm cell nuclei ($\sim$4 μm) (Rotman *et al.* 2005). About 2% of the undeveloped ovules were fertilized, although some appeared to contain a single fertilization event; in such ovules a developing endosperm was evident but there was no visible embryo, while in others an arrested embryo and endosperm were present in the same undeveloped ovule (data not shown). Seed set analysis in mature siliques of self crosses showed 20–25% unfertilized ovules and a lower percentage of white aborted ovules (10–15%), likely fertilized but arrested later during seed development. We recovered a homozygous *pei2* plant (data not shown), in which seed set was reduced to 20%. These seeds were randomly positioned along the siliques, suggesting that the phenotype was not caused by abnormal pollen tube growth. However, the homozygous plant also had a high percentage of aborted pollen and defects in embryo sac development, indicating that the homozygous mutant exhibits sporophytic defects in early stages of gametogenesis. We therefore analyzed the pollen phenotype in heterozygous plants.

Although this category of mutants is perhaps the most interesting, we could not definitively assign a tagged gene to any of the *pei* mutants. In *pei2* the insertion is located in an intergenic region of 700 bp, between a phosphoenolpyruvate carboxylase (PPC3) and a gene encoding a *TTL2* (*Tetratricopeptide-repeat Thioredoxin-Like 2*) protein (Table 1). Although the 3′-UTR of *TTL2* is not annotated, it is likely that the insertion falls in this region, perhaps regulating transcript stability (Figure 4).

DISCUSSION

Arabidopsis genes under gametophytic selection: We characterized 67 Arabidopsis *Ds* insertion lines showing male transmission defects. These lines expand the set of mutations previously described as affecting male gametophyte function. The mutations predominantly affected development after pollen mitosis I and therefore affect the expression of "late" male gametophytic factors (Mascarenhas 1990). The mutations identified affect pollen development (51%) or postpollination stages, namely pollen germination (12%) and tube growth (28%). In addition we describe a new class of male

