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Genetic Ablation of Floral Cells in Arabidopsis

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A chimeric toxic gene consisting of the diphtheria toxin A chain gene fused to a promoter previously shown to direct pistil- and anther-specific expression was used to genetically target cell killing in transgenic Arabidopsis. Flowers of Arabidopsis transformants that carried the toxic gene fusion had distinct structural defects. The papillar cells at the stigma surface were stunted and were biosynthetically inactive. Anther development was also impaired by toxic gene expression, leading to abnormalities in anther dehiscence, pollen morphology, and pollen germination. The combined defects of pistil and anther rendered transformants that carried the toxic gene fusion self-sterile. However, the transformants were cross-fertile with untransformed plants: the viable pollen of ablated plants was rescued by wild-type stigmas, and, strikingly, the ablated papillar cells allowed the growth of wild-type pollen.

INTRODUCTION

Genetic ablation methodology, in which a toxic gene is placed under the control of a tightly regulated promoter, is a powerful tool for the analysis of developmental processes and the role specific cell types play in complex tissues. The genetically targeted death of specific cells has been achieved by this approach in mammalian systems (Breitman et al., 1987; Palmiter et al., 1987; Behringer et al., 1988) and plants (Mariani et al., 1990, 1992; Thorsness et al., 1991). In one application of this method, we directed expression of the diphtheria toxin A chain (DT-A), a potent inhibitor of translation (reviewed in Collier, 1977), from the promoter of the *S Locus Glycoprotein* (*SLG*) gene, a gene derived from the self-incompatibility locus of Brassica. The *SLG* promoter is active exclusively in specific tissues of the pistil and anther (Nasrallah et al., 1988; Kandasamy et al., 1989; Sato et al., 1991; Thorsness et al., 1991; Toriyama et al., 1991) and is thus well suited for investigating aspects of pollination biology. Tobacco plants transformed with the *SLG::DT-A* gene fusion exhibited dramatically altered floral development and fertility (Thorsness et al., 1991).

Our analysis of different transgenic hosts that expressed a reporter gene fusion of the *SLG* promoter to the *Escherichia coli* β-glucuronidase (*GUS*) gene has shown that the precise pattern of *SLG* promoter activity is species dependent (Sato et al., 1991; Thorsness et al., 1991; Toriyama et al., 1991). In transgenic tobacco, GUS activity was detected at high levels in the transmitting tissue of the style and ovary of flowers and in mature pollen grains (Thorsness et al., 1991). In contrast, in transgenic Arabidopsis, the *SLG* promoter was active in two different cell types: at high levels in the stigmatic papillar cells that are displayed at the surface of the pistil in mature buds and flowers, and at much lower levels in the tapetum, a cell layer that plays a nutritive role in pollen development, in young buds at 6 days before flower opening (Toriyama et al., 1991). This pattern of *SLG* promoter activity predicted that the consequences of *SLG::DT-A* expression in Arabidopsis would be very different from those observed in tobacco. Further, the toxic gene fusion should be useful for analyzing the pollination process in Arabidopsis, and in particular, for investigating the role of papillar cells in this genus. In crucifers, it is at the surface of these cells that a series of events are set in motion that result in successful pollination. After pollen germination and the elongation of the pollen tube into the papillar cell wall, the directed growth of the pollen tube through the transmitting tissue of the style ultimately results in fertilization of the ovules. A papillar cell is thought to act as a selective barrier that allows only the growth of appropriate pollen. This selectivity is most dramatically manifested during intraspecific pollinations in genera such as Brassica, which exhibit self-incompatibility, a self-recognition phenomenon that prevents self-fertilization. In self-fertile genera, such as Arabidopsis, in which self-incompatibility is not known to occur, the role of papillar cells in the early events of pollination is not understood.

In this report, we show that expression of the *SLG::DT-A* fusion in transgenic Arabidopsis plants affected papillar cell growth and anther development. Directed cell killing by this toxic gene fusion altered the fertility of transformants. Transgenic plants were self-sterile, but the defects in papillar cells and in pollen were complemented in reciprocal crosses to wild-type plants.

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RESULTS

Toxic Gene Expression Results in Morphological Abnormalities in Stigmatic Papillae and Pollen

A chimeric gene was constructed that fused the *DT-A* gene to a 3.65-kb SLG_{13} gene promoter fragment (Thorsness et al., 1991). This toxic gene fusion was carried between the T-DNA borders of the binary vector BIN19 (Bevan et al., 1984; Thorsness et al., 1991) and was introduced into Arabidopsis by Agrobacterium-mediated transformation of root segments (Valvekens et al., 1988). In a parallel experiment, the SLG_{13}::*GUS* reporter gene fusion (Thorsness et al., 1991) was introduced into Arabidopsis. As previously observed in tobacco transformation experiments, mature SLG_{13}::*DT-A* and SLG_{13}::*GUS*–transformed plants were obtained at similar frequencies, indicating that the SLG_{13}::*DT-A* fusion was not expressed during transformation and subsequent growth of the plant body. The consequences of toxic gene expression were analyzed in 13 independent primary transformants, as described below. In addition, for two of these transformants, the analysis was performed in progeny plants over three subsequent generations. Because SLG_{13}::*DT-A* transformants were self-sterile (see below), progeny plants were generated by crosses to untransformed control plants. DNA gel blot hybridization analysis demonstrated that multiple linked copies of the *DT-A* sequence were stably integrated in the genomes of these progeny plants (data not shown).

