

## Five Gametophytic Mutations Affecting Pollen Development and Pollen Tube Growth in *Arabidopsis thaliana*

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### ABSTRACT

Mutant analysis represents one of the most reliable approaches to identifying genes involved in plant development. The screening of the Versailles collection of *Arabidopsis thaliana* T-DNA insertion transformants has allowed us to isolate different mutations affecting male gametophytic functions and viability. Among several mutated lines, five have been extensively studied at the genetic, molecular, and cytological levels. For each mutant, several generations of selfing and outcrossing have been carried out, leading to the conclusion that all these mutations are tagged and affect only the male gametophyte. However, only one out of the five mutations is completely penetrant. A variable number of T-DNA copies has integrated in the mutant lines, although all segregate at one mutated locus. Two mutants could be defined as "early mutants": the mutated genes are presumably expressed during pollen grain maturation and their alteration leads to the production of nonfunctional pollen grains. Two other mutants could be defined as "late mutant" since their pollen is able to germinate but pollen tube growth is highly disturbed. Screening for segregation ratio distortions followed by thorough genetic analysis proved to be a powerful tool for identifying gametophytic mutations of all phases of pollen development.

**P**OLLEN development can be considered as an ideal system not only for the analysis of important processes in plant reproduction but also for studying different aspects of plant biology such as cell fate determination, cellular differentiation, intercellular and intracellular signaling, and polar growth. Male sporogenesis begins with the division of a diploid sporophytic cell that gives rise to the tapetal initial and the sporogenous cell. The latter cell first undergoes several mitoses and then pollen mother cells undergo meiosis and mitosis (pollen mitosis I, PMI) to originate pollen grains containing two cells: a larger vegetative cell and a smaller generative cell. The generative cell undergoes another mitotic division (pollen mitosis II) to give rise to both sperm cells. The vegetative cell controls the further development of the pollen grain and the growth of the pollen tube in the style until the delivery of both sperm cell nuclei to the embryo sac (for reviews see McCORMICK 1993; BEDINGER *et al.* 1994; PREUSS 1995; FRANKLIN-TONG 1999). This complex sequence of events comprises incompletely understood phenomena such as the establishment of the cell polarity necessary for the asymmetric mitotic division (PMI), nuclear migration mediated

by components of the cytoskeleton, synthesis of the pollen tube cell wall, and the dynamic interaction between the pollen and the style leading to cell-cell recognition at fertilization.

The isolation of mutants for each phase of microgametogenesis is a fundamental step in dissecting this developmental program. Nevertheless, among reproductive mutants isolated so far, gametophytic mutations affecting genes active in pollen are rare (YANG and SUNDARESAN 2000). The analysis of several mutant collections highlights that gametophytic mutants are much less easy to score than sterile mutants, probably because they show no direct and/or easily observable mutant phenotype, and theoretically no homozygous plants for the mutation can be obtained. The screening of the Versailles collection of *Arabidopsis thaliana* T-DNA insertion transformants, which was focused on the isolation of mutations affecting gametophytic viability and function, has shown that only 0.2% of the transformants (8 out of 15,861 lines scored) presented putatively tagged defective male gametophyte mutations (BONHOMME *et al.* 1998b). A similar picture arises from the screening of other collections of T-DNA insertion mutants since HOWDEN *et al.* (1998) isolated only 1 line out of 1000 that could be considered as a fully defective male gametophytic mutant. FELDMANN *et al.* (1997) have obtained comparable results even if a higher percentage of fully male gametophyte-defective mutants (2 out of 142) was found.

Among the limited number of gametophytic mutants

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that have been identified, only a few have been characterized at the biochemical level (reviewed by TWELL 1994; TWELL and HOWDEN 1998; and see below) and no gene has been cloned so far. *Scp* (*sidecar pollen*) was the first male gametophytic mutant described in *A. thaliana* obtained from the screening of a mutant collection generated by fast neutron bombardment mutagenesis (CHEN and McCORMICK 1996). Mutated plants produce aborted pollen and a low percentage of pollen grains with an extra cell, which shows vegetative cell identity. So *scp* affects both pollen viability and the number of cell divisions. Interestingly, the expression and the gametophytic penetrance of *scp* varies in different genetic backgrounds (CHEN and McCORMICK 1996). In a program of transposon insertion mutagenesis in maize, Sari-Gorla and collaborators identified two male gametophytic mutants called *gametophytic male sterile-1* and *-2* (*gaMS-1* and *gaMS-2*, SARI-GORLA *et al.* 1996, 1997). Cytological characterization of the *gaMS-1* mutant shows that the mutated gene acts during or immediately after the first pollen mitosis (PMI) and leads to the production of nonfunctional pollen grains (SARI-GORLA *et al.* 1996). The *gaMS-2* mutant pollen grains show no differentiation between the vegetative and the generative nuclei. However, both nuclei undergo further division leading to an alteration in number and location of nuclei in mature pollen grains (SARI-GORLA *et al.* 1997). In the *Arabidopsis limpet pollen* (*lip*) mutant, the generative cell fails to migrate into the pollen grain after PMI such that the sperm cell remains against the pollen wall. As suggested by the authors, *lip* could encode a  $\beta$ (1-3)-glucanase required for degradation of the transient callose wall, which separates the vegetative and generative cell after PMI. Alternatively, *lip* may encode a factor involved in the presumptive cytoplasmic reorganization required for cell migration (HOWDEN *et al.* 1998). Another mutant, *gemini pollen 1*, is affected in pollen mitosis I and shows an incompletely penetrant phenotype resulting in equal, unequal, or partial divisions. *GEMINI POLLEN 1* is defined as a gametophytically expressed factor required for correct nuclear migration and spatial coordination of karyokinesis and cytokinesis at PMI (PARK *et al.* 1998).

Taken together, the data obtained from the analysis of these mutants identify some putative gene functions that are necessary during the early phases of microgametogenesis. These functions, if altered, affect the number of cell divisions and the differentiation of vegetative and generative nuclei. Until now, few mutations of gametophytically acting genes affecting pollen tube germination and growth have been found. Several complex cellular processes are involved in pollen tube growth including exocytosis, cytoplasmic streaming, and modifications of cytoskeletal organization (TAYLOR and HEPLER 1997; YANG 1998; FRANKLIN-TONG 1999). A male gametophytic mutant showing reduced or very slow pollen tube growth has been isolated from the Versailles *A. thaliana*

T-DNA transformants collection (*Ttd8*, BONHOMME *et al.* 1998a,b). *Ttd8* mutant plants produce half normal pollen and half pollen grains, which are unable to grow a tube *in vitro* or can produce only very short pollen tubes. A few pollen tubes are still able to reach and fertilize the ovules located close to the stigma, leading to an incomplete penetrance of the mutation (BONHOMME *et al.* 1998a,b). Using a segregation distortion assay based on the multimarker chromosome 1 in *Arabidopsis*, GRINI *et al.* (1999) have also isolated a male gametophytic line (*mad4*) in which only 51% of the pollen tubes are able to travel down the ovary (GRINI *et al.* 1999).