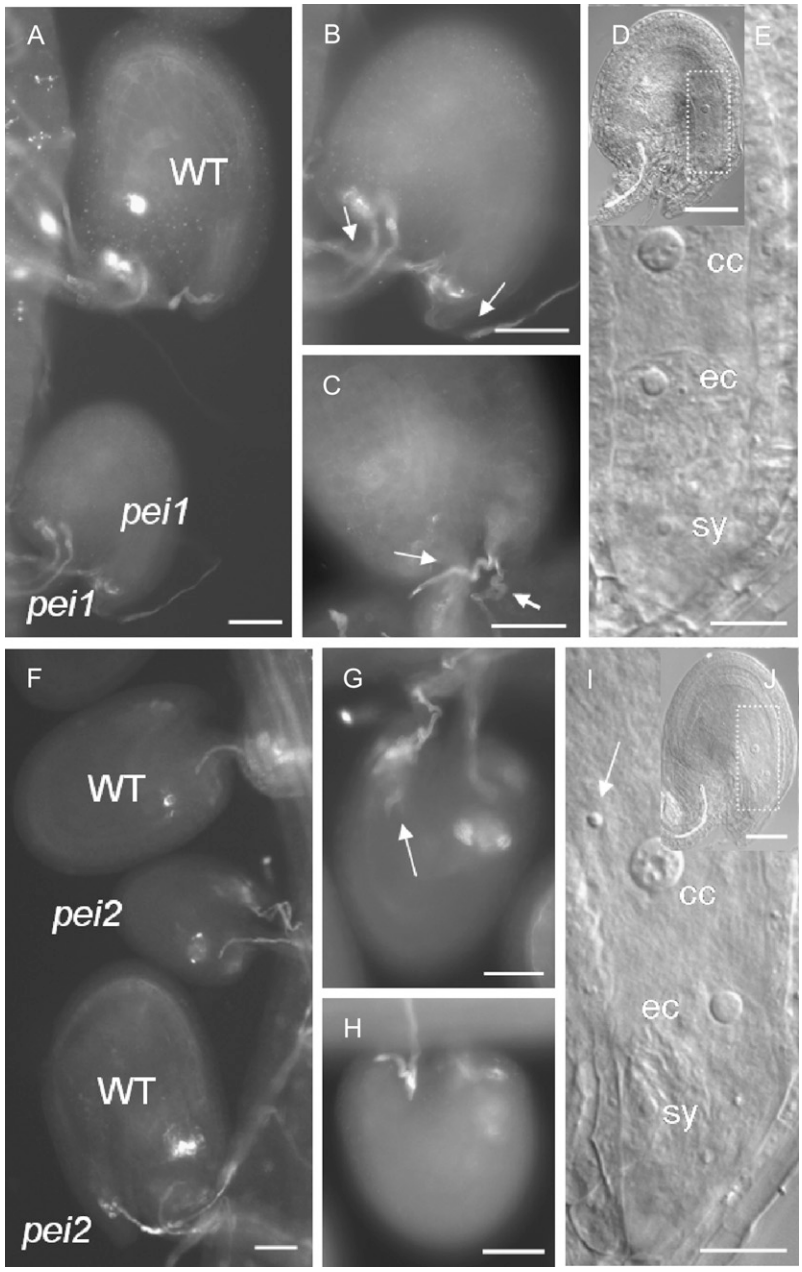

FIGURE 3.—Phenotypes of pollen tube–embryo sac interaction (PEI) and fertilization defective mutants. Representative images are shown of 3 DAP pistils from reciprocal crosses performed between wild-type females and *pei*/+ pollen. Images show DAB stain (left in each panel) and ovule clearings of the same cross (right in each panel). (A–E) *pei1*/+: (A) DAB-stained pistil showing one wild-type developed ovule and a small mutant ovule targeted by pollen tubes; (B) detail of a mutant ovule with two pollen tubes in the micropyle region; (C) wild-type developed ovule where two pollen tubes appear to enter the ES; (D) cleared mutant (unfertilized) ovule, with the ES delimited by a discontinuous rectangle; (E) magnified ES region showing central cell (cc), polarized egg cell (ec), and intact synergid (sy). (F–J) *pei2*/+: (F) pistil section showing two wild-type ovules and a mutant undeveloped ovule; (G and H) two mutant ovules, where pollen tube overgrowth inside the ES (arrow) is shown in H and normal pollen tube reception is shown in G; (J) cleared mutant ovule with delimited ES region, magnified in I; and (I) unfertilized ES where cc, eg, and sy nuclei are visible and an extra nucleus is visible above the cc nucleus (arrow). Bars: E and I, 20 μm; A–D, F–H, and J, 50 μm.

gametophytic mutants showing defects in pollen tube interactions with the embryo sac or fertilization (3%).

Genetic analysis showed that about half of the lines analyzed had a predominant male gametophytic defect, while the other half also affected the female gametophyte. The presence of both classes is likely the result of the selection criteria used in this screen, since we deliberately selected lines with Kan segregation ratios of 1:1 or <1:1, therefore including lines affecting both gametophytes. An overlap in gene expression between both gametophytes has been reported (PAGNUSSAT *et al.* 2005; JOHNSTON *et al.* 2007), indicating that the genes involved may perform general functions during gametophyte development.

Only half of the 67 lines showed no transmission through pollen and thus were considered fully penetrant male gametophytic mutants. Mutations with incomplete penetrance might be due to variable expressivity, due to insertions located in regulatory regions, or might indicate that the affected genes influence but are not vital for gametophyte function (CHEN and MCCORMICK 1996; BONHOMME *et al.* 1998; PROCISSI *et al.* 2001).

Despite the associations found between the insertion location and the tagged genes we failed to detect the corresponding transcripts in wild-type mature pollen in five lines (Figure 4). Three of these lines affect early stages of pollen development (*pdd* mutants) so their transcripts might be absent from mature pollen; indeed we were able to detect the transcripts for two of them in developing inflorescences (Figure 4). For *pgd4* the transcript is expressed in early stages of pollen development (HONYS and TWELL 2004), but is absent

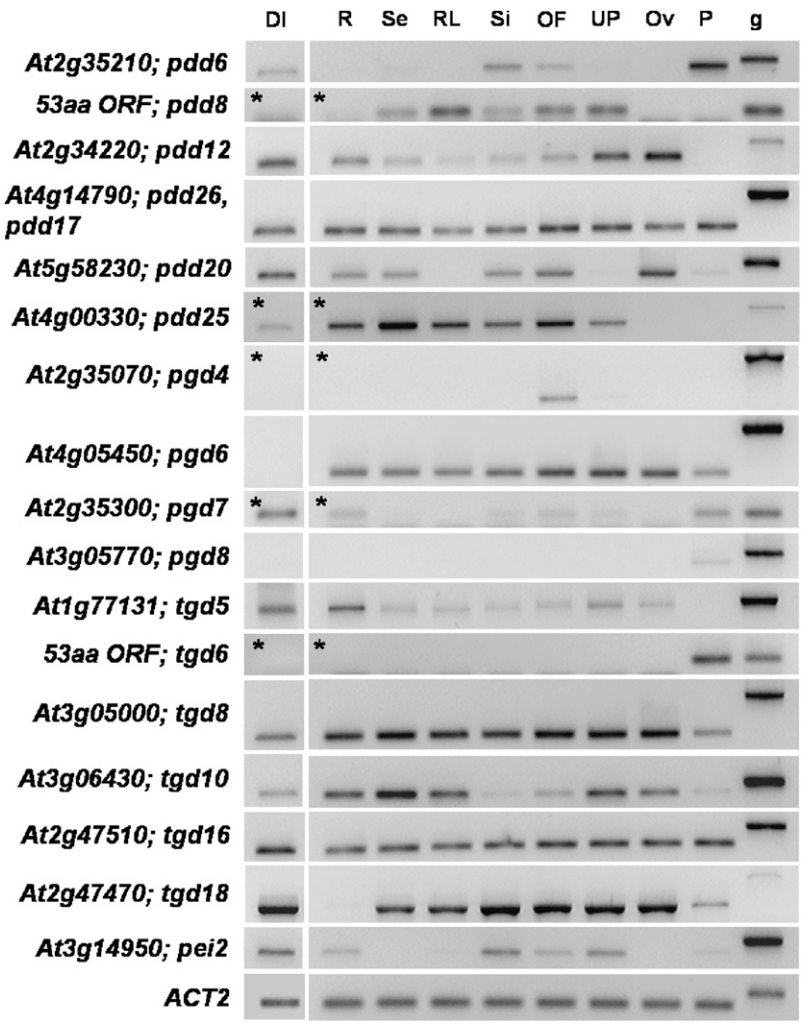

FIGURE 4.—Expression of candidate genes associated with the *Ds* insertion lines. The expression pattern of each gene was determined by RT–PCR in different tissues in wild-type Columbia: DI, developing inflorescences; R, roots; Se, seedlings; RL, rosette leaves; Si, siliques; UP, unpollinated pistil; Ov, ovules; and P, pollen. The actin gene *ACT2* was used as an internal control. The Arabidopsis Genome Initiative identification number of the candidate gene and the name of the associated mutation are indicated at the left. PCR reactions were performed with 32 cycles unless marked with an *, where twice as much cDNA and 45 cycles were used. g denotes genomic DNA.