Expression of the toxic gene fusion had profound effects on specific tissues of the Arabidopsis flower, but not on other tissues of transformants. In the pistil, *DT-A* expression blocked normal development of the papillar cells which form the surface of the stigma. Figure 1 shows that, when compared to untransformed controls (Figure 1A), the pistils in flowers of SLG_{13}::*DT-A* transformants had normal ovaries and styles but stunted stigmatic papillae (Figure 1B). Figure 2 shows that the stunted papillae were three- to fivefold shorter in transformants (Figure 2B) than in untransformed controls (Figure 2A). All transformants exhibited a similar defect in papillar cell elongation. When analyzed by light microscopy, the stunted papillae of SLG_{13}::*DT-A* transformants were not obviously necrotic. However, ultrastructural analysis of ablated stigmas revealed clear cytological abnormalities. Figure 3 shows that the cytoplasm of many papillar cells and some subepidermal basal cells was deteriorated and appeared as an electron dense mass, while other papillae exhibited an abnormal clustering of organelles within the cytoplasm.

DT-A expression also affected anther development. However, the severity of the defect varied considerably between transformants and even between flowers of an individual transformant. In general, anther dehiscence occurred later in SLG_{13}::*DT-A* transformants than in untransformed controls, and under the growth conditions used, anthers failed to dehisce

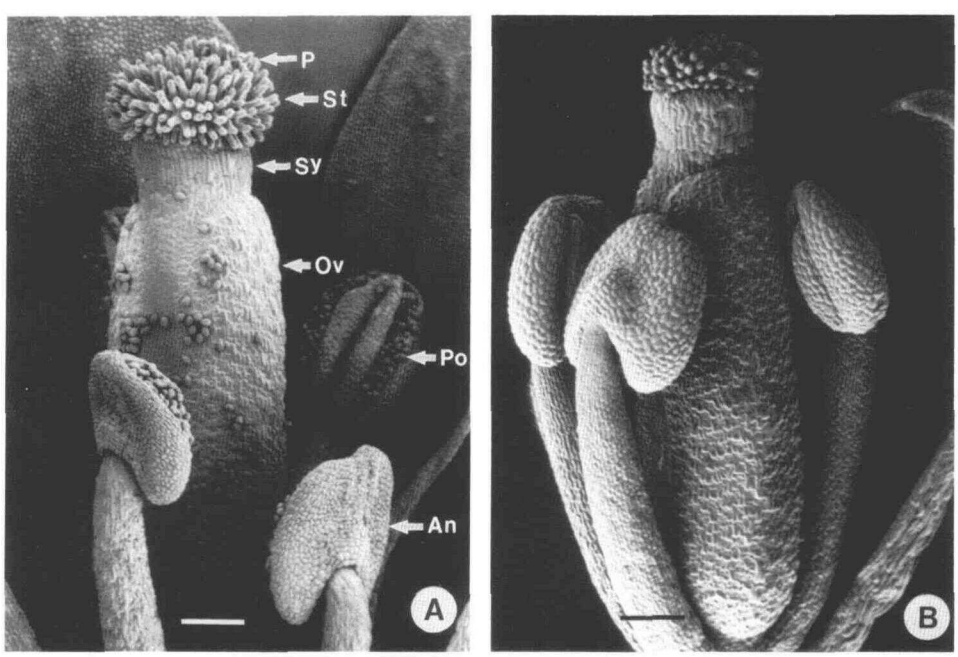

Figure 1. Scanning Electron Microscopic Analysis of Wild-Type and Ablated Flowers.

(A) Flower from an untransformed control exhibiting elongated papillae and dehisced anthers. An, anther; Ov, ovary; P, papillar cell; Po, pollen; St, stigma; Sy, style. Bar = 100 μm.

(B) Flower from an SLG_{13}::*DT-A* transformant showing stunted papillae and delayed dehiscence of anthers. Bar = 100 μm.

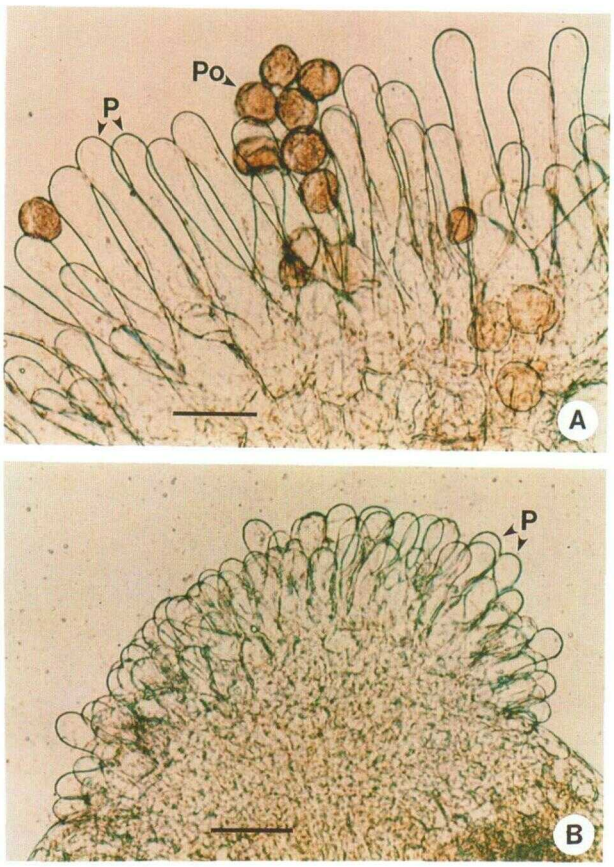

Figure 2. Light Microscopic Analysis of the Stigmatic Surface in Wild-Type and Ablated Flowers.