Finally, very little information concerning the interaction between pollen tubes and female tissues during fertilization is available, and no male gametophytic mutant affecting this process has been described so far.

In this article we present a comparative analysis of five male gametophytic *A. thaliana* mutants. These mutants have been isolated on the basis of the altered segregation for kanamycin resistance carried on the T-DNA insertion as reported in BONHOMME *et al.* (1998b). Genetic analysis together with molecular characterization of the mutant lines indicates that all mutations are presumably tagged. All mutants are defective male gametophytic mutants, although only one out of the five is fully penetrant. Cytological studies show that different steps of pollen development are affected, leading to new mutant phenotypes, in particular concerning pollen tube growth.

## MATERIALS AND METHODS

**Plant material and genetic analysis:** The *Ttd* (T-DNA transmission defect) lines of *A. thaliana* (L.) Heynh., geographic race Wassilevskija (Ws), were isolated from the Versailles collection of T-DNA insertion mutants produced by Agrobacterium-mediated transformation as described in BECHTOLD *et al.* (1993). Plants were transformed with a T-DNA construct (pGKB5 binary vector) that carries both *bar* and *nptII* selection markers (BOUCHEZ *et al.* 1993).

All five transformants (*Ttd34*, *-38*, *-40*, *-41*, and *-42*) were chosen because they showed a confirmed 1:1 segregation ratio for the kanamycin resistance character at T2 generation (BONHOMME *et al.* 1998b; our unpublished data). Their T-DNA transmission rate was then studied for three to five generations, in selfing and crosses, checking the segregation of Kan<sup>R</sup> vs. Kan<sup>S</sup> seedlings 10 days after sowing seeds *in vitro* on *A. thaliana* medium (ESTELLE and SOMERVILLE 1987) containing kanamycin (100 mg/liter). At each generation, 6–12 Kan<sup>R</sup> sister plants from each of the five *Ttd* lines were selfed and used in cross experiments independently; results have been pooled per generation in Tables 1 and 2. The progeny of one or two backcrossed plants was selected for constituting the next generation. Subsequent generations have been indicated as G1, G2, and so on. Wild-type plants (Ws) were used as pollinators when the *Ttd* lines were used as maternal parent after manual emasculation. A male-sterile (*ms*) mutant, obtained via chemical mutagenesis [ethyl methanesulfonate (EMS)] in the Columbia ecotype, was used as maternal parent when the *Ttd* lines were used as pollinator. The mutation in

the *ms* line prevents anthers from dehiscing and thus allows pollen grains to reach the stigma. The mutation has been mapped on chromosome 3 at 2 cM from the *nga162* marker (C. HORLOW, unpublished results).

**Seed set assay:** Green expanded siliques from selfed mutant and wild-type adult plants were collected. The longest mature siliques were cleared in 70% ethanol for 24 hr and then dissected to count the developing seeds under a binocular microscope. The average numbers of seeds were calculated out of five or more siliques from 10 or more plants of each *Ttd* line.

**Cytological studies:** Anthers were dissected under a binocular microscope and pollen was gently squashed in staining solution under a coverslip. Pollen grains were observed under a light microscope (Wild M420 microscope with Wild HPS 48/52 photoautomat and interchangeable 35-mm magazine with data back, Leitz) after Alexander's staining (ALEXANDER 1969) and under UV epi-illumination after 4',6-diamidino-2-phenylindole (DAPI) staining, following the procedure of PARK *et al.* (1998).

*In vitro* growth of pollen tubes was tested according to HODGKIN (1983). Germination and pollen tube growth was estimated for several hundred grains, 5 hr after placing the pollen on solid preheated (at 37°) medium at 26° in the shade. Observations were done under light microscope.

For confocal laser scanning microscopy, a glass slide was covered with a thin layer of solid pollen germination medium (HODGKIN 1983). Pollen was tapped onto the medium and left to germinate for 5 hr at 26° in the shade. Germinated pollen grains were incubated with 200  $\mu$ l of FM 1-43 (5  $\mu$ g/ml; Molecular Probes, Eugene, OR) and viewed using a Leica TCS-NT laser scanning microscope with an Argon/Krypton laser (Omnichrome, Chino, CA). FM 1-43 is a vital dye of membranes used in neurobiology (BETZ *et al.* 1992) that we successfully tested on plant cells (O. GRANDJEAN, unpublished results). The preparations were excited at 488 nm and a BP530/30 filter was used. Photomicrographs were taken on Elite Chrome 160T Kodak film. Pictures were processed using Adobe Photoshop 5.5 software.

**Molecular characterization:** For Southern blot analysis, DNA was extracted from plantlets or leaves as described in BOUCHEZ *et al.* (1996). DNA (1–3  $\mu$ g) was digested using *Eco*RI, *Eco*RV, or *Pst*I restriction endonucleases (Amersham, Buckinghamshire, UK). The fragments were separated on a 0.8% agarose gel in 1 $\times$  TBE buffer and blotted onto nylon membrane (Gene-screens+) as described in AUSUBEL *et al.* (1990) using 0.4 N NaOH as transfer solution.

Hybridization experiments were done in 0.5 M Na<sub>2</sub>HPO<sub>4</sub> pH 7.2, 7% SDS, and 1 mM EDTA at 65°. Washes were performed according to AUSUBEL *et al.* (1990). Probes were purified and labeled with <sup>32</sup>P using the Pharmacia (Piscataway, NJ) Oligo-labeling kit.

## RESULTS

**Genetic analysis of five male gametophytic mutant lines:** *Transmission of the kanamycin resistance character for several generations of selfing and crossing:* The screening of the Versailles collection of *A. thaliana* T-DNA insertion transformants identified eight putative gametophytic mutants (BONHOMME *et al.* 1998b). Two of these (*Ttd34* and *Ttd38*) and three new ones identified following the same protocol (*Ttd40*, *-41*, and *-42*) were selected for subsequent analysis. All five transformants showed a 1:1 segregation ratio for the kanamycin resistance character brought by the T-DNA in the first selfing progeny (see

BONHOMME *et al.* 1998b and data not shown). Such a transmission defect of the selection marker to the progeny indicates that a T-DNA sequence is presumably inserted in a gene essential for the formation of viable gametes. A detailed analysis of T-DNA transmission rate for the five mutant lines has been carried out for at least three generations.

