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Pollen Tube and Root-Hair Tip Growth Is Disrupted in a Mutant of *Arabidopsis thaliana*[1]

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The expansion of both root hairs and pollen tubes occurs by a process known as tip growth. In this report, an *Arabidopsis thaliana* mutant (*tip1*) is described that displays defects in both root-hair and pollen-tube growth. The root hairs of the *tip1* mutant plants are shorter than those of the wild-type plants and branched at their base. The *tip1* pollen-tube growth defect was identified by the aberrant segregation ratio of phenotypically normal to mutant seeds in siliques from self-pollinated, heterozygous plants. Homozygous mutant seeds are not randomly distributed in the siliques, comprising only 14.4% of the total seeds, 5.3% of the seeds from the bottom half, and 2.2% of the seeds from the bottom quarter of the heterozygous siliques. Studies of pollen-tube growth in vivo showed that mutant pollen tubes grow more slowly than wild-type pollen through the transmitting tissue of wild-type flowers. Co-segregation studies indicate that the root-hair and pollen-tube defects are caused by the same genetic lesion. Based on these findings, the *TIP1* gene is likely to encode a product involved in a fundamental aspect of tip growth in plant cells.

The morphogenesis of plant cells is largely dependent upon the proper control of the direction and extent of cellular expansion. A well-characterized mode of plant cell expansion is tip growth, whereby new cell growth is limited to a single growing point and leads to the formation of a tubular-shaped cell. Two cell types that are known to display tip growth are pollen tubes and root hairs (Schnepf, 1986; Steer and Steer, 1989; Heath, 1990).

The polarized growth of root hairs and pollen tubes is similar in several respects. Ultrastructural studies have revealed a characteristic organization of the cytoplasmic contents in these cells, with exocytotic Golgi vesicles and ER elements at the apex, the larger organelles (Golgi bodies, ER, and mitochondria) in the subapical zone, and the enlarging central vacuole at the proximal part of the cells (Schnepf, 1986). Also, the growth of each cell is associated with, and dependent upon, a continuous influx of Ca^{2+} at the apex and an internal Ca^{2+} gradient (Reiss and Herth, 1979; Clarkson et al., 1988; Schiefelbein et al., 1992). Furthermore, normal root-hair and pollen-tube morphogenesis requires a functional actin cytoskeleton (Heath, 1990).

Because of the similarity in the growth of root hairs and pollen tubes, it is possible that genes exist whose products are required for the expansion of both cell types. Numerous studies have demonstrated that a significant proportion of the genes expressed in pollen are also expressed in vegetative tissue (Ottaviano and Mulcahy, 1989). Using isozyme analyses (Tanksley et al., 1981; Sari Gorla et al., 1986) and RNA hybridization studies (Willing et al., 1984; Willing and Mascarenhas, 1984), the degree of overlap between gametophytic and sporophytic gene expression has been estimated at 40 to 70%. Although these studies provide a general understanding of the level of genetic overlap in the sporophytic and gametophytic phases of plant development, they do not provide detailed information on the specific sporophytic cells or tissues where the pollen genes are expressed. In particular, there has been no report of the identification or characterization of genes expressed in both pollen tubes and root hairs.

One strategy to identify genes essential for the tip-growth process is to identify mutations that simultaneously alter growth of both root-hair and pollen-tube cells. A large collection of root-hair morphology mutants in *Arabidopsis thaliana* has previously been generated and partially characterized (Schiefelbein and Somerville, 1990). In this report a mutant that displays defects in both root-hair formation and pollen-tube growth is described from this collection. The product of the affected gene is suggested to play an important role in tip growth in each cell type.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The mutant lines of *Arabidopsis thaliana* (L.) Heynh. described in this report are derived from the Columbia ecotype. The ethyl methanesulfonate mutagenesis that generated the *tip1* mutant has been described (Estelle and Somerville, 1987; Schiefelbein and Somerville, 1990). All comparative studies were conducted with plants that had been backcrossed to the Columbia wild type for at least two generations.