(A) Stigma papillae of a flower from an untransformed control.
(B) Stigma papillae of a flower from an *SLG*$_{13}$::*DT-A* transformant. Papillar cell length is highly reduced relative to the untransformed control.
P, papillar cells; Po, pollen. Bars = 20 μm.

in some cases. Less pollen was released from anthers of transformants than from anthers of untransformed controls, even in anthers that were fully dehisced. Toxic gene expression did not lead to visible aberrations in the sporophytically derived cells of the anther. Figure 4 shows the microscopic analysis of anther sections at various stages of flower bud development in an untransformed control plant (Figures 4A to 4C) and an *SLG*$_{13}$::*DT-A* transformant (Figures 4D to 4F). The major tissues of the anther such as the epidermis, endothecium, and tapetum developed normally in ablated flowers. However, pollen development was visibly affected in *SLG*$_{13}$::*DT-A* transformants. Although no differences were observed between transformants and the wild type in anthers at the tetrad stage (Figures 4A and 4D) and at the early uninucleate microspore stage (Figures 4B and 4E), the effects of *DT-A* expression were clearly evident in anthers containing mature trinucleate pollen (Figures 4C and 4F). A significant proportion of pollen grains were apparently nonfunctional as judged by their reduced size and collapsed appearance.

Toxic Gene Expression Affects the Ability of Pollen to Germinate in Vitro

To assay the viability of pollen from *SLG*$_{13}$::*DT-A* transformants, we performed in vitro pollen germination experiments. Four plants representing third-generation progeny from one primary transformant were analyzed in detail, together with an untransformed control. For each plant, pollen from four or five flowers was germinated in vitro. The results of this analysis are shown in Figure 5. Pollen from all flowers of the untransformed control exhibited nearly 100% germination efficiency (Figure 5A). In contrast, among the *SLG*$_{13}$::*DT-A* transformants, in vitro germination efficiency was typically between 25 and 50%, although extremes of 0 and 75% germination efficiency were also observed. Pollen tubes of those grains of *SLG*$_{13}$::*DT-A* transformants that did germinate (Figures 5B and 5C) were typically shorter than those of untransformed controls and occasionally exhibited bizarre branched morphologies (Figure 5D). Collapsed grains never germinated, whereas normal grains germinated with variable frequency.

Ablated Flowers Are Self-Sterile, but Pollen Tube Growth Is Allowed in Reciprocal Crosses to Untransformed Plants

To characterize the effects of *SLG*$_{13}$::*DT-A* expression on the pollination responses of transformants, intergeneric and intraspecific pollinations were performed, and pollen tube growth into the pistil was visualized with the dye, aniline blue. We observed no change in the response of ablated stigmas in "wide" pollinations to Brassica species: on ablated and wild-type stigmas alike, Brassica pollen hydrated and germinated, but pollen tubes did not invade the papillar cell wall (data not shown). In intraspecific pollinations, different responses were obtained after selfing *SLG*$_{13}$::*DT-A* transformants and in crosses to untransformed wild-type plants. The pollination behavior of ablated flowers is summarized in Table 1 and representative micrographs are shown in Figure 6. Pollen of untransformed control plants efficiently germinated and generated extended pollen tubes into pistils of both untransformed controls and *SLG*$_{13}$::*DT-A* transformants (Figures 6A and 6B). Similarly, pollen of *SLG*$_{13}$::*DT-A* transformants generated extended pollen tubes into the pistil of untransformed control plants (Figure 6C), although in some cases the efficiency of germination was somewhat reduced relative to a wild-type cross. Strikingly, in some crosses between pistil and pollen of *SLG*$_{13}$::*DT-A* transformants, the efficiency of pollen germination was dramatically reduced, and pollen tube growth did not extend into the ablated pistil (Figure 6D). However, the extent of this phenotype was variable in multiple trials of the same