At each generation, 6–12 sister plants from each *Ttd* line, hemizygous for the T-DNA, have been selfed and used as pollen donor or maternal parent in reciprocal crosses (Table 1). T-DNA transmission rate was estimated as Kan<sup>R</sup> plantlet frequency in the progeny. Table 1 shows that all *Ttd* lines, except *Ttd42*, produced ~50% Kan<sup>R</sup> plants at each selfing generation. These results confirmed the preliminary data (BONHOMME *et al.* 1998b and unpublished results) even though there was some variability of the values among sister plants and among different generations analyzed. Indeed, transmission rates in selfing sometimes went much beyond 50% (59% for *Ttd34* at the G4 generation and 57% for *Ttd40* at the G4 generation), but never indicated a normal transmission of the T-DNA. Self-progeny of hemizygous *Ttd42* plants contained 61% Kan<sup>R</sup> plants in the G2 generation instead of the theoretical value of 50%. This value was confirmed in the subsequent generation (62% Kan<sup>R</sup> in G3). However, crossing data still indicated a transmission defect in this line (see below).

When hemizygous mutant plants were used as male, the mean percentage of Kan<sup>R</sup> plantlets in the progeny ranged from 0% (*Ttd38* line, at any generation) to 9% (*Ttd41* line in G2). These Kan<sup>R</sup> progeny were unexpected in the hypothesis of no male transmission of the T-DNA. We named these plants escaped Kan<sup>R</sup> plants (BONHOMME *et al.* 1998b). The percentage of escaped Kan<sup>R</sup> plants was very stable in the *Ttd40* line, among sister plants (see lowest and highest transmission rate) and from one generation to the other (0.2 to 0.4%). However, the mean T-DNA transmission rate varied from one generation to the next in the *Ttd34* line (1% in G1 and G3, 0.3% in G4), in the *Ttd41* line (9% in G2 vs. 4% in G4 and 1% in G5), and in the *Ttd42* lines (1% in G2 and 3% in G3). Lowest and highest values of transmission rates reported in Table 1 indicate that the number of escaped Kan<sup>R</sup> plants scored in the progeny of each sister plant was much more variable in these three lines and could vary, for example, from 0 Kan<sup>R</sup> plantlets out of 200 sown seeds to 15 Kan<sup>R</sup> out of 200 seeds in the progeny of two *Ttd41* sister plants used to pollinate male-sterile plants. The occurrence of these escaped plants suggests that four out of the five *Ttd* lines (*Ttd34*, *-40*, *-41*, and *-42*) are characterized by an incomplete penetrance of the mutation at the gametophytic level. Only the *Ttd38* line showed absolutely no T-DNA transmission through the pollen and could be considered as a fully penetrant gametophytic mutant.

When hemizygous mutant plants were used as female, the T-DNA transmission rate was ~50% for all *Ttd* lines

**TABLE 1**  
**Stability of T-DNA transmission rate to the progeny for five putative male gametophytic mutants**

Line	T-DNA transmission (%)				
	G1	G2	G3	G4	G5
<i>Ttd34</i> selfed	<u>54</u> 42–61 (1220)	<u>49</u> 39–58 (667)	<u>52</u> 45–57 (2349)	<u>59</u> 52–70 (2288)	—
<i>Ttd34</i> × WT	<u>46</u> 42–50 (845)	—	<u>50</u> 44–56 (1790)	<u>50</u> 40–61 (283)	—
ms line × <i>Ttd34</i>	<u>1</u> 0–5 (1598)	—	<u>1</u> 0–5 (2392)	<u>0.3</u> 0–2 (793)	—
<i>Ttd38</i> selfed	<u>48</u> 42–60 (1140)	<u>48</u> 41–52 (2193)	—	<u>51</u> 39–67 (2588)	<u>48</u> 40–54 (1485)
<i>Ttd38</i> × WT	—	<u>49</u> 42–54 (1820)	<u>51</u> 37–59 (1526)	<u>48</u> 41–67 (1529)	—
ms line × <i>Ttd38</i>	—	<u>0</u> (1526)	—	<u>0</u> (1350)	—
<i>Ttd40</i> selfed	<u>49</u> 44–54 (1733)	<u>50</u> 42–65 (1199)	<u>49</u> 40–57 (2340)	<u>57</u> 53–60 (1711)	—
<i>Ttd40</i> × WT	<u>49</u> 38–55 (937)	—	<u>46</u> 36–61 (1383)	<u>57</u> 51–58 (135)	—
ms line × <i>Ttd40</i>	<u>0.4</u> 0–1 (1737)	—	<u>0.2</u> 0–0.5 (3029)	<u>0.3</u> 0–1 (979)	—
<i>Ttd41</i> selfed	<u>51</u> 45–58 (1169)	—	<u>55</u> 50–61 (795)	<u>54</u> 50–66 (1668)	<u>50</u> 42–53 (2293)
<i>Ttd41</i> × WT	—	<u>54</u> 41–69 (255)	<u>50</u> 45–52 (373)	<u>52</u> 45–60 (785)	<u>51</u> 46–55 (1285)
ms line × <i>Ttd41</i>	—	<u>9</u> 0–15 (742)	—	<u>4</u> 0–14 (1791)	<u>1</u> 0–5 (1961)
<i>Ttd42</i> selfed <sup>a</sup>	<u>54</u> 49–67 (2428)	<u>61</u> 57–67 (2700)	<u>62</u> 58–68 (2007)	—	—
<i>Ttd42</i> × WT	—	<u>49</u> 42–57 (1435)	<u>57</u> 50–75 (131)	—	—
ms line × <i>Ttd42</i>	—	<u>1</u> 0–2 (2916)	<u>3</u> 0–14 (825)	—	—

For each *Ttd* line, 6 to 12 sister plants have been analyzed in selfing and crossing at each generation. Percentage of T-DNA transmission has been calculated for each sister plant as  $100 \times \text{Kan}^R \times \text{Kan}^S / (\text{Kan}^R + \text{Kan}^S)$ . Mean values of these percentages are reported (underlined), as well as the lowest and highest values of transmission rate obtained. Total numbers of plantlets scored are indicated in parentheses. G, generation, see MATERIALS AND METHODS; ms, male sterile; —, not determined.

<sup>a</sup> For this line, plants homozygous for the T-DNA have been found in each selfing generation (see text).

(Table 1). Surprisingly, percentages sometimes exceeded 50% (57% in G4 for *Ttd40*, and 57% in G3 for *Ttd42*), but in these cases the effective number of counted plantlets was quite low (135 and 131, respectively). These results strongly suggest that the mutations act specifically in male gametophytes and do not compromise the function of the female reproductive structure. This was confirmed by counting seeds in the siliques after selfing of hemizygous plants. For all five lines, analysis of seed set showed no significant differences with wild type (not shown), indicating the absence of female gametophyte abortion despite the presence of the T-DNA insertion in the genome.