from mature pollen, suggesting that the insertion might have altered the expression pattern of the gene in a way that compromised pollen germination. For *tgd5* transcription might be induced at later stages during pollen tube growth, since *de novo* transcription of pollen-expressed genes is known to occur during pollen germination and tube growth (WANG *et al.* 2008)

Mutants affecting pollen development: Differentiation of the male gametophyte is characterized by a relatively simple series of cellular events (MCCORMICK 2004). Some previously described pollen development mutants, *i.e.*, *sidecar pollen* (CHEN and MCCORMICK 1996), *limpet pollen* (HOWDEN *et al.* 1998), and *gemini pollen1* (PARK *et al.* 1998), affect the asymmetric cell division after PMI, which is necessary for the determination of vegetative and generative cell fate. Most of the *pdd* mutations predominantly affected cellular processes occurring during the transition from PMI to PMII. The *pdd6* and *pdd34* phenotypes are similar to *limpet pollen*, in that the engulfment and inward positioning of the generative cell were prevented after PMI, but in *pdd6* and *pdd34* no sperm cells were formed and pollen collapsed early on. The phenotype of *pdd6* indicates that an ADP ribosylation factor (ARF) GTPase-activating protein (ARF-GAP; AGD10) performs an important role during PMII. ARF-GAPs are small GTPases with well-established roles in vesicle trafficking (VERNOUD *et al.* 2003). The Arabidopsis *AGD10* gene affects root hair development and pollen tube elongation (SONG *et al.* 2006). Those authors recovered homozygous lines and did not report a pollen abortion phenotype or the female gametophytic defect previously described (PAGNUSSAT *et al.* 2005); however, we analyzed the same *Ds* insertion line and noted a pollen abortion phenotype and a female gametophytic defect. It is possible that *agd10* mutants have pleiotropic cellular effects, affecting protein secretion and transport during gametophyte development and pollen tube elongation.

The *pdd20* insertion is located in the *MULTICOPY SUPPRESSOR of IRA1* (*MSI1*) gene, a component of the MEA/FIE polycomb group complex. MSI1 is believed to function during the cell cycle, since *msi1* pollen grains fail to undergo PMII (CHEN *et al.* 2008), although generative cell fate was not affected and mature pollen grains contained an undivided but still functional generative cell. The *pdd20* phenotype we described is consistent with previous observations, which demonstrated male (CHEN

et al. 2008) and female (Guitton and Berger 2005; Pagnussat *et al.* 2005) developmental defects.

After PMI the expression of late pollen genes is detected and continues as pollen matures, creating the stable pool of RNAs thought to be essential for germination and pollen tube growth (Mascarenhas 1990). The *pdd17* and *pdd26* insertions in the 5′-UTR of a gene encoding a nuclear-encoded DExH box RNA helicase (SUV3) support the importance of RNA metabolism during this transition. RNA helicases perform functions during RNA splicing, RNA transport, ribosome biogenesis, translation, and RNA decay (de la Cruz *et al.* 1998) and mutations in these genes cause specific developmental defects in plants (Jacobsen *et al.* 1999; Willmann 2001; Gong *et al.* 2002; Western *et al.* 2002). Calcium/calmodulin-dependent kinases play important roles in protein phosphorylation in eukaryotes (Hardie 1999). The disruption of *CRKC* in *pdd25* and of an unknown protein in *pdd12* supports a possible role for these proteins during pollen development, since the transcripts were detected in developing inflorescences. Although the insertions in *pdd8* and *pdd30* were associated with the presence of two unannotated small ORFs in their vicinity, we failed to detect those transcripts in mature pollen or in developing inflorescences. Therefore the function of these two ORFs in pollen development remains to be determined.

Mutants showing defects in pollen germination and tube growth: Upon maturation, pollen grains are released from anthers in a desiccated and quiescent state and then undergo a process of metabolic activation, which involves water uptake and reorganization of the plasma membrane, before germination and pollen tube growth. *In vivo*, pollen coat lipids perform critical functions during hydration (Preuss *et al.* 1993; Lush *et al.* 1998), although gametophytic factors are involved in controlling the initial stages of germination (Golovkin and Reddy 2003; Steinebrunner *et al.* 2003). The *pgd7* mutant might represent a new class of gametophytic factors required during rehydration after pollen grains are released from anthers. The insertion is located in the 3′-UTR of *At2g35300*, which encodes a LEA protein. LEA proteins contain hydrophilic side chains thought to interact with and protect hydrophilic proteins during desiccation by causing protein aggregation (Goyal *et al.* 2005). LEA protein expression is common in desiccated seeds, but decreases significantly upon germination (Hong-Bo *et al.* 2005). In addition to mRNAs (Mascarenhas 1990; Becker *et al.* 2003; Honys and Twell 2003), the mature pollen grain also stores a large number of proteins (Holmes-Davis *et al.* 2005). Given this association, the unique phenotype of *pgd7* might be caused by deregulated LEA protein expression that is essential to protect proteins during pollen desiccation.