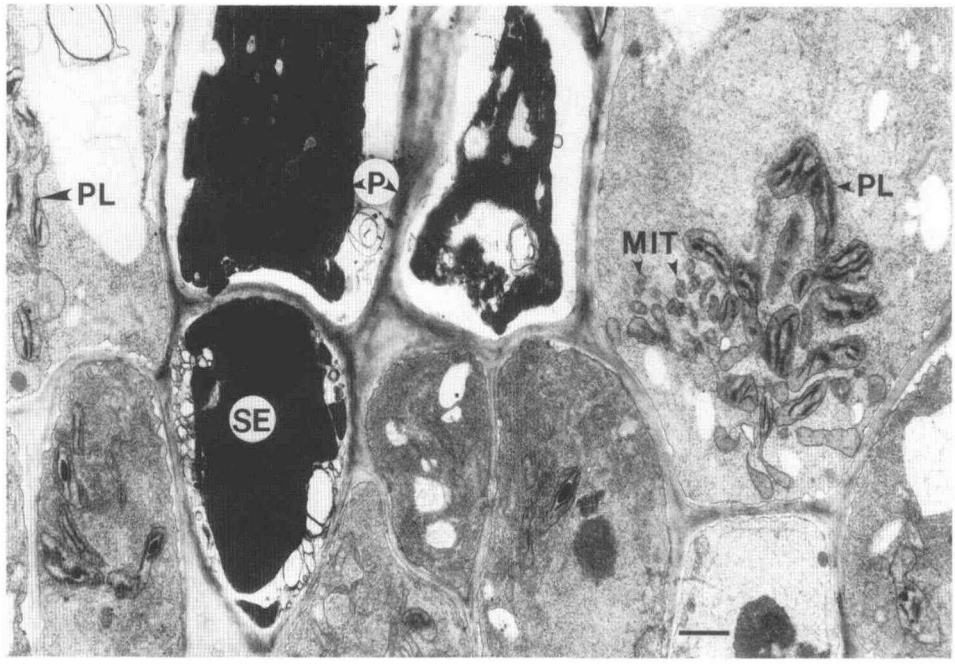

Figure 3. Ultrastructural Analysis of the Ablated Stigma of an *SLG$_{13}$::DT-A* Transformant.

The cytoplasm in two papillar cells and a subepidermal basal cell is deteriorated and appears as an electron dense mass. Other papillar cells have an abnormal accumulation of cytoplasmic organelles. MIT, mitochondria; P, papillar cells; PL, plastids; SE, subepidermal basal cell. Bar = 1 μm.

cross. These patterns of pollen tube elongation corresponded with observed patterns of seed set in *SLG$_{13}$::DT-A* transformants. Whereas *SLG$_{13}$::DT-A* transformants were self-sterile, crosses between transformants and untransformed controls produced kanamycin-resistant seed as well as progeny with the ablated phenotype. Thus, despite the observed cytological aberrations, papillar cells were able to support pollen tube growth. In addition, despite the in vitro pollen germination defects, the viable pollen grains that carried the toxic gene fusion were able to function in the plant and effect fertilization.

Ablated Papillar Cells Are Biochemically Inactive

Because the stigmas of *SLG$_{13}$::DT-A* transformants functioned in supporting the growth of pollen tubes, it was important to determine if the ablated papillae were biochemically active. Plants that carried both the *SLG$_{13}$::DT-A* toxic gene fusion and the *SLG$_{13}$::GUS* reporter gene fusion (Toriyama et al., 1991) were generated by crossing pollen from an *SLG$_{13}$::GUS* transformant to the pistils of an *SLG$_{13}$::DT-A* transformant. Segregation of the two fusions was analyzed in 15 progeny plants by DNA gel blot hybridization, and expression of GUS activity in the stigma papillae was analyzed by histochemical staining. Table 2 shows that each of the seven progeny plants that carried the toxic gene fusion exhibited the ablated phenotype of shortened papillar cells. Of these, six plants also carried the *SLG$_{13}$::GUS* reporter gene fusion; however, none of these double transformants expressed GUS activity. In contrast, all of the eight progeny that carried only the reporter gene fusion expressed GUS activity in the papillae. This inability of double transformants to express GUS activity demonstrated that *DT-A* expression in the ablated papillae inhibited protein synthesis and rendered these cells biochemically inactive.

DISCUSSION

Expression of *DT-A* from the Brassica *SLG* gene promoter caused defects in specific tissues of Arabidopsis flowers. In the pistil, the stigma papillae were stunted. At the ultrastructural level, cytological abnormalities were observed in both the papillar cells and the subtending basal cells; they were evident as extensive cytoplasmic deterioration in some cells and as abnormal cytoplasmic organellar accumulation in other cells. *SLG* promoter activity in the basal cells had not been noted in our analysis of *SLG$_{13}$::GUS* Arabidopsis transformants, which were examined only at the light microscopic level (Toriyama et al., 1991), but had been observed in Brassica by ultrastructural immunocytochemical studies aimed at localizing the *SLG* gene product (Kandasamy et al., 1989).

The papillae of *SLG$_{13}$::DT-A* transformants were biosynthetically inactive, because ablated stigmas that carried the

SLG$_{13}$::*GUS* fusion did not express GUS activity. Despite these effects of *DT-A* expression, the pistils of *SLG*$_{13}$::*DT-A* transformants were able to support the growth of wild-type pollen tubes and to produce seed. These results indicate that biosynthetically active papillae are not required for pollination in Arabidopsis. Seed set was typically lower in *SLG*$_{13}$::*DT-A* transformants than in untransformed controls. However, because DT-A is not secreted and is not internalized by eukaryotic cells in the absence of the diphtheria toxin B chain (Pappenheimer and Gill, 1972; Collier, 1977), the reduced seed set may reflect a reduced ability of pollen to adhere to the shortened papillae, rather than to a more specific toxic effect of *DT-A* on pollen tube growth.