The use of a male-sterile line as maternal parent allowed a gain of considerable time in crossing experiments. However, because the ms line background (Columbia, Col0) was different from the *Ttd* lines background (Wassilevskija, Ws), each *Ttd* line has been used as pollinator of WT-Col0, WT-Ws, and ms line. Results reported in Table 2 confirmed the low transmission rate to the ms line for all five lines. No  $\text{Kan}^R$  progeny could be obtained when using the *Ttd38* line as male, and the T-DNA transmission rate remained <1% with *Ttd40* as pollinator, whatever the female plant. However, mean T-DNA transmission rates, which were equivalent when using WT-Ws or WT-Col0 as female, were clearly higher than the transmission rate to the ms line, particularly in *Ttd34*, *Ttd41*, and *Ttd42* lines. Mean T-DNA transmission ratio reached 16% in *Ttd42*, and even 28% for one

sister plant (48  $\text{Kan}^R$ :122  $\text{Kan}^S$ ). For all four incompletely penetrant mutations (*Ttd34*, -40, -41, and -42), variable transmission ratios were observed from one sister plant to the other, as already noted for Table 1 results.

*Occurrence of homozygous plants:* Plants homozygous for the mutations are unexpected when gametophytic mutations are fully penetrant. However, homozygous plants for the T-DNA have been found for two *Ttd* lines. Two homozygous *Ttd34* plants out of 23 were found in G4

**TABLE 2**  
**Comparison of T-DNA transmission rate (%) to wild-type and male-sterile lines**

Pollinator	Female lines		
	Ws	Col0	ms line
<i>Ttd34</i>	<u>2</u> 0–6 (552)	<u>2</u> 0–5 (563)	<u>0</u> (1021)
<i>Ttd38</i>	<u>0</u> (776)	<u>0</u> (1005)	<u>0</u> (925)
<i>Ttd40</i>	<u>0.5</u> 0–1 (848)	<u>0.6</u> 0–1 (894)	<u>0</u> (483)
<i>Ttd41</i>	<u>9</u> 4–20 (741)	<u>16</u> 1–22 (950)	<u>3</u> 1–4 (750)
<i>Ttd42</i>	<u>16</u> 5–28 (423)	<u>16</u> 10–20 (744)	<u>1</u> 0–3 (634)

For each *Ttd* line, four sister plants have been used as pollinators of WT plants (Ws and Col0 ecotypes) and male-sterile plants (ms, Columbia ecotype). Percentage of T-DNA transmission has been calculated for each sister plant as  $100 \times \text{Kan}^R / (\text{Kan}^R + \text{Kan}^S)$ . Mean values of these percentages are reported (underlined), as well as the lowest and highest values of transmission rate obtained. Total numbers of plantlets scored are indicated in parentheses.

self-generation, showing very short siliques but otherwise normal aspect of the sporophyte. A high frequency of homozygous *Ttd42* plants has been found in each generation: 3 out of 22 and 2 out of 12 plants, respectively, in G1 and G2. To obtain a more reliable figure for the proportion of homozygous plants in the case of the *Ttd42* line, the self-progeny of 95 individual sister plants originating from a hemizygous mother plant were analyzed (G3 self-generation). Eleven of the 95 (11.4%) were found to be homozygous for the T-DNA insertion. No phenotypic difference could be observed between hemizygous and homozygous *Ttd42* plants, except in the seed set. Siliques from homozygous *Ttd42* plants showed a significant proportion of aborted seeds: seed set was 37.5 on average *vs.* 51.5 in wild-type siliques and 51 in hemizygous *Ttd42*.

No homozygous plants were observed in *Ttd40* and *Ttd41* progeny, although the percentage of transmission through the pollen is not null in both these lines.

*Analysis of escaped kanamycin-resistant plants:* To determine how escaped Kan<sup>R</sup> plants transmitted the T-DNA to their progeny, several of these plants were analyzed in self-pollination (Figure 1). Variable transmission rates for different escaped plants were observed for each *Ttd* line, but figures obtained still reflected a transmission defect of the kanamycin resistance to the progeny. Con-

trary to selfing data presented in Table 1, the number of plantlets scored to determine these values were not pooled for sister plants and usually did not exceed 200. For *Ttd34*, *Ttd40*, and *Ttd41* lines, the majority of escaped plants showed a T-DNA transmission rate in selfing of between 40 and 60%, even when these escaped plants were quite numerous (*Ttd41*, G4 generation). The escaped plants originating from the *Ttd42* line showed a T-DNA transmission rate often slightly higher than 60% and thus very comparable to the selfing transmission ratio of mother plants.

**Molecular analysis of five gametophytic mutant lines:** In Southern blot experiments using as probe a fragment overlapping the T-DNA left (LB) or right (RB) border (Figure 2B), it is possible to determine the T-DNA insertion pattern of a mutant line, *i.e.*, the number of integrated T-DNA copies. An internal portion of the T-DNA was also used as probe (KAN probe in Figure 2B) to establish possible complex configurations.

Southern blot of the five mutant lines highlighted the insertion of a variable number of T-DNA copies (Figure 2C and Figure 3). Only two lines (*Ttd34* and *Ttd42*) contained a single copy of T-DNA, which is very likely a complete one, as all three probes gave one hybridization signal. Other lines showed complex arrays with multiple fragments. Three T-DNA copies would be