For growth of plants in Petri dishes, seeds were surface-sterilized, placed on the surface of agarose-solidified medium, chilled at 4°C, and incubated in a vertical orientation as previously described (Schiefelbein and Somerville, 1990). Root-hair phenotypes were scored by examining the seedling roots after 4 to 6 d of growth under these conditions. The growth of *Arabidopsis* plants in soil has been described (Schiefelbein and Somerville, 1990).

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Analysis of Seeds from Heterozygous Siliques

Plants heterozygous for a particular root-hair mutation were allowed to self-pollinate. Siliques at a mature stage of development, but prior to dehiscence, were removed from plants. Using a razor blade, the siliques were cut horizontally at the approximate center to generate a top half and bottom half. The number of phenotypically normal and mutant seeds from these two portions of the siliques was determined by plating seeds on agarose-solidified medium and observing the root-hair phenotype after 4 to 6 d of growth. Seeds from 12 to 20 siliques were tested from each plant.

Pollen Assays

Anthers were removed from wild-type or mutant plants and used to transfer pollen to the stigma of wild-type flowers. The stigma surface was completely saturated with pollen. At 4, 5, or 6.5 h after pollination, the stigmas were detached and, to stop pollen-tube growth, were either plunged into liquid nitrogen for 1 min or immersed overnight in 70% ethanol. Stigmas were then placed in a drop of 0.05% (w/v) water-soluble aniline blue (Polysciences, Warrington, PA) in 50 mM sodium phosphate buffer (pH 8.5). Samples were squashed prior to mounting on glass slides and observing with a Leitz Orthoplan microscope equipped with a Ploempak 2.1 fluorescence vertical illuminator, HBO 200 mercury burner, and Neofluor objectives. Photomicrographs were recorded on Kodak Ektachrome tungsten slide film at ASA 160, or Kodak TMAX at ASA 400 for black and white prints.

For the in vitro pollen-tube growth assays, pollen was transferred from anthers to stigmas and then applied to the surface of a slide covered with the following pollen growth medium: 15% (w/v) Suc, 0.4 mM $Ca(NO_3)_2$, 0.4 mM H_3BO_3, 1% (w/v) agarose (Carpenter et al., 1992). The stigma was placed within 5 mm of the pollen grains, approximately 5 μL of the pollen growth medium (without agarose) was applied over the pollen and stigma tissue, and the slide was placed in a humid chamber. Pollen germination and tube growth was observed after 2 to 6 h of incubation.

Co-Segregation Test

Selfed (F_3) seed was collected from 60 individual F_2 plants (without sacrificing the entire plant) that were derived from a cross between a homozygous *tip1* mutant plant and a wild-type plant. The seedlings from these seeds were examined for their root-hair phenotypes. The F_3 seed lots that gave rise to segregating populations identified F_2 plants heterozygous for the root-hair defect. Four siliques were removed from each of these heterozygous plants and analyzed in the top half/bottom half silique test described above.

Morphological Analyses

The roots of wild-type and mutant seedlings were examined with a Wild M420 Makroskop and photomicrographs were recorded on Kodak TMAX ASA 100 black and white print film. For scanning EM, pieces of agarose bearing 5-d-old seedlings were removed from Petri plates, moistened, and immediately transferred to the specimen chamber of an environmental scanning electron microscope (Electroscan model E3) and examined.

The primary root measurements were made on 5-d-old seedlings grown in Petri dishes on agarose-solidified medium as described above. Epidermal cell length was determined from mature root epidermal cells that lacked root hairs and was visualized under a Leitz Laborlux S microscope.

RESULTS

Isolation of the *tip1* Mutant

Fifteen root-hair mutants generated by ethyl methanesulfonate treatment (Schiefelbein and Somerville, 1990) were crossed with wild-type plants and analyzed in the F_1 and F_2 for the segregation ratios. One root-hair mutant line (designated *tip1*) was selected for detailed study because it displayed an aberrant segregation ratio in the F_2, indicating a possible defect in gametophyte function (described below).

The *tip1* mutant plants produce root hairs that are approximately one-tenth the length of wild-type hairs (Fig. 1). The morphology of the mutant root hairs is characterized by an unusual branching pattern, with two or three branches attached to a central stalk (Fig. 2). The mutant root hairs form at the apical end of the epidermal cell (data not shown), the position where root hairs normally emerge in wild-type plants (Schiefelbein and Somerville, 1990). The phenotype of the mutant hairs indicates that the *tip1* mutation causes a disruption in root-hair elongation (tip growth), which generates the shorter, branched hairs.