DT-A expression also caused defects in anther development and pollen function. The severity of these defects varied between transformants and between flowers of a single

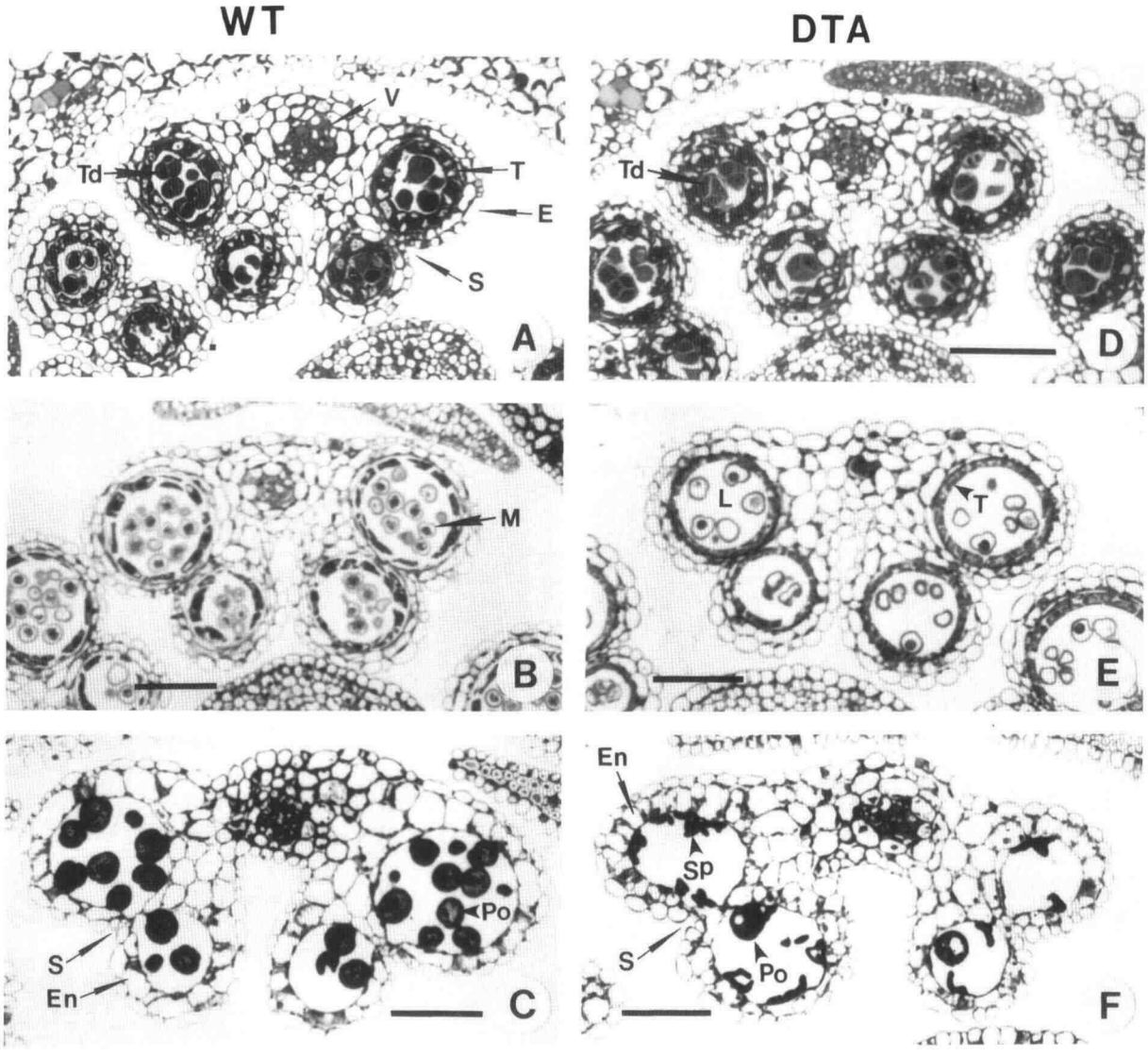

Figure 4. Anther Development in an Untransformed Plant and in an *SLG*$_{13}$::*DT-A* Transformant.

Anther sections were obtained and processed for microscopy, as described in Methods. WT, untransformed wild-type control; DTA, *SLG*$_{13}$::*DT-A* transformant; E, epidermis; En, endothecium; L, locule; M, microspore; Po, pollen; S, stromium; SP, sterile pollen grains; T, tapetum; Td, tetrad; V, vascular bundle.

(A) to **(C)** Untransformed anthers at the tetrad stage of meiosis **(A)**, at the uninucleate microspore stage **(B)**, and at the trinucleate pollen stage **(C)**.
(D) to **(F)** Transformed anthers at the tetrad stage of meiosis **(D)**, at the uninucleate microspore stage **(E)**, and at the trinucleate pollen stage **(F)**.
Bars = 50 μm.