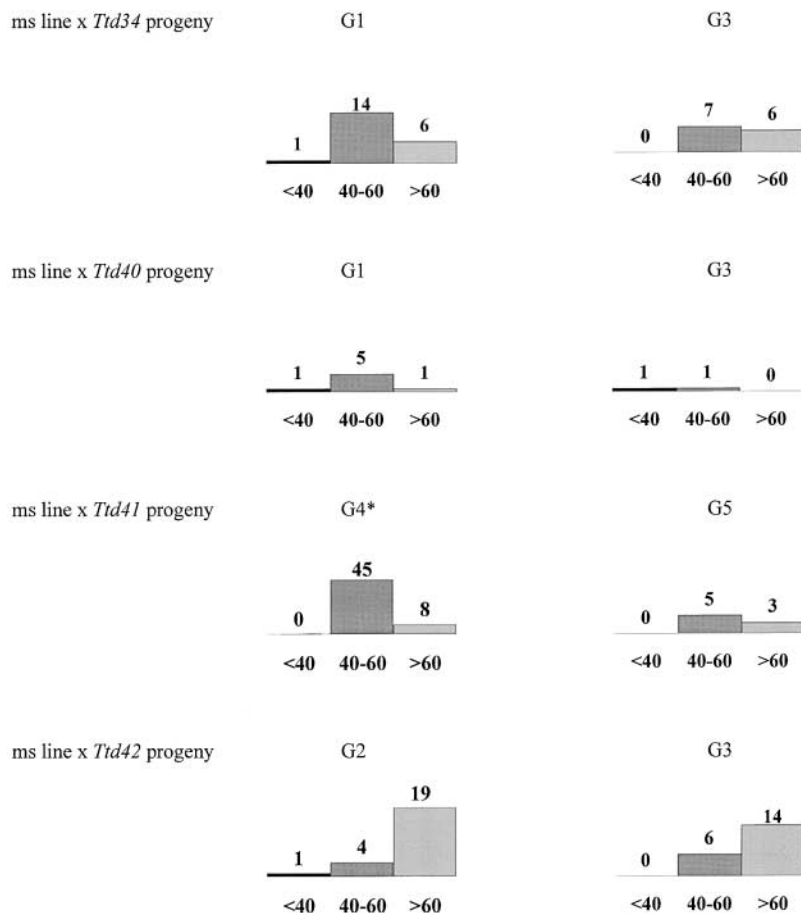
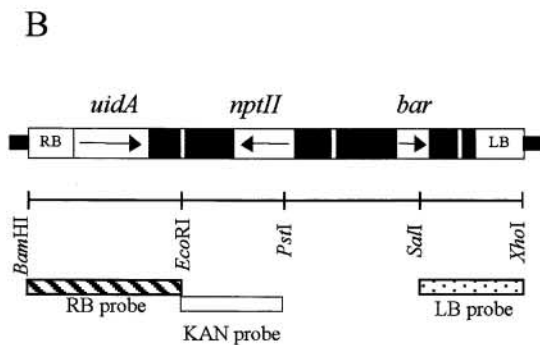
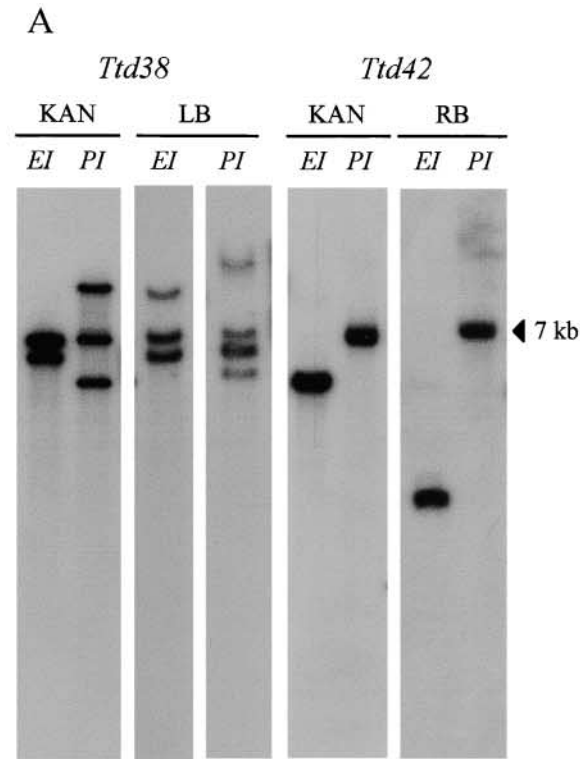


FIGURE 1.—T-DNA transmission to selfing progeny for escaped Kan<sup>R</sup> plants. The T-DNA transmission rate was calculated for escaped plants obtained at different generations, noted above the diagrams. Bar height is proportional to the number of escaped plants showing a T-DNA transmission rate in selfing lower than 40% (solid bars), between 40 and 60% (dark gray bars), or higher than 60% (light gray bars). The number of plants is reported above the bars. (\*) For the progeny of *Ttd41*, the scale is different from the others as the number of analyzed plants was considerably higher.

present in the *Ttd38* line according to RB and KAN probe hybridization results, and the pattern with LB probe even suggests the presence of an extra truncated

T-DNA copy (Figure 2, A and C). However, precise analysis of hybridization patterns leads to a more complex situation: the hybridization of a 7-kb fragment (size of the T-DNA) common to all unique-site-enzyme profiles with all three probes suggests the integration of two T-DNA copies organized in a direct tandem repeat (Figure 2A and BONHOMME *et al.* 1998b). A 12-kb *EcoRI* fragment and a 20-kb *PstI* fragment hybridize with both RB and LB probes and likely indicate another LB-RB junction involving truncated T-DNA copies as the KAN probe does not reveal these fragments (Figure 2A). Finally, integration of an extra copy, devoid of RB border, is suggested by a 6-kb *EcoRI* fragment highlighted by both LB and KAN probes. This complex pattern is conserved even after two backcrosses (Figure 2A and data not shown). The hybridization pattern is also complex for the *Ttd40* and *Ttd41* lines (Figure 2C and Figure 3). We observed, in the *Ttd40* line, two T-DNA hybridizing fragments with the RB probe (Figure 3) and three with both LB and KAN probes. Finally, for the *Ttd41* line, we observed two T-DNA hybridizing fragments after hybridization with the internal probe and four when the external probes were used (Figure 3). These results suggested the presence of complete and deleted T-DNA copies. No change was observed in the T-DNA insertion pattern of the *Ttd* lines after backcrosses or in some escaped plants chosen in each *Ttd* line progeny because their T-DNA transmission rate in selfing was close to 60% (Figure 3).

**Cytological characterization of gametophytic mutant phenotypes:** To analyze pollen viability, mature pollen grains from *Ttd* lines were stained with Alexander's solution (ALEXANDER 1969). Wild-type pollen grains appeared red and round in shape (BONHOMME *et al.* 1998a). In a hemizygous plant harboring a fully penetrant male gametophytic mutation, 50% of the pollen grains are expected to show a mutant phenotype, if any. No differences were seen between *Ttd34*, *Ttd41*, and



**C**

line	RB	KAN	LB
<i>Ttd34</i>	1	1	1
<i>Ttd38</i>	3	3	4
<i>Ttd40</i>	2	3	3
<i>Ttd41</i>	4	2	4
<i>Ttd42</i>	1	1	1

**FIGURE 2.**—T-DNA insertion pattern of *Ttd* lines. (A) Genomic DNA in *Ttd38* and *Ttd42* lines was digested with *EcoRI* (EI) and *PstI* (PI) and hybridized with probes shown in B. A first *Ttd38* line DNA extraction (at G1 generation) was used for hybridization with KAN probe and a second DNA extraction after two backcrosses (at G3 generation) was used for hybridization with LB probe. *Ttd42* DNA was extracted at the G2 generation and hybridized with KAN and RB probes. (B) T-DNA map showing the fragments used as probe in Southern blot experiments. The RB probe is a *BamHI-EcoRI* fragment. The LB probe is a *SalI-XhoI* fragment. The KAN probe is a *EcoRI-PstI* fragment. Restriction sites are unique, except for *BamHI*. (C) Genomic DNA has been restricted with *EcoRI*, *EcoRV*, and *PstI* and hybridized with probes shown in B. Numbers of T-DNA copies deduced from the number of signals obtained with different probes are indicated. Note that for *Ttd38*, hybridization with LB probe highlights four LB borders integrated when digesting with *EcoRV* or *PstI* enzymes and only three when restriction is made with *EcoRI* (see pattern in A).