To determine whether the *tip1* mutation affects one of the four root-hair loci previously characterized (*RHD1*, *RHD2*, *RHD3*, or *RHD4*; Schiefelbein and Somerville, 1990), comple-

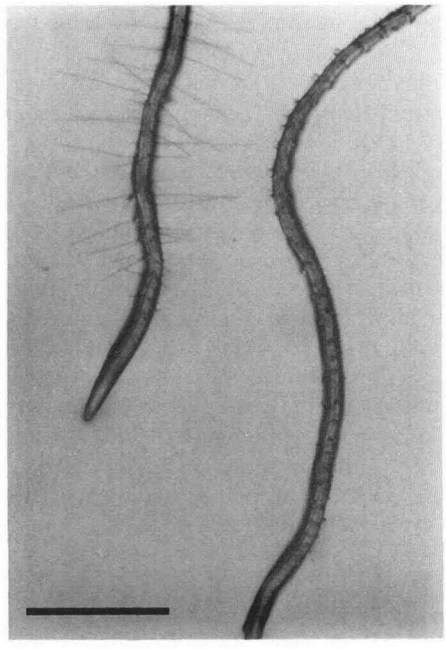

Figure 1. Comparison of wild-type (left) and *tip1* mutant (right) roots growing on the surface of an agarose-solidified nutrient medium. Bar = 1 mm.

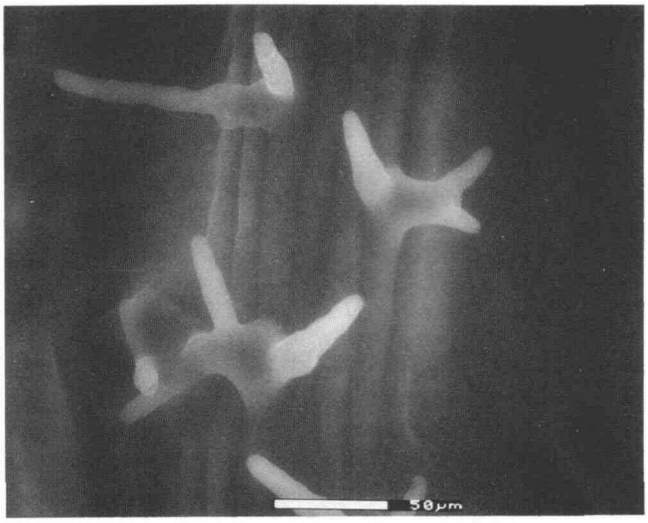

Figure 2. Morphology of *tip1* mutant root hairs. Morphological details of untreated *tip1* mutant root hairs visualized by environmental scanning EM. Note the multiple branches that arise from a common site. Bar = 50 μm.

mentation tests were performed. The homozygous *tip1* mutant plants were cross-pollinated with each of the other mutants and the F_1 plants were examined for their root-hair phenotype. In each instance, the F_1 plants produced hairs indistinguishable from the wild type, indicating that the *tip1* mutation resides in a complementation group different from *rhd1-rhd4*.

When the *tip1* mutant was crossed to the wild type, the F_1 plants possessed normal root hairs, indicating that the mutation is recessive. The F_2 progeny displayed a significant deviation from the expected 3:1 ratio of phenotypically normal to mutant plants for a recessive mutation (125 wild type:21 mutant, $\chi^2 = 8.78$, $P < 0.01$). The deviation indicates a deficiency in the number of homozygous *tip1* mutant seeds.

Distribution of Normal and Mutant Seeds in Heterozygous Siliques

The deficiency in the number of *tip1* mutants in the F_2 population could be due to any number of factors, including an inability of mutant embryos to develop normally or an inability of gametes bearing mutant alleles to function properly. To distinguish between the various possibilities, the distribution of phenotypically normal and mutant seeds within the siliques of heterozygous plants was examined. The general approach employed here has been described in detail by Meinke (1982). In essence, a nonrandom distribution of mutant seeds along the length of heterozygous siliques indicates that either a nonrandom distribution of mutant ovules exists within the silique or there is delayed germination or abnormal growth of mutant pollen tubes from the stigma to the ovules.