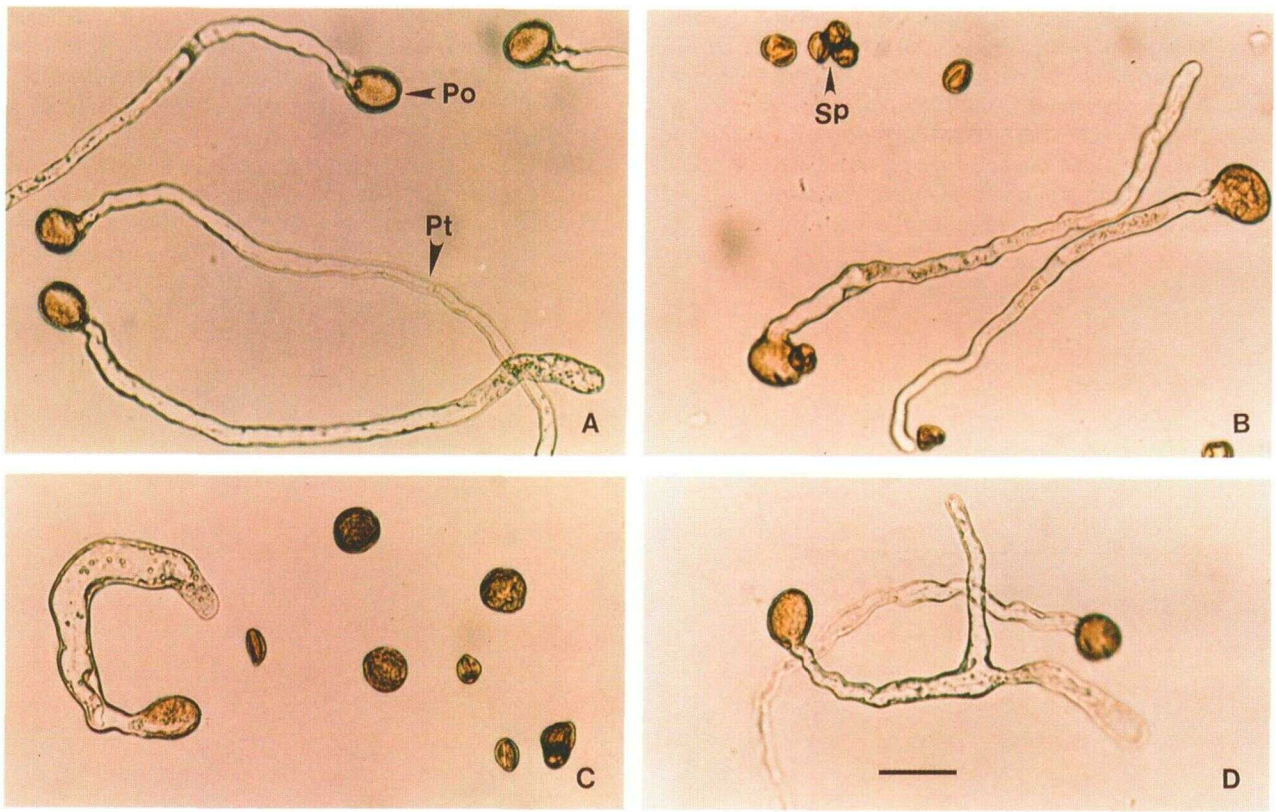

Figure 5. In Vitro Pollen Germination.

(A) Pollen of an untransformed control. Note that the pollen grains are spherical, germinate efficiently, and generate extended pollen tubes.
(B) to **(D)** Pollen of three third generation *SLG_{13}::DT-A* transformants. Note that the pollen is a mix of spherical and smaller collapsed grains. Only the spherical grains germinate, and they often generate stunted or abnormal pollen tubes.
Po, pollen; Pt, pollen tube; Sp, sterile pollen. Bar = 20 μm.

transformant. However, even in the most severely affected plants, the cells of the tapetum were not killed and did not exhibit any obvious cytological aberrations. It is at least theoretically possible that *SLG_{13}::DT-A* expression does lead to tapetal cell death, and that neighboring cells are subsequently recruited to replace the killed cells. We do not believe this to be the case, however, because the *SLG* promoter becomes active relatively late in tapetal cell development. Furthermore, experiments by others (Koltunow et al., 1990; Mariani et al., 1990) in which toxic gene expression was directed to the tapetal cells under the control of a highly active promoter resulted in the complete destruction of the tapetum in transgenic anthers. Rather, it is likely that the differences we observed in penetrance of the ablated phenotype in the stigmas and anthers of *SLG_{13}::DT-A* transformants reflect the relative strengths of *SLG_{13}* promoter activity in these organs.

SLG gene expression in the anther of Arabidopsis is quite low, as judged by expression of the *SLG_{13}::GUS* fusion (Toriyama et al., 1991). It is possible that some threshold level of toxin was required for cell killing, and that level was attained only in some flowers. In Arabidopsis, the *SLG_{13}::GUS* fusion is expressed only sporophytically in the tapetal cells of the anther and not in individual microspores (Toriyama et al., 1991). This is consistent with the observation that the *SLG_{13}::DT-A* fusion can be transmitted to progeny via pollen grains. Weak

Table 1. Summary of Pollination Responses in Wild-Type and Ablated Flowers

	Stigma	Anther	Pollination Response
Self-pollination			
Wild-type flowers (+)	+	+	Fertile[a]
Ablated flowers (abl)	abl	abl	Sterile
Cross-pollination			
[abl] ♀ × [+] ♂	abl	+	Fertile[a]
[+] ♀ × [abl] ♂	+	abl	Fertile[a]

[a] Pollen germination, pollen tube development, invasion of papillar cells, fertilization, and seed set allowed.