Upon pollen hydration metabolic activation requires energy production and biosynthetic capacity. Such an energy requirement was demonstrated in the double knockout of two pollen-expressed apyrases, in which pollen germination was impaired (Steinebrunner *et al.* 2003). A mitochondrial adrenodoxin-like ferrodoxin (MFDX1; *pgd6*) involved in redox-dependent energy pathways as an electron carrier (Takubo *et al.* 2003) may perform a similar function during pollen germination.

After germination the pollen grain forms a tube that elongates at the apex. Targeted vesicle fusion and coordination of the actin cytoskeleton and signaling pathways are essential to establish and maintain pollen tube growth and apical polarity. Therefore, it is not surprising that some genes identified in the *tgd* mutant class, as previously reported for other mutants (Sanderfoot *et al.* 2001; Johnson *et al.* 2004), involve components of membrane trafficking machinery. For example, in *tgd8*, the correct delivery of cell wall materials at the pollen tube tip might be compromised by deregulation of a gene encoding a TRAPP component, a multisubunit vesicle tethering factor belonging to the Bet3 family, which is involved in ER-to-Golgi vesicle trafficking (Sacher *et al.* 1998). The *tgd19* phenotype might represent disruption of a signaling pathway required during pollen tube growth and guidance and pollen tube–embryo sac communication, but as *tgd19* is associated with a deletion, the phenotype might be additive from disruption of one or more pollen-expressed genes.

The maintenance of pollen tube polarity is controlled at different levels, as demonstrated by the *tgd1*, *tgd14*, and *tgd7* mutants. In *tgd14* and *tgd7* the insertions fall in intergenic regions and according to the Tiling array (Yamada *et al.* 2003) there are no predicted small secreted proteins or small RNAs in these regions. In *tgd7* the insertion is located between genes encoding a BAP28-related protein and a Rab GDP dissociation inhibitor with similarity to guanosine diphosphate dissociation inhibitor 1 (GDI1). The biological function for BAP28 has been studied only in vertebrates, where it is involved in ribosome biogenesis (Azuma *et al.* 2006), but roles for Rab GTPases in vesicle trafficking and actin cytoskeletal organization during pollen tube growth are established (Cole and Fowler 2006). Similarly for *tgd14* the insertion is between genes encoding a zinc finger family protein and a pollen-specific (Pina *et al.* 2005) ENTH domain protein, with possible functions in vesicle secretion. Defects in the regulation of pollen tube growth are associated with a small unannotated ORF encoding a pollen-specific secreted protein of 53 aa (*tgd6*) and with a glycogenin glycosyltransferase pseudogene (*tgd5*).

Mutants affected in pollen tube–embryo sac interactions and fertilization: The *pei* mutations define a new class of male gametophytic factors required for pollen tube–embryo sac interactions, likely affecting pollen tube reception and discharge of sperm cells or gamete fusion. Typically, female gametophytic mutations show 50% reduced seed set, but in the *pei* mutants seed set is variable, since each fertilization event is strictly dependent on the genotype of the pollen tube that first

reaches the ovule. In wild type, once entry to the ovule is achieved, pollen tube growth is arrested in a synergid cell, a process termed pollen tube reception (Huck *et al.* 2003). Signals from the embryo sac regulate this process and mutants were found in which pollen tube reception is disrupted, resulting in pollen tube overgrowth and lack of sperm discharge (Huck *et al.* 2003; Rotman *et al.* 2003). More recently a loss-of-function mutation, *abstinence by mutual consent* (*amc*) (Boisson-Dernier *et al.* 2008), described a similar phenotype, but in *amc* reception is impaired only when an *amc* pollen tube reaches an *amc* embryo sac. Another example is the *aca9* mutant, in which pollen tubes grow more slowly and also fail to discharge sperm cells into the embryo sac; however, *aca9* affects only the male gametophyte (Schiott *et al.* 2004). In the *pei1* and *pei2* mutants more than one pollen tube was observed in the micropyle of single ovules but those ovules remained unfertilized, indicating a defect either in pollen tube reception or in gamete fusion. Usually a single pollen tube grows toward an ovule. The limitation of one pollen tube per ovule might result from the termination of attraction signals emitted by the ovule once the embryo sac is fertilized (Higashiyama *et al.* 2001), or by the release of an inhibitory signal once a pollen tube is growing in the funiculus, or by a fertilized embryo sac (Shimizu and Okada 2000). This phenomenon is thought to prevent polyspermy (Shimizu and Okada 2000) and therefore to prevent the formation of polyploids with imbalanced paternal and maternal genomes. It is possible that in the *pei* mutants the mutant pollen tubes fail to discharge the sperm cells, allowing a second pollen tube to reach the ovule. However, in *pei2* this might not be true, and therefore this mutant is particularly interesting. In ovules targeted by two pollen tubes, we often observed the presence of two extra small nuclei inside the central cell, suggesting that sperm cells were released inside the embryo sac. If so, two hypotheses might explain this observation: both sperm cells fused with the central cell but failed to undergo karyogamy, or alternatively a mutant pollen tube discharged the sperm cells but the signal usually produced to prevent polyspermy failed to inhibit a second pollen tube from discharging sperm cells into the embryo sac. Interestingly the *pei2* insertion is located in an intergenic region, likely in the 3′-UTR of *TTL2*. Proteins containing tetratricopeptide repeat (TPR) motifs are known to mediate specific interactions with partner proteins, either forming active multiprotein complexes or acting as cochaperones involved in the folding of substrates (Blatch and Lassle 1999); therefore we suggest that this protein might function in assembly of proteins required for gamete fusion.