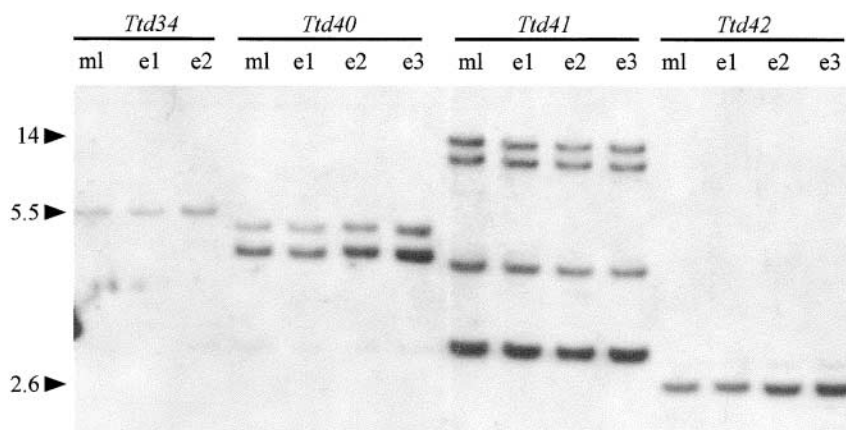


FIGURE 3.—T-DNA insertion pattern is similar in mutant lines and escaped plants. DNA was extracted from *Ttd* mutant lines (ml) and escaped progeny (noted e1 to e3) of these lines, which were chosen with particularly high T-DNA transmission rates in selfing. Genomic DNA was digested with *Eco*RI and hybridized with RB probe (see Figure 2B).

*Ttd42* and wild-type pollen grains (data not shown). For both *Ttd38* (Figure 4D) and *Ttd40* (Figure 4H) lines, half of the pollen grains showed practically no staining of the cytoplasm and only the wall was present, stained in green. Pollen development, from uninucleate microspore stage to mature grain stage, has been followed for both lines in epifluorescence microscopy, after staining with DAPI. In both lines, no abnormality was noted at the uninucleate microspore stage (Figure 4, A and E). At the binucleate stage, however, two populations could be distinguished: beside normal binucleate pollen, smaller grains were observed, either looking empty (Figure 4B) or containing what looked like a microspore-like nucleus (Figure 4F). In WT anthers, 81% of pollen grains were the same size and showed a clear vegetative diffuse nucleus and bright sperm nucleus at binucleate stage, while only 55 and 53% of the pollen population had reached this stage in *Ttd38* and *Ttd40*, respectively. At this stage, empty pollen grains were observed in WT and mutant lines at certain proportions (18% in WT, 31% in *Ttd38*, and 18% in *Ttd40*), but <1% of mononucleate microspores were observed in WT, while 14 and 29%, respectively, of microspores were still uninucleate in *Ttd38* and *Ttd40* lines [counting done on 240 (WT), 150 (*Ttd38*), and 550 (*Ttd40*) microspores/pollen grains]. At mature stage, in both *Ttd38* and *Ttd40* lines, half of the pollen grains had followed a normal development and showed three nuclei (two bright sperm nuclei and one more diffuse vegetative nucleus), and half of the pollen grains were smaller, with no visible nucleus, or sometimes diffusely stained (Figure 4, C and G), as if they had not properly undergone PMI. These aborted grains are likely T-DNA-carrying gametophytes that appeared collapsed after Alexander's staining. Fifty percent ( $n = 1165$ ) of *Ttd38* pollen grains and 49% ( $n = 1334$ ) of *Ttd40* pollen grains showed such an abnormal nuclear phenotype. These data are in agreement with results obtained from genetic analysis, showing for both these lines that the gametophytic mutation is completely or almost completely penetrant (0% of male transmission for the *Ttd38* line and 0.5%, on average, for the *Ttd40* line). As with Alexan-

der's staining, *Ttd34*, *Ttd41*, and *Ttd42* mutant lines produced pollen grains indistinguishable from wild type after DAPI staining (data not shown).

To test if pollen grains were still able to germinate and grow pollen tubes, *in vitro* pollen germination experiments were performed. Half of the *Ttd34* pollen tubes (Figure 5B) were shorter than wild-type ones (Figure 5A) and twisted, while half of the *Ttd42* pollen tubes (Figure 5E) showed a variable phenotype. Some pollen tubes were shorter than wild-type pollen tubes and some were twisted; it was possible to observe, in particular, some long tubes showing branched tips (see arrows, Figure 5E). A closer confocal laser scanning microscopy inspection after staining by FM 1-43 confirmed a strong alteration of pollen tube shape. In the *Ttd34* line, very short pollen tubes were observed, characterized by swelling and enlargements of the tube (Figure 5C). Some pollen tubes showed such a severe phenotype that it was impossible to recognize the tube tip and the growth direction. *Ttd42* pollen tubes (Figure 5F) were often as long as wild-type ones, but they showed more than one tip. Moreover, it was possible to note long tubes with big bulges along the shank of the tube. The majority of pollen tubes presented the mutant phenotype in homozygous *Ttd34* and *Ttd42* lines.

In *in vitro* experiments, 82% ( $n = 648$ ) of *Ttd41* (Figure 5D) pollen grains were able to germinate and pollen tubes were indistinguishable from wild-type ones (Figure 5A). Despite the absence of homozygous plants for this mutant line, which would make the observations easier, first *in vivo* pollination experiments have been performed, but no abnormalities have been highlighted so far (data not shown). Thus no mutant pollen phenotype has been observed at any stage for the *Ttd41* line.

## DISCUSSION

**All five gametophytic mutations studied are tagged and affect only male gametophytes, but only one is completely penetrant:** Our previous results (BONHOMME *et al.* 1998b) had shown that the segregation distortion criterion alone is not sufficient for isolating gameto-