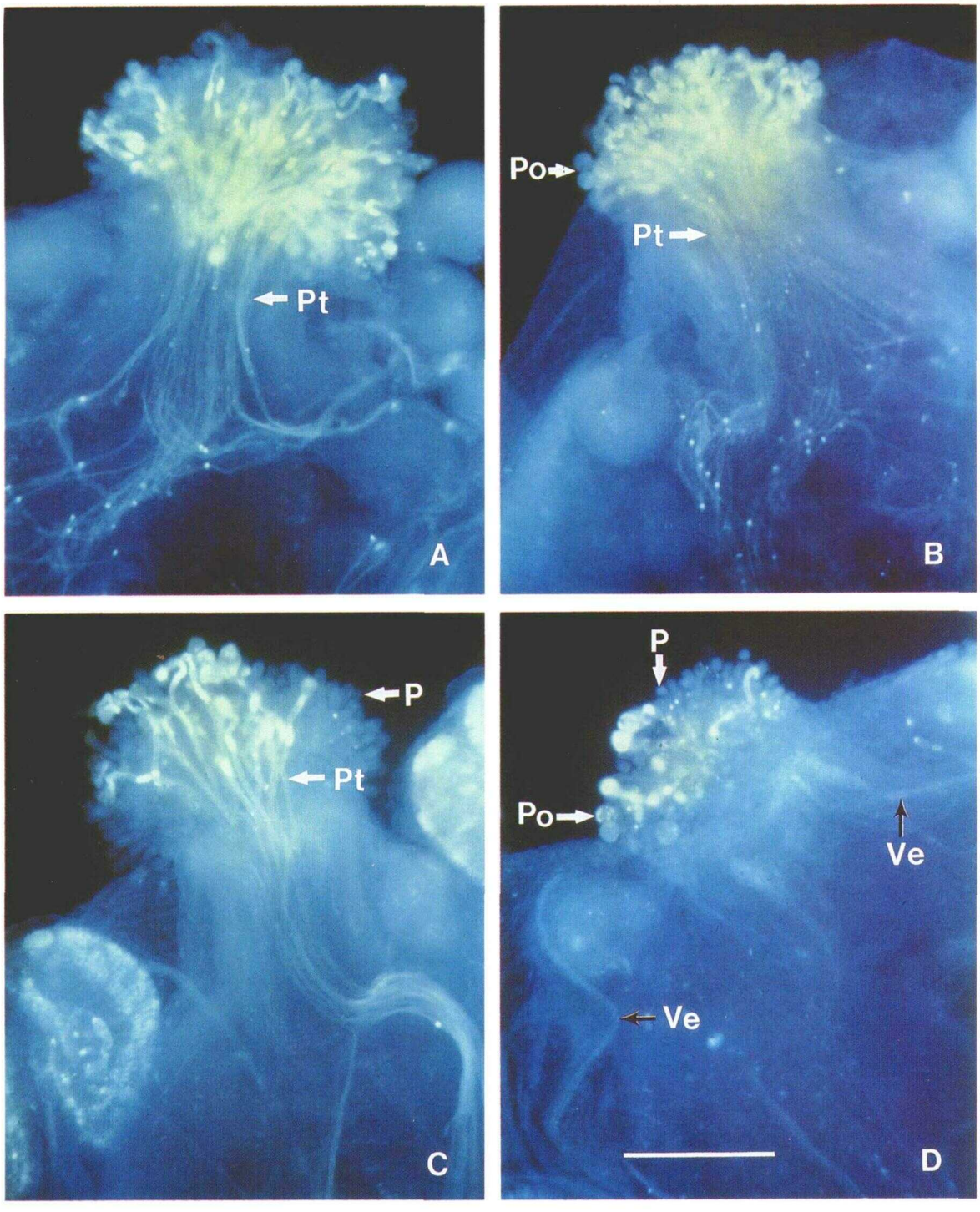

Figure 6. Fluorescence Microscopy of Pollen Tube Growth in Pistils.

(A) Untransformed control stigma pollinated with untransformed control pollen. Most grains are germinated, and pollen tubes extend into the style.
(B) *SLG_{13}::DT-A* stigma pollinated with untransformed control pollen. Most grains are germinated, and pollen tubes extend into the style.
(C) Untransformed control stigma pollinated with *SLG_{13}::DT-A* pollen. Many grains are germinated, and pollen tubes extend into the style.
(D) *SLG_{13}::DT-A* stigma pollinated with *SLG_{13}::DT-A* pollen. Few grains are germinated, and pollen tubes do not extend into the style.
P, papillar cells; Po, pollen; Pt, pollen tubes; Ve, vascular element. Bar = 100 μm.

Table 2. Gene Segregation in the Progeny of a Cross between *SLG$_{13}$::DT-A* and *SLG$_{13}$::GUS* Primary Transformants

Progeny Plant	Papillar Length	GUS Activity[a]	*DT-A* DNA[b]	*GUS* DNA[b]
4-2	Normal	+	−	+
4-4	Stunted	−	+	+
4-5	Normal	+	−	+
4-6	Normal	+	−	+
4-8	Normal	+	−	+
4-9	Normal	+	−	+
4-10	Normal	+	−	+
4-11	Stunted	−	+	+
4-12	Normal	+	−	+
4-13	Stunted	−	+	−
4-14	Stunted	−	+	+
4-16	Stunted	−	+	+
4-18	Normal	+	−	+
4-19	Stunted	−	+	+
4-20	Stunted	−	+	+

[a] GUS activity in papillar cells was identified by histochemical staining. + indicates staining; −, no staining.