The segregation ratio of phenotypically normal to mutant seeds in the top half and bottom half of siliques from plants heterozygous for the *tip1* mutation, or the *rhd1*, *rhd2*, *rhd3*, or *rhd4* mutations (as controls), are summarized in Table I. The segregation ratios observed in the two halves of the *rhd1-rhd4* heterozygotes do not deviate significantly from the expected ratio of 3:1 (i.e. 25% mutant seeds). However, seeds from the top and bottom halves of siliques from the *tip1* heterozygotes displayed segregation ratios significantly different from 3:1 ($P < 0.01$ for top half, $P < 0.001$ for bottom half). Furthermore, the bottom halves of the *tip1* heterozygous siliques possess a smaller proportion of mutant seeds than the top halves; only 22% of the *tip1* mutant seeds in these siliques are located in the bottom half.

To characterize the aberrant ratio of phenotypically normal to mutant seeds in these siliques further, segregation ratios were calculated for seeds from the top one-fourth and bottom one-fourth of *tip1* heterozygous siliques. The segregation ratio in the top one-fourth was 128 wild type:30 mutant (19.0% mutant, $\chi^2=3.05$, $P > 0.05$), whereas the ratio in the bottom one-fourth was 174 wild type:4 mutant (2.2% mutant, $\chi^2 =49.15$, $P < 0.001$). These experiments demonstrate that a nonrandom distribution of mutant seeds exists in these siliques and that the deficiency of mutant seeds is most pronounced in the lowest portion of the silique.

Pollen-Tube Growth Analyses

The aberrant segregation ratios observed in the siliques from *tip1* heterozygotes is consistent with the notion that *tip1* mutant pollen tubes grow abnormally and are unable to compete effectively with wild-type pollen to fertilize ovules

Table I. *Segregation ratio of phenotypically normal to mutant seeds*

The proportions of phenotypically normal and mutant seeds were determined from the top half and bottom half of siliques from heterozygous plants.

Silique Source[a]	Top Half			Bottom Half		
	Total seeds	Percent mutant	χ^{2b}	Total seeds	Percent mutant	χ^{2b}
rhd1/+	229	21.8	1.22	211	23.7	0.19
rhd2/+	317	24.9	0.00	326	22.7	0.92
rhd3/+	243	25.1	0.00	183	24.0	0.09
rhd4/+	236	20.8	2.26	243	21.4	1.68
tip1/+	352	18.5	8.02	300	5.3	61.88

[a] A total of 12 to 20 siliques was tested from each line. [b] χ^2 calculated based on 3:1 ratio of phenotypically normal to mutant; critical χ^2 value is 3.84 (for $P = 0.05$).

near the base of the silique. Alternatively, it is possible that ovules bearing the *tip1* mutant allele are preferentially located near the upper portion of the silique. To distinguish between these possibilities, the growth of wild-type and mutant pollen tubes was analyzed in vivo.

Wild-type flowers were hand-pollinated with either mutant or wild-type pollen, and after 4, 5, or 6.5 h of incubation, the pollen-tube length was examined by squashing and staining with aniline blue to detect callose. The results showed that the growth of the mutant pollen tubes through the transmitting tissue and into the ovaries was significantly retarded compared with wild-type pollen tubes at each time point tested. For example, after 4 h, the lengths of the mutant pollen tubes were about one-half the lengths of wild-type pollen tubes. At this time point, two distinct groups of aniline blue-staining papillae were visible in wild-type pollen tubes; one group near the stigma and one group near the upper portion of ovaries (Fig. 3). In contrast, mutant pollen tubes had produced only the first group of callose papillae near the stigma and no visible callose staining near the ovaries (Fig. 3). After 6.5 h, wild-type pollen tubes contained a third group of callose papillae located further into the set of ovaries, whereas the mutant pollen tubes displayed only two sets of callose papillae (in equivalent positions to the 4-h wild-type pollen tubes; data not shown).