Chromosomal rearrangements in *Ds* insertion lines: Our results indicate that *Ds* insertion lines, even those that exhibit straightforward genetic behavior, may contain an unexpectedly high frequency of rearrangements. It is often the case that gametophytic mutations are not directly associated with the integration event, and chromosomal rearrangements are known to cause gamete lethality and reduced gametophytic transmission (Ray *et al.* 1997). Such chromosomal rearrangements can interfere with reverse genetic analyses and provide misleading information about the molecular basis of mutant phenotypes (Bonhomme *et al.* 1998). Moreover, the current location of a *Ds* element is not always what causes the mutant phenotype, as genomic footprints left in neighboring regions during the transposition event can underlie the phenotype (Escobar-Restrepo *et al.* 2007). Although *Ds* insertion lines in general contain a single and intact transposed element (Tissier *et al.* 1999; Ito *et al.* 2002), we identified 18 lines in which the mutant phenotypes were possibly caused by complex patterns of insertions or multiple insertions and not by a single disruption in a haploid-expressed gene (supplemental Table 1). In some of these lines the recovered flanking sequences were homologous to binary vector sequences, suggesting that the transposition event could have occurred within the donor T-DNA itself (Raina *et al.* 2002). These lines were therefore excluded from further analysis.

T-DNA integration has been associated with major chromosomal rearrangements such as small deletions, inversions, translocations, and duplications (Laufs *et al.* 1999; Forsbach *et al.* 2003), but, to date, few studies have reported such genomic rearrangements in *Ds* transposants in Arabidopsis (Oh *et al.* 2003; Page *et al.* 2004). In 9 of the 39 *Ds* insertion lines for which we recovered both flanking sequences, the 5′ and 3′ *Ds* flanking sequences did not map to the same chromosomal location, and the *Ds* flanks were separated by 26–525 kb, suggesting that the integration event was accompanied by a large deletion. Genomic deletions in *Ds* transposants are expected to occur at a low frequency (1%) and on average they are ~160 kb (Page *et al.* 2004). Our collection is admittedly a biased sample, but deletions appear to be rather frequent; these may have been inadvertently selected for when screening for gametophytic mutations with strongly aberrant transmission ratios. Although the deletion of several genes is expected to compromise plant viability, most mutations, even those presenting large deletions, such as *pdd13* (525 kb) and *pgd2* (200 kb) were still transmitted through one or both gametophytes, as reported for other known deletion mutants (Oh *et al.* 2003; Zhang and Peterson 2005; Ding *et al.* 2007). However, we cannot discard the possibility that other rearrangements, *e.g.*, inversions, or two linked *Ds* elements, contribute to this class. For several other lines the left and right borders of the *Ds* insertion were located on different chromosomes. It is known that reciprocal translocations can cause gametophytic lethality. Tetrad analysis allowed us to discard this type of aberration in 2 independent lines (SGT01710 and SGT03441) (supplemental Table 1), since in such cases equal proportions of tetrads with four viable pollen grains with balanced chromosomes and tetrads with four nonviable pollen

grains that carry chromosomal deficiencies would be produced (Ray *et al.* 1997). But other lines (*pdd14*, *pdd27*, *pdd29*, *pdd33*, *tgd4*, and *ppi1*) showed a stable pattern of abortion (2:2) or normal pollen morphology, although the flanking sequences mapped to different chromosomes. Internal chromosomal translocations caused by incomplete or aborted integration (Tax and Vernon 2001) might be associated with some of these *Ds* insertions. For example, in *tgd17* the TAIL–PCR recovered only one flanking sequence, mapping the *Ds* insertion to the *CUT1* gene. *CUT1* encodes a condensing enzyme required for elongation of C24 very-long-chain fatty acids and *cut1* mutants show waxless (*eceriferum*) stems and siliques as well as conditional male sterility (Millar *et al.* 1999). Since the *cut1* mutation is a sporophytic mutation and no apparent defects were reported in heterozygous plants, it is possible that in *tgd17* a chromosomal rearrangement caused the duplication of *CUT1* and that the gametophytic defect is due to disruption of a different gene, at the left border of the *Ds* insertion. While the occurrence of translocations has seldom been reported for *Ds* insertions, for these and for lines in which one flanking sequence was recovered, we cannot rule out that truncations of *Ds* elements might have occurred during the integration process.

Despite all these cases where the mutant phenotypes could not be directly associated with the disruption or alteration in expression of a particular gene, functions during male gametophyte development were associated with genes in which the encoded proteins are involved in diverse biological functions, such as cell wall secretion, RNA metabolism, protein regulation, stability, or assembly, energy metabolism, and signaling. Our results also indicate that several unannotated small proteins or regulatory molecules located in intergenic regions play roles during male gametophyte development and function.

We thank the members of our lab for helpful discussions and David Hantz and Julie Calfas for excellent greenhouse assistance. We also thank University of California (Berkeley) undergraduates Cynthia Foust, Hanjun Kim, Chris Leong, Raymond Lew, Doris Lo, Tanida Rojchanakasetchai, Zoya Derebenskiy, and Amy Hassenberg for technical assistance. This work was supported by National Science Foundation 2010 program grant no. 0313501.