[b] The presence of *DT-A* or *GUS* DNA was determined by DNA gel blot hybridization analysis. + indicates presence of the transgene; −, absence of the transgene.

toxin expression in the tapetum, while not resulting in the death of tapetal cells, might partially impair tapetal cell function and pollen development and still allow normal development of some grains. Those grains that develop normally would not express toxin gametophytically and so would be able to transmit the fusion gene to progeny through fertilization.

Although both pistil and pollen of *SLG$_{13}$::DT-A* transformants were functional, we were unable to obtain seed after self-pollination, apparently due to the combined defects of pistil and anther. These results hint at the presence of functionally equivalent factors at the surfaces of papillar cells and pollen grains. Whether these factors are provided by a functional tapetum or a functional papillar cell, their presence at the pollen–papillar cell interface would allow pollen tube development to proceed. In any event, the pollen phenotype of the *SLG$_{13}$::DT-A* transformants is reminiscent of the "conditional male fertility" phenotype observed in chalcone synthase–deficient petunia plants. These plants produce flavonoid-deficient pollen that does not function in self-crosses but functions on wild-type stigmas (Taylor and Jorgensen, 1992). Conditional male fertility has been attributed to the inability of the sporophyte, presumably the tapetum, to provide the developing pollen grains with the flavonoid(s) required for normal pollen function.

The observation that ablated pistils remained fertile in appropriate crosses can be contrasted with the results of *SLG$_{13}$::DT-A* expression in transgenic tobacco and transgenic Brassica. In transgenic tobacco, the ablation of the transmitting tissue resulted in female sterility (Thorsness et al., 1991). In transformants of the self-fertile oilseed rape cultivar Westar, the ablation of papillar cell function resulted in female sterility of mature flowers (Kandasamy et al., 1993). The different consequences of papillar cell ablation in Brassica and Arabidopsis indicate that the mechanisms by which papillar cells perceive pollen differ in these two crucifers despite the similar paths followed by pollen tubes (Elleman et al., 1992). It should be noted that the papillar cell surface in Arabidopsis as well as Brassica is uniquely conducive to pollen germination and early pollen tube growth because under normal circumstances pollen tubes do not emerge and grow efficiently on the surfaces of organs other than stigmas (Lolle and Cheung, 1993). Our data predict that in Arabidopsis, any factors that may be required for pollen germination and early pollen tube development, as well as factors involved in intraspecific versus interspecific pollen recognition, are highly stable molecules. These factors would be synthesized early in the development of papillar cells, prior to the onset of *SLG$_{13}$::DT-A* expression and shortly after these cells differentiate.

METHODS

Plant Growth and Transformation

The *SLG$_{13}$::DT-A* toxic gene fusion plasmid pMKT17 (Thorsness et al., 1991) was introduced into *Arabidopsis thaliana* strain C24 by *Agrobacterium tumefaciens*–mediated transformation of root segments (Valvekens et al., 1988). Root segments were inoculated with Agrobacterium strain pCIB542/A136 (derived from helper plasmid pEHA101; Hood et al., 1986) carrying pMKT17, and transgenic plants were selected on media containing kanamycin. To test transformation efficiencies, plasmid pMKT8, which carries the *SLG$_{13}$::GUS* reporter gene fusion (Thorsness et al., 1991; Toriyama et al., 1991), was transformed in parallel. In some experiments, seed from transformants was germinated on media containing 50 mg/L kanamycin.

Light Microscopy

To visualize papillar cells with the light microscope, pistils were dissected from flowers, softened in 0.1 N NaOH, and examined under bright-field illumination.

To examine the structure of anthers, flower buds at various stages of development were fixed in a mixture of 4% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, at 20°C for 3 hr. The samples were washed in buffer and postfixed in buffered 1% (w/v) OsO4 at 4°C overnight. After washing in buffer, the samples were dehydrated in an ethanol series and embedded in Spurr's epoxy resin. Tissue sections 0.5- to 1-μm thick were cut with glass knives, stained with Toluidine blue and observed and photographed with an optical microscope (Zeiss, Oberkochen, Germany).

Scanning and Transmission Electron Microscopy

Samples were prepared for scanning and transmission electron microscopy, as described in Thorsness et al. (1991).

Histochemical GUS Assays

GUS activity was localized using a histochemical assay (Jefferson et al., 1987). Intact flowers were incubated at 37°C in 2 mM 5-bromo-4-chloro-3-indoyl β-D-glucuronide, 0.1 mM $NaPO_4$, pH 7.0, 0.5% Triton X-100 for 24 hr. Flowers were rinsed in 70% ethanol and observed microscopically.

Pollen Analysis

Isolated pollen grains were germinated in vitro in 10% sucrose, 0.01% boric acid, 3 mM calcium nitrate. Grains were incubated in microtiter wells at 25°C for 24 hr. Growth of pollen tubes was analyzed by light microscopy.

For pollination assays, mature buds or flowers were mounted in agar and stigmas were brushed with pollen from isolated anthers. After 3 hr, flowers were softened in 1 N NaOH and stained in decolorized aniline blue (Kho and Baer, 1968). Pollen tube development was analyzed by fluorescence microscopy.

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