The shorter lengths of the mutant pollen tubes were not due to a delay in germination, because in vivo observations at early time points (1 h after incubation) revealed no difference in the ability of wild-type and mutant pollen grains to germinate. There was also no noticeable difference in the morphology of the pollen tubes produced by the wild-type and *tip1* mutant pollen. The *tip1* mutant plants did appear to produce a smaller amount of pollen than the wild type; this was not a factor in these experiments because the stigma surface was saturated with donor pollen in each instance.

Observations at the later time points of this in vivo analysis indicated that the mutant pollen tubes were capable of growing to the base of the gynoecium. To examine further whether mutant pollen is in fact able to fertilize ovules on the lower portion of the gynoecium, wild-type flowers pollinated with wild-type or mutant pollen were allowed to mature, and the number of seeds produced in each silique was examined. The data from this experiment showed that flowers pollinated by mutant pollen generated as many seeds as flowers pollinated by wild-type pollen (Table II). This indicates that, in the absence of competing wild-type pollen, the *tip1* mutant pollen is able to fertilize a normal complement of ovules within the silique.

The growth of wild-type and mutant pollen tubes was also analyzed in vitro, using a standard pollen growth medium (see "Materials and Methods"). Under these conditions, the germination and growth of the *tip1* mutant pollen could not be distinguished from that of the wild-type pollen. Each type of pollen grain germinated at a rate that varied from approximately 5 to 50%. The length of the pollen tubes generated by each type of pollen varied from 20 to 200 μm. In addition, there was no visible difference in the morphology of the pollen tubes, and no branched pollen tubes were observed.

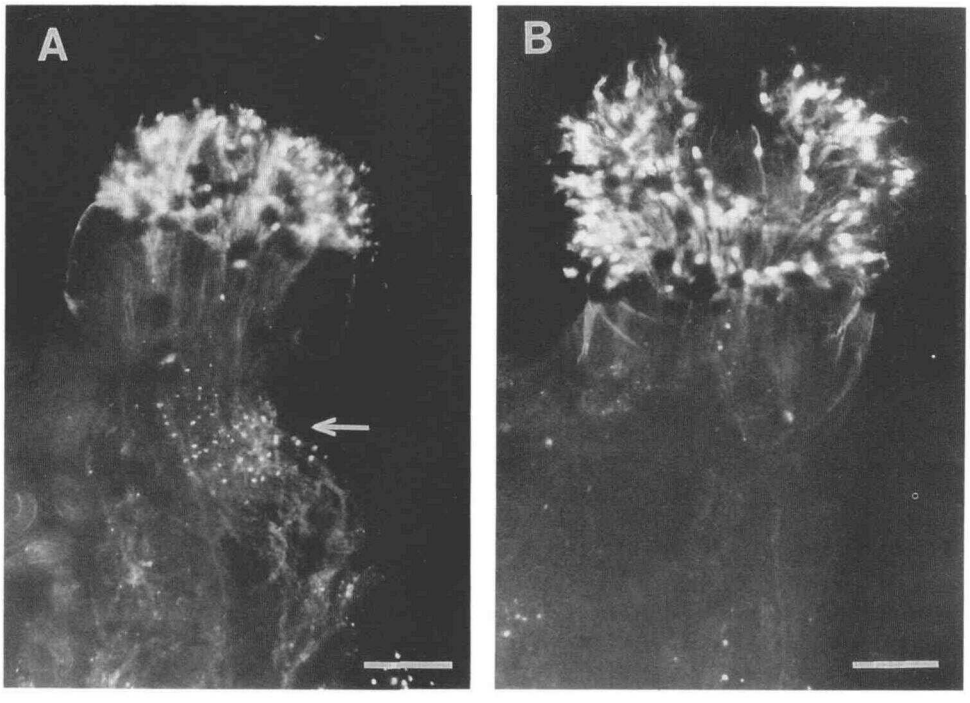

Figure 3. Growth of pollen tubes from wild-type (A) and *tip1* mutant (B) plants 4 h after application of pollen to stigmas of wild-type flowers. Stigmas were frozen in liquid nitrogen, rewarmed, squashed to expose pollen tubes, and stained with aniline blue to reveal callose papillae. Note that wild-type pollen tubes have generated a second group of callose papillae (arrow) that is absent from the mutant pollen. Bars = 100 μm.