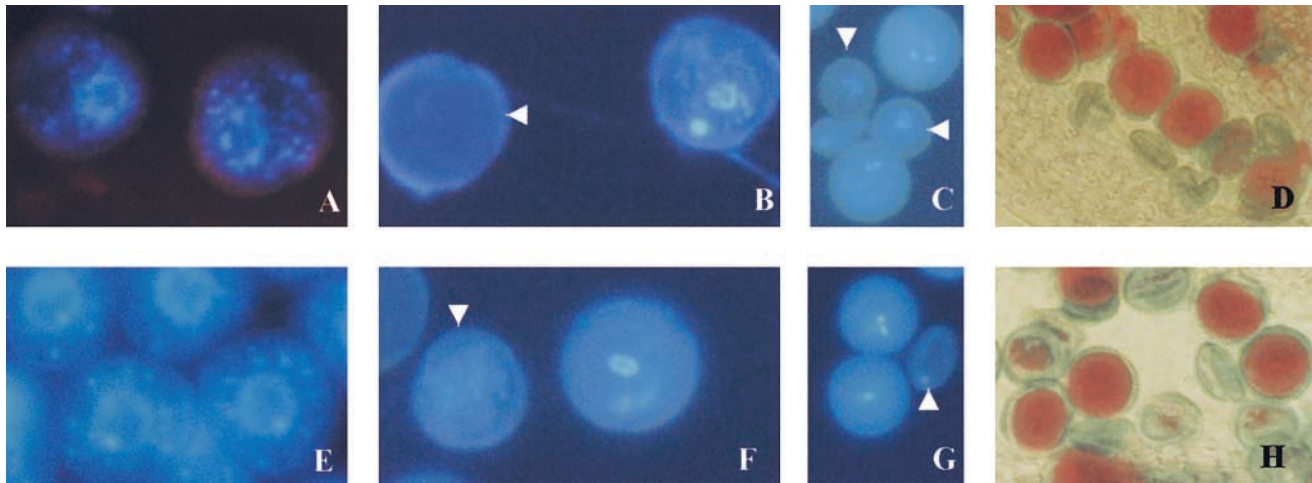


FIGURE 4.—Pollen phenotype of hemizygous *Ttd38* (A–D) and *Ttd40* (E–H) lines. (A–C and E–G) Microspore and pollen development analyzed following DAPI staining. All cells look normal and identical at the uninucleate microspore stage for both *Ttd38* (A) and *Ttd40* (E) lines. Later on, smaller grains showing no visible nuclei (white arrowhead in B, *Ttd38*) or unique diffuse nucleus (white arrowhead in F, *Ttd40*) are observed beside normal binucleate pollen grains. At mature stage, half of the grains are trinucleate and identical to wild type, while the other half are much smaller, collapsed, and sometimes showing traces of stained chromatin (white arrowheads in C and G). (D and H) Mature pollen stained with Alexander's solution. For both lines, half of the pollen is red and round in shape (wild-type phenotype) while the other half is stained green (pollen walls) and displays a squashed shape with sporadic red staining.

phytic mutations: other genetic events such as chromosomal mutations and/or rearrangements also lead to gametophyte abortion, and thorough analysis of T-DNA transmission to the progeny is necessary for confirming first observations. All mutants presented in this article are gametophytic in their mode of inheritance and action. The transmission defect of the T-DNA to the progeny has been observed and confirmed in each studied generation. In addition, for each *Ttd* line, the mutated phenotype could not be segregated from the T-DNA(s) after several rounds of selfing and backcrossing, indicating that the mutations are very likely tagged.

The analysis of several mutant collections highlights a strong background of low-penetrance mutations affecting the viability of both male and female gametophytes (FELDMANN *et al.* 1997; BONHOMME *et al.* 1998b; HOWDEN *et al.* 1998; GRINI *et al.* 1999). Even though in higher plants the developmental programs of both male

and female organs are substantially independent from each other, it has been proven that genes exist whose products could be specifically active in both (DROUAUD *et al.* 2000). However, all mutants analyzed in this work can be considered as only male defective gametophytic mutants, which lead to absence of T-DNA transmission through the male gametes and normal transmission through the female ones. This normal transmission through the embryo sac also argues for the fact that true gametophytic mutations were isolated, rather than chromosomal aberrations such as translocations or deletions.

Throughout the study of T-DNA transmission rate to the progeny by the *Ttd* lines, variations have been observed, from one generation to the next, and between sister plants. First, growth and crossing conditions might be different from one generation to the other, which can explain part of the variability observed. Second, the

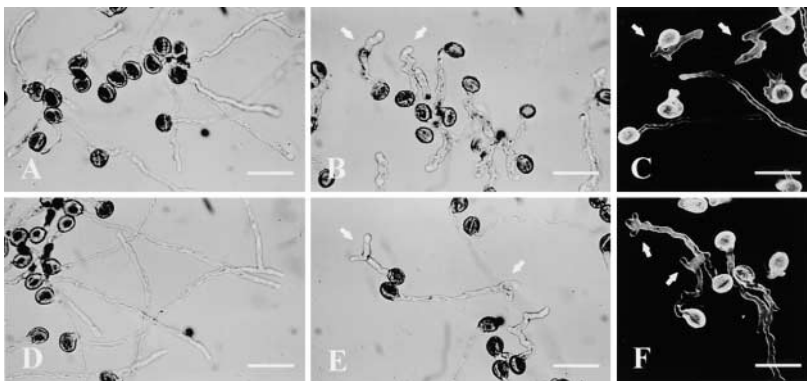


FIGURE 5.—*In vitro* pollen tube growth. For mutant lines, plants hemizygous for the T-DNA have been used. Half of the pollen is thus expected to show a mutant phenotype. (A) Wild-type pollen tube growth in which usually 85–90% of the pollen grains germinate. (B) *Ttd34* pollen tubes are shorter and twisted (indicated by white arrows). (D) *Ttd41* pollen tubes are indistinguishable from wild-type ones. (E) *Ttd42* pollen tubes are branched at the tip (indicated by white arrows). Confocal laser scanning microscopy of *Ttd34* (C) and *Ttd42* (F) pollen tubes after staining with FM1-43 (see MATERIALS AND METHODS). Mutant pollen tubes are indicated by white arrows. Bar, 25  $\mu$ m.



comparison of T-DNA transmission rate through the male to two different WT ecotypes and to the *ms* line used in this work suggests that the *ms* mutation in some way interferes with pollen viability/pollen tube growth because the transmission rates to this line are much lower than to WT emasculated plants. Alternatively, one can imagine a kind of positive effect of WT flower bud emasculating, which has to be done early prior to anthesis. Pollen grains deposited on emasculated flowers might encounter slightly different conditions (linked to different timing) compared to pollen grains deposited on an *ms* line stigma. This effect is much stronger for lines carrying pollen tube defects than for “early” mutations. In this comparison, as well as in the whole analysis of T-DNA transmission rate, variations observed underline the strong influence of a number of endogenous and exogenous factors on pollination success.

Only one out of the five selected *Ttd* lines (*Ttd38*) showed a total lack of T-DNA transmission through the pollen and thus could be considered as a fully defective gametophytic mutant. Conversely, all other mutations analyzed in this work and many others obtained from different *A. thaliana* populations are not completely penetrant (FELDMANN *et al.* 1997; BONHOMME *et al.* 1998b; HOWDEN *et al.* 1998). To explain incomplete penetrance, we can hypothesize that the mutant phenotype is expressed strongly in most of the pollen grains but weakly in some. It is possible to assume that in all four lines the affected genes are not “essential” genes. This observation reinforces the picture proposed by BONHOMME *et al.* (1998b) that essential genes could be relatively rare if compared to the number of pollen expressed genes and that genes gametophytically expressed might be preferentially duplicated. Recent studies have shown that large portions of the Arabidopsis genome are duplicated and reshuffled (BLANC *et al.* 2000; ARABIDOPSIS GENOME INITIATIVE 2000). Incomplete penetrance is also easier to understand for the three lines *Ttd34*, *-41*, and *-42*, carrying late mutations, than for early mutations. We can underline that many more escaped Kan<sup>R</sup> plants were obtained for the *Ttd41* line, the mutation of which acts the latest, probably during interaction between pollen tube and female tissues. Variable segregation ratio has been observed when escaped Kan<sup>R</sup> plants have been analyzed in selfing. This result is expected for *Ttd42*, since it is equivalent to the ratio obtained in selfing of the mother plant. For the three other lines, the ratios obtained, although higher than expected (*i.e.*, higher than the percentage obtained when selfing the mother plants), still indicate that these escaped plants present a transmission defect and do not reveal any change in the state of the mutation.