LITERATURE CITED

Azuma, M., R. Toyama, E. Laver and I. B. Dawid, 2006 Perturbation of rRNA synthesis in the *bap28* mutation leads to apoptosis mediated by p53 in the zebrafish central nervous system. J. Biol. Chem. **281:** 13309–13316.

Becker, J. D., L. C. Boavida, J. Carneiro, M. Haury and J. A. Feijo, 2003 Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. Plant Physiol. **133:** 713–725.

Blatch, G. L., and M. Lassle, 1999 The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays **21:** 932–939.

Boavida, L. C., and S. McCormick, 2007 Temperature as a determinant factor for increased and reproducible in vitro pollen germination in *Arabidopsis thaliana*. Plant J. **52:** 570–582.

Boavida, L. C., A. M. Vieira, J. D. Becker and J. A. Feijo, 2005 Gametophyte interaction and sexual reproduction: how plants make a zygote. Int. J. Dev. Biol. **49:** 615–632.

Boisson-Dernier, A., S. Frietsch, T. H. Kim, M. B. Dizon and J. I. Schroeder, 2008 The peroxin loss-of-function mutation *abstinence by mutual consent* disrupts male-female gametophyte recognition. Curr. Biol. **18:** 63–68.

Bonhomme, S., C. Horlow, D. Vezon, S. de Laissardiere, A. Guyon *et al.*, 1998 T-DNA mediated disruption of essential gametophytic genes in *Arabidopsis* is unexpectedly rare and cannot be inferred from segregation distortion alone. Mol. Gen. Genet. **260:** 444–452.

Chen, Y. C., and S. McCormick, 1996 *sidecar pollen*, an Arabidopsis thaliana male gametophytic mutant with aberrant cell divisions during pollen development. Development **122:** 3243–3253.

Chen, Z., J. L. Tan, M. Ingouff, V. Sundaresan and F. Berger, 2008 *Chromatin assembly factor 1* regulates the cell cycle but not cell fate during male gametogenesis in *Arabidopsis thaliana*. Development **135:** 65–73.

Christensen, C. A., S. Subramanian and G. N. Drews, 1998 Identification of gametophytic mutations affecting female gametophyte development in *Arabidopsis*. Dev. Biol. **202:** 136–151.

Cole, R. A., and J. E. Fowler, 2006 Polarized growth: maintaining focus on the tip. Curr. Opin. Plant Biol. **9:** 579–588.

de la Cruz, J., D. Kressler, D. Tollervey and P. Linder, 1998 *Dob1p (Mtr4p)* is a putative ATP-dependent RNA helicase required for the 3′ end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. EMBO J. **17:** 1128–1140.

Ding, L., L. M. Fan and S. M. Assmann, 2007 *pgd1*, an *Arabidopsis thaliana* deletion mutant, is defective in pollen germination. Sex Plant Reprod. **20:** 137–149.

Dresselhaus, T., 2006 Cell-cell communication during double fertilization. Curr. Opin. Plant Biol. **9:** 41–47.

Drews, G. N., and R. Yadegari, 2002 Development and function of the angiosperm female gametophyte. Annu. Rev. Genet. **36:** 99–124.

Edwards, K., C. Johnstone and C. Thompson, 1991 A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349.

Escobar-Restrepo, J. M., N. Huck, S. Kessler, V. Gagliardini, J. Gheyselinck *et al.*, 2007 The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. Science **317:** 656–660.

Feldmann, K. A., D. A. Coury and M. L. Christianson, 1997 Exceptional segregation of a selectable marker (KanR) in Arabidopsis identifies genes important for gametophytic growth and development. Genetics **147:** 1411–1422.

Forsbach, A., D. Schubert, B. Lechtenberg, M. Gils and R. Schmidt, 2003 A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. Plant Mol. Biol. **52:** 161–176.

Golovkin, M., and A. S. Reddy, 2003 A calmodulin-binding protein from *Arabidopsis* has an essential role in pollen germination. Proc. Natl. Acad. Sci. USA **100:** 10558–10563.

Gong, Z., H. Lee, L. Xiong, A. Jagendorf, B. Stevenson *et al.*, 2002 RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. Proc. Natl. Acad. Sci. USA **99:** 11507–11512.

Goyal, K., L. J. Walton and A. Tunnacliffe, 2005 LEA proteins prevent protein aggregation due to water stress. Biochem. J. **388:** 151–157.

Guitton, A. E., and F. Berger, 2005 Loss of function of *MULTICOPY SUPPRESSOR OF IRA 1* produces nonviable parthenogenetic embryos in *Arabidopsis*. Curr. Biol. **15:** 750–754.

Hardie, D. G., 1999 Plant protein serine/threonine kinases: classification and functions. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50:** 97–131.

Heslop Harrison, J., Y. Heslop Harrison and K. R. Shivanna, 1984 The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. Theor. Appl. Genet. **67:** 367–375.

Higashiyama, T., S. Yabe, N. Sasaki, Y. Nishimura, S. Miyagishima *et al.*, 2001 Pollen tube attraction by the synergid cell. Science **293:** 1480–1483.

Holmes-Davis, R., C. K. Tanaka, W. H. Vensel, W. J. Hurkman and S. McCormick, 2005 Proteome mapping of mature pollen of *Arabidopsis thaliana*. Proteomics **5:** 4864–4884.

Hong-Bo, S., L. Zong-Suo and S. Ming-An, 2005 LEA proteins in higher plants: structure, function, gene expression and regulation. Colloids Surf. B Biointerfaces **45:** 131–135.