Table II. *Characteristics of wild-type and tip1 mutant plants*

Additional morphological and physiological properties of the wild-type and *tip1* mutant plants.[a]

Characteristic	Wild Type	*tip1*
Seed accumulation[b] (number per silique)	40.5 ± 4.0	39.0 ± 8.9
Primary root length[c] (cm)	1.41 ± 0.15	1.40 ± 0.08
Root epidermal cell length[d] (mm)	0.29 ± 0.05	0.27 ± 0.04

[a] Values indicate the mean ± SD. [b] Wild-type flowers were hand-pollinated with the indicated pollen source (n = 8). [c] Primary root measured after 5 d of growth (n = 6). [d] Mature, nonhair-bearing root epidermal cells measured after 5 d of growth (n = 10).

Co-Segregation Analysis of Root-Hair and Pollen-Tube Defects

The presence of a detectable root-hair and pollen-tube phenotype in the *tip1* mutant line suggested that a single mutation may be responsible for both defects. To test this hypothesis, a co-segregation analysis was conducted on F_2 plants derived from a cross between the *tip1* mutant and wild type. Among 60 F_2 plants tested, a total of 28 plants were identified as being heterozygous for the mutation that causes the root-hair defect. Siliques from these 28 plants were then tested for the segregation ratio of phenotypically normal to root-hair mutant seeds in the top half/bottom half silique test described above. If the mutation that causes the root-hair defect was not linked to the mutation that causes the pollen-tube defect, some of these F_2 plants would be expected to display a normal (3:1) segregation ratio for the root-hair phenotype in seeds from both the top half and bottom half of the heterozygous siliques. On the other hand, if a single mutation were responsible for both phenotypes, seeds from the siliques of each of these 28 plants should exhibit the aberrant segregation ratio characteristic of the original *tip1* mutant line. The results of this analysis are summarized in Table III, and they show that seeds from the bottom half of the siliques from each line displayed a significant deviation from the 3:1 ratio ($P < 0.01$). These data support the hypothesis that the two phenotypes are the result of a single mutation at the *tip1* locus.

Other Properties of *tip1* Mutant Plants

To determine whether the *tip1* mutation affects other aspects of *Arabidopsis* development, various cells and tissues of the mutant plants were examined. No significant difference was detected between the wild type and the *tip1* mutant in the primary root length, the morphology of the leaf trichomes, the morphology of the stigma, or the general morphology of the floral organs (Table II). Although the *tip1* mutation causes abnormal expansion of root hair cells, the expansion of hairless root epidermal cells is not affected (Table II).

DISCUSSION

The isolation and characterization of the *tip1* mutant described in this report indicates that a single mutation leads to abnormal cell expansion in the root hairs and pollen tubes. In *tip1* root hairs, tip growth appears to be affected at an early stage, shortly after the initiation of hairs, and branched root hairs are generated. Each of the individual branches grows to about one-tenth of the length of wild-type root hairs. In the *tip1* pollen tubes, the rate of tip growth is reduced in vivo, although the morphology of the tubes does not appear to be affected. These results indicate that the *TIP1* gene product plays an important role during tip growth in both root hairs and pollen tubes.

At this stage it is difficult to explain the different effects of the *tip1* mutation on the growth and morphology of the root hair and pollen tube. One possibility is that root-hair tip growth is more sensitive to mutations in the *TIP1* gene, and therefore its growth is more severely affected. A detailed understanding of these effects will have to await the biochemical analysis of the gene's product. Based on our current understanding of tip growth, the *TIP1* product may be involved in any one of several processes, including the control of Ca^{2+} levels within the cell, the coordination of the actin filament arrays, or the synthesis of specific cell wall components. The further biochemical and cellular characterization of *TIP1* should enhance our understanding of tip growth in plants and contribute to the use of root-hair development as a model for studies of plant cell morphogenesis (Schiefelbein et al., 1993).