**Isolation of early and late gametophytic mutants:** Two out of the five mutants (*Ttd38* line and *Ttd40* line) showed nonfunctional pollen grains in their anther. As we suppose that dead pollen grains correspond to

T-DNA-carrying gametophytes, both these mutants could be defined as “early mutants” of the pollen development program. Because different gametophytic mutations cannot be compared to each other by an allelism test, at the present time we cannot rule out the possibility that both these mutants, showing a similar phenotype, could be allelic. Microspore and pollen development observations in *Ttd38* and *Ttd40* lines suggest that putatively tagged genes act at PMI. Collapsed phenotype of mature pollen in both *Ttd* lines distinguish these mutations from *scp*, *lip*, or *gem1* Arabidopsis mutations, where several classes of pollen phenotypes were described at mature stage (CHEN and McCORMICK 1996; HOWDEN *et al.* 1998; PARK *et al.* 1998). However, also for *Ttd* lines, it is difficult to recognize a single mutant phenotype at binucleate stage (presence of empty and mononucleate cells), when mutations are supposed to interfere with gametogenesis.

Until now most early gametophytic mutations studied showed a low degree of penetrance at the gametophytic level, while both *Ttd* lines were fully (*Ttd38*) or almost fully (*Ttd40*) penetrant. When a heterozygous *scp* plant was used as a male, the transmission of *scp* relative to the wild type was only 37%, and homozygous *scp* plants could be obtained, which produced 20% normal pollen (CHEN and McCORMICK 1996). Similarly, homozygous *geminipollen 1* mutants showed only 40% of aberrant pollen (PARK *et al.* 1998), and 25% of the *limpet pollen* mutant showed abnormal phenotype (HOWDEN *et al.* 1998). T-DNA transmission rates through the female gametophyte indicate that *Ttd38* and *Ttd40* mutants are only male gametophytic. The female transmission rate of *gem 1* mutation shows that GEM 1 action is not restricted to the male gametophyte and may act during megagametophyte development and/or function (PARK *et al.* 1998). Similarly, GRINI *et al.* (1999) isolated three lines showing variable defects in both male and female gametophytic development. Several mutants display a very high variability and a broad range of different mutant phenotypes (CHEN and McCORMICK 1996; PARK *et al.* 1998). These pleiotropic mutation effects make it difficult to distinguish between primary and secondary effects and to identify a specific gene function. *Ttd38* and *Ttd40* genes could also affect plant tissues other than pollen, but this could not be verified because homozygous plants have not been found. Therefore, the isolation of the mutated gene will be the only way to provide additional insights into their possible functional role at both gametophytic and sporophytic levels.

In this work we also describe the isolation of three male gametophytic mutants, which could be defined as “late mutant.” All three mutations, *Ttd34*, *Ttd41*, and *Ttd42*, concern only the male gametophyte although not completely penetrant. The affected genes clearly act after pollen maturation, two out of three during pollen tube growth. *In vitro* pollen tube growth experiments showed that *Ttd34* and *Ttd42* pollen grains are

able to germinate and that the mutations interfere later with pollen tube elongation. Only two mutants affecting pollen tube development have been described in *A. thaliana* (BONHOMME *et al.* 1998a; GRINI *et al.* 1999). Both these mutant lines also show altered transmission of the mutation via the male gamete only and incomplete penetrance. Pollen tubes, although delayed relative to wild type or arrested shortly after germination, can reach and fertilize the ovules located close to the stigma. This must also occur in *Ttd34* and *Ttd42* lines for which homozygous plants have been obtained. However, *Ttd34* and *Ttd42* lines show new phenotypes of twisted and branched pollen tubes, characterized by a severe alteration of the growth direction.

Both *Ttd34* and *Ttd42* homozygous progeny do not show any visible alteration of the sporophyte (except the difference in seed set between homozygous and hemizygous plants), which is expected since male fertility of homozygous plants is very low. Even without competition with wild-type pollen tubes, only few ovules can be fertilized by mutated pollen tubes. Nevertheless, we do not know if these alleles are null alleles. So the possibility that the genes have a sporophytic or megagametophytic function that would not be impaired in the *Ttd* lines cannot be ruled out.

The *Ttd41* line shows no obvious abnormalities because the majority of its pollen is viable and able to germinate *in vitro* in a way similar to wild type. We can suppose that the mutated gene acts after the completion of the pollen development program, probably during the interaction between pollen tube and pistil tissues at pollination. The successful germination and tube growth of compatible pollen is a complex phenomenon, requiring cell-cell recognition, interactions, and very likely the production of diffusible molecules by pollen and/or transmitting female tissues (HÜLSKAMP *et al.* 1995a,b; PREUSS 1995). At this stage of the analysis, it is difficult to hypothesize which aspect of these processes could be impaired in the *Ttd41* line. In addition to *in vivo* studies, the scoring of homozygous plants would be a fundamental step toward understanding the action of the mutated gene and examining the possibility that the mutation can produce any sporophytic effect.

**New genes and new functions in pollen development:** Molecular analysis of mutant lines using T-DNA fragments as probes shows that only two lines (*Ttd34* and *Ttd42*) out of the five carry a single complete T-DNA copy. Our data are in agreement with recent results, obtained during the analysis of several T-DNA insertion transformant collections (FELDMANN *et al.* 1997; BONHOMME *et al.* 1998b; HOWDEN *et al.* 1998), demonstrating that a high number of lines is characterized by a complex pattern of integration. In our case, even if three lines carry more than one T-DNA integration and probably truncations of T-DNA sequence, all T-DNA copies behave like a mutation at one locus. Moreover, escaped Kan<sup>R</sup> plants conserve the same T-DNA integration pat-

terns. Isolation of the sequences flanking the left and right T-DNA borders is in progress. This will be the beginning of the complete characterization of the disrupted sequences and the isolation of the tagged genes.

The pollen tube is characterized by a polar growth confined to a specific domain of the cell, called "tip growth," a well-known mode of plant cell elongation whereby new cell growth is limited to a single point end and leads to the formation of a tubular-shaped cell (YANG 1998; FRANKLIN-TONG 1999). The discovery of two mutants able to produce abnormal pollen tubes (*Ttd34* and *Ttd42*) and carry one T-DNA copy only should allow the isolation and study of new genes likely involved in pollen tip growth for which very few mutants are known so far.

The analysis through several generations of male gametophytic mutant lines generated by insertional mutagenesis is an efficient way to identify, isolate, and characterize genes involved in microgametogenesis. Moreover, this analysis is a fundamental tool in dissecting not only the pollen development program but also in analyzing other aspects of plant reproduction such as pollen-pistil interaction and fertilization. New genes, undiscovered by other strategies, will be isolated and are expected to complete the whole gametogenesis study.

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