Honys, D., and D. Twell, 2003 Comparative analysis of the *Arabidopsis* pollen transcriptome. Plant Physiol. **132:** 640–652.

Honys, D., and D. Twell, 2004 Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. Genome Biol. **5:** R85.

Howden, R., S. K. Park, J. M. Moore, J. Orme, U. Grossniklaus *et al.*, 1998 Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in Arabidopsis. Genetics **149:** 621–631.

Huck, N., J. M. Moore, M. Federer and U. Grossniklaus, 2003 The *Arabidopsis* mutant *feronia* disrupts the female gametophytic control of pollen tube reception. Development **130:** 2149–2159.

Ito, T., R. Motohashi, T. Kuromori, S. Mizukado, T. Sakurai *et al.*, 2002 A new resource of locally transposed dissociation elements for screening gene-knockout lines in silico on the *Arabidopsis* genome. Plant Physiol. **129:** 1695–1699.

Jacobsen, S. E., M. P. Running and E. M. Meyerowitz, 1999 Disruption of an RNA helicase/RNAse III gene in *Arabidopsis* causes unregulated cell division in floral meristems. Development **126:** 5231–5243.

Johnson, M. A., K. von Besser, Q. Zhou, E. Smith, G. Aux *et al.*, 2004 Arabidopsis *hapless* mutations define essential gametophytic functions. Genetics **168:** 971–982.

Johnson, S. A., and S. McCormick, 2001 Pollen germinates precociously in the anthers of *raring-to-go*, an *Arabidopsis* gametophytic mutant. Plant Physiol. **126:** 685–695.

Johnson-Brousseau, S. A., and S. McCormick, 2004 A compendium of methods useful for characterizing *Arabidopsis* pollen mutants and gametophytically-expressed genes. Plant J. **39:** 761–775.

Johnston, A. J., P. Meier, J. Gheyselinck, S. E. Wuest, M. Federer *et al.*, 2007 Genetic subtraction profiling identifies genes essential for *Arabidopsis* reproduction and reveals interaction between the female gametophyte and the maternal sporophyte. Genome Biol. **8:** R204.

Lalanne, E., and D. Twell, 2002 Genetic control of male germ unit organization in *Arabidopsis*. Plant Physiol. **129:** 865–875.

Lalanne, E., C. Michaelidis, J. M. Moore, W. Gagliano, A. Johnson *et al.*, 2004 Analysis of transposon insertion mutants highlights the diversity of mechanisms underlying male progamic development in Arabidopsis. Genetics **167:** 1975–1986.

Laufs, P., D. Autran and J. Traas, 1999 A chromosomal paracentric inversion associated with T-DNA integration in *Arabidopsis*. Plant J. **18:** 131–139.

Liu, C. M., and D. W. Meinke, 1998 The *titan* mutants of *Arabidopsis* are disrupted in mitosis and cell cycle control during seed development. Plant J. **16:** 21–31.

Liu, Y. G., N. Mitsukawa, T. Oosumi and R. F. Whittier, 1995 Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J. **8:** 457–463.

Lush, W. M., F. Grieser and M. Wolters-Arts, 1998 Directional guidance of *Nicotiana alata* pollen tubes in vitro and on the stigma. Plant Physiol. **118:** 733–741.

Martin, F. W., 1959 Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol. **34:** 125–128.

Mascarenhas, J. P., 1990 Gene activity during pollen development. Annu. Rev. Plant Physiol. **41:** 317–331.

McCormick, S., 2004 Control of male gametophyte development. Plant Cell **16**(Suppl.): S142–S153.

Millar, A. A., S. Clemens, S. Zachgo, E. M. Giblin, D. C. Taylor *et al.*, 1999 *CUT1*, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell **11:** 825–838.

Murashige, T., and F. Skoog, 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant **15:** 473.

Murray, M. G., and W. F. Thompson, 1980 Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8:** 4321–4325.

Oh, S. A., S. K. Park, I. Jang, R. Howden, J. M. Moore *et al.*, 2003 *halfman*, an *Arabidopsis* male gametophytic mutant associated with a 150 kb chromosomal deletion adjacent to an introduced *Ds* transposable element. Sex. Plant Reprod. **16:** 99–102.

Page, D. R., C. Kohler, J. A. da Costa-Nunes, C. Baroux, J. M. Moore *et al.*, 2004 Intrachromosomal excision of a hybrid *Ds* element induces large genomic deletions in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **101:** 2969–2974.

Pagnussat, G. C., H. J. Yu, Q. A. Ngo, S. Rajani, S. Mayalagu *et al.*, 2005 Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. Development **132:** 603–614.

Park, S. K., R. Howden and D. Twell, 1998 The *Arabidopsis thaliana* gametophytic mutation *gemini pollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. Development **125:** 3789–3799.

Pina, C., F. Pinto, J. A. Feijo and J. D. Becker, 2005 Gene family analysis of the *Arabidopsis* pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant Physiol. **138:** 744–756.

Preuss, D., B. Lemieux, G. Yen and R. W. Davis, 1993 A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization. Genes Dev. **7:** 974–985.

Procissi, A., S. de Laissardiere, M. Ferault, D. Vezon, G. Pelletier *et al.*, 2001 Five gametophytic mutations affecting pollen development and pollen tube growth in *Arabidopsis thaliana*. Genetics **158:** 1773–1783.

Raina, S., R. Mahalingam, F. Chen and N. Fedoroff, 2002 A collection of sequenced and mapped *Ds* transposon insertion sites in *Arabidopsis thaliana*. Plant Mol. Biol. **50:** 93–110.

Ray, S. M., S. S. Park and A. Ray, 1997 Pollen tube guidance by the female gametophyte. Development **124:** 2489–2498.

Rotman, N., F. Rozier, L. Boavida, C. Dumas, F. Berger *et al.*, 2003 Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*. Curr. Biol. **13:** 432–436.

Rotman, N., A. Durbarry, A. Wardle, W. C. Yang, A. Chaboud *et al.*, 2005 A novel class of MYB factors controls sperm cell formation in plants. Curr. Biol. **15:** 244–248.

Sacher, M., Y. Jiang, J. Barrowman, A. Scarpa, J. Burston *et al.*, 1998 TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. EMBO J. **17:** 2494–2503.

Sanderfoot, A. A., M. Pilgrim, L. Adam and N. V. Raikhel, 2001 Disruption of individual members of *Arabidopsis* syntaxin gene families indicates each has essential functions. Plant Cell **13:** 659–666.

Schiott, M., S. M. Romanowsky, L. Baekgaard, M. K. Jakobsen, M. G. Palmgren *et al.*, 2004 A plant plasma membrane Ca2+ pump is required for normal pollen tube growth and fertilization. Proc. Natl. Acad. Sci. USA **101:** 9502–9507.

Shimizu, K. K., and K. Okada, 2000 Attractive and repulsive interactions between female and male gametophytes in *Arabidopsis* pollen tube guidance. Development **127:** 4511–4518.

Siddiqi, I., G. Ganesh, U. Grossniklaus and V. Subbiah, 2000 The *dyad* gene is required for progression through female meiosis in *Arabidopsis*. Development **127:** 197–207.

Siebert, P. D., A. Chenchik, D. E. Kellogg, K. A. Lukyanov and S. A. Lukyanov, 1995 An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res. **23:** 1087–1088.

Smyth, D. R., J. L. Bowman and E. M. Meyerowitz, 1990 Early flower development in *Arabidopsis*. Plant Cell **2:** 755–767.

Song, X. F., C. Y. Yang, J. Liu and W. C. Yang, 2006 RPA, a class II ARFGAP protein, activates ARF1 and U5 and plays a role in root hair development in Arabidopsis. Plant Physiol. **141:** 966–976.

Springer, P. S., 2000 Gene traps: tools for plant development and genomics. Plant Cell **12:** 1007–1020.

Steffen, J. G., I. H. Kang, J. Macfarlane and G. N. Drews, 2007 Identification of genes expressed in the *Arabidopsis* female gametophyte. Plant J. **51:** 281–292.

Steinebrunner, I., J. Wu, Y. Sun, A. Corbett and S. J. Roux, 2003 Disruption of apyrases inhibits pollen germination in *Arabidopsis*. Plant Physiol. **131:** 1638–1647.

Sundaresan, V., P. Springer, T. Volpe, S. Haward, J. D. Jones *et al.*, 1995 Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. Genes Dev. **9:** 1797–1810.

Takubo, K., T. Morikawa, Y. Nonaka, M. Mizutani, S. Takenaka *et al.*, 2003 Identification and molecular characterization of mitochondrial ferredoxins and ferredoxin reductase from *Arabidopsis*. Plant Mol. Biol. **52:** 817–830.

Tax, F. E., and D. M. Vernon, 2001 T-DNA-associated duplication/translocations in *Arabidopsis*. Implications for mutant analysis and functional genomics. Plant Physiol. **126:** 1527–1538.

Tissier, A. F., S. Marillonnet, V. Klimyuk, K. Patel, M. A. Torres *et al.*, 1999 Multiple independent defective suppressor-mutator transposon insertions in *Arabidopsis*: a tool for functional genomics. Plant Cell **11:** 1841–1852.

Vernoud, V., A. C. Horton, Z. Yang and E. Nielsen, 2003 Analysis of the small GTPase gene superfamily of *Arabidopsis*. Plant Physiol. **131:** 1191–1208.

Wang, Y., W. Z. Zhang, L. F. Song, J. J. Zou, Z. Su *et al.*, 2008 Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in *Arabidopsis*. Plant Physiol. **148:** 1201–1211.

Western, T. L., Y. Cheng, J. Liu and X. Chen, 2002 HUA ENHANCER2, a putative DExH-box RNA helicase, maintains homeotic B and C gene expression in *Arabidopsis*. Development **129:** 1569–1581.

Willmann, M. R., 2001 Unravelling PTGS: SDE3—an RNA helicase involved in RNA silencing in *Arabidopsis*. Trends Plant Sci. **6:** 344–345.

Yadegari, R., and G. N. Drews, 2004 Female gametophyte development. Plant Cell **16**(Suppl.): S133–S141.

Yamada, K., J. Lim, J. M. Dale, H. Chen, P. Shinn *et al.*, 2003 Empirical analysis of transcriptional activity in the *Arabidopsis* genome. Science **302:** 842–846.

Yu, H. J., P. Hogan and V. Sundaresan, 2005 Analysis of the female gametophyte transcriptome of *Arabidopsis* by comparative expression profiling. Plant Physiol. **139:** 1853–1869.

Zhang, J., and T. Peterson, 2005 A segmental deletion series generated by sister-chromatid transposition of *Ac* transposable elements in maize. Genetics **171:** 333–344.

Communicating editor: T. P. Brutnell