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A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization

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Plants distinguish among the pollen grains that land on the stigma, permitting only compatible pollen to fertilize egg cells. To investigate these cell–cell interactions, *Arabidopsis* mutations that affect pollen–pistil communication were isolated. A male-sterile mutation that disrupts pollen–pistil interactions by eliminating the extracellular pollen coat (tryphine) is described here. Stigma cells that contact the mutant pollen produce callose, a carbohydrate synthesized in response to foreign pollen. The mutant pollen fails to germinate because it does not absorb water from the stigma, yet germinates in vitro, indicating it is viable. The defect is also conditional; high humidity results in pollen hydration and successful fertilization. Analysis of mature, mutant pollen indicated that it is deficient in long-chain lipids and has none of the lipoidic tryphine normally present on its surface. Immature mutant pollen grains have aberrant tryphine that disappears during pollen development. The sterile plants also lack stem waxes, and pollen from other wax-defective (*eceriferum*) mutants with reduced fertility has few of the lipid droplets normally present in tryphine. These results demonstrate that tryphine is critical for pollen–stigma interactions and suggest that tryphine lipids are required for fertilization, either by directly signaling the stigma or by stabilizing other tryphine components.

[*Key Words*: Pollen; *Arabidopsis*; compatibility; lipids; tryphine; *eceriferum*]

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Fertilization in flowering plants involves many cell–cell interactions, including adhesion of pollen grains to the stigma surface, growth of pollen tubes through the pistil, migration of sperm cells within the pollen tube, and, ultimately, fusion of the sperm with egg cells; yet few of the molecules required for these interactions have been identified. As a first step in understanding these cell-signaling events, several sterile *Arabidopsis* mutants blocked in early steps of fertilization were identified. In this paper, one such mutant, defective in the interaction between pollen grains and stigma cells, is described.

The signaling events that occur during fertilization presumably require direct contact between cells and rely on molecules present in low abundance. Few of these molecules have been identified through in vitro reconstitution experiments, in part because manipulation of reproductive tissues is technically difficult. In contrast, genetic approaches depend solely on the function of molecules, rather than on their abundance, and allow cell–cell communication events to be identified and characterized in living plants. Because most plant species are hermaphrodites (producing both sperm and eggs), mutant plants defective in either the male or the female reproductive process can be obtained and propagated readily.

Arabidopsis thaliana is well-suited to a genetic approach to fertilization. The advantages of its