The *tip1* mutant, with its unusual root-hair/pollen-tube phenotype, was the only one of its kind identified in this genetic screen, of a total of 15 root-hair mutants examined. However, this should not be taken as an accurate reflection of the extent of genetic overlap between root-hair and pollen-tube tip growth. The experimental approach used in this study tends to underestimate the actual degree of gene overlap, since mutant alleles of genes essential for pollen-tube growth would be transmitted less frequently (if at all) to the progeny. A potential solution to this problem is the isolation of conditional mutants (e.g. temperature-sensitive mutants) or leaky mutants that would enable some pollen tubes to develop. In this light, it is possible that the *tip1* mutant described here is a leaky mutant and is able to express some level of gene activity. The isolation and characterization of additional *tip1* mutant alleles should serve to address this possibility.

Although a high level of gametophytic-sporophytic gene overlap has been detected in plants (Ottaviano and Mulcahy, 1989), relatively few specific loci have been identified by mutation that affect development in both the gametophytic and sporophytic phases. Meinke (1982, 1985) has described several embryo-lethal mutants of *Arabidopsis* that appear to alter male gametophyte function. A number of defective endosperm mutants of maize have also been shown to affect pollen development (Jones, 1928; Ottaviano et al., 1987). However, the *tip1* mutant described here appears to be the only known example of a mutant that affects morphogenesis of tip-growing cells in both the gametophytic and sporophytic generations.

Table III. *Segregation ratio of phenotypically normal to tip1 mutant seeds*

The proportions of phenotypically normal and root-hair mutant seeds were determined from the top half and bottom half of siliques from heterozygous (*tip1*/+) F_2 plants.

F_2 Plant[a]	Top Half			Bottom Half		
	Total seeds	Percent mutant	χ^{2b}	Total seeds	Percent mutant	χ^2
2	84	21.4	0.57	79	2.5	21.27
4	47	19.1	0.86	53	3.8	12.74
7	91	17.6	2.67	60	1.7	17.42
10	69	17.4	2.13	51	3.9	12.08
11	49	16.3	1.97	61	4.9	13.12
17	97	27.8	0.42	85	3.7	20.90
18	69	24.6	0.01	63	3.2	16.01
19	53	13.2	3.93	40	0.0	13.33
20	54	16.7	2.00	64	3.1	16.33
21	82	18.3	1.97	41	2.4	11.13
23	73	17.8	2.01	50	2.0	14.11
24	81	21.0	0.70	48	4.2	11.11
25	112	16.1	4.76	87	2.3	23.91
26	51	15.7	2.36	53	3.8	12.74
27	56	25.0	0.00	55	3.6	13.39
29	62	19.4	1.05	42	4.8	9.17
32	115	15.7	5.36	58	1.7	16.76
36	75	22.7	0.22	55	3.6	13.39
46	57	15.8	2.58	53	3.8	12.74
48	62	14.5	3.63	55	1.8	15.76
52	40	15.0	2.13	29	3.5	7.18
53	109	17.4	3.33	96	3.1	24.50
56	86	20.9	0.76	54	3.7	13.06
62	83	12.0	7.43	57	3.5	14.04
68	65	15.4	3.21	58	5.2	12.16
69	40	22.5	0.13	41	4.9	8.85
77	86	19.8	1.26	52	5.8	10.26
82	69	13.0	5.26	60	3.3	15.02
Cumulative	2017	18.4	46.9	1600	3.3	401.4

[a] A total of four siliques was tested from each line. [b] χ^2 calculated based on 3:1 ratio of wild type to mutant; critical χ^2 value is 3.84 (for P = 0.05).

The identification of genes, like *TIP1*, that affect pollen-tube growth rates and contribute to nonrandom fertilization events is of considerable interest in plant population genetics. In several cultivated species, pollen-tube growth rates have been positively correlated with the growth rate of the sporophytes (Mulcahy and Mulcahy, 1987). Using different pollen donors in *Hibiscus*, Snow and Spira (1991) demonstrated that nonrandom fertilization due to variation in pollen-tube growth rates can occur in natural populations. Thus, pollen-tube competition and genes that affect pollen-tube growth may play an important role in the evolution of natural plant populations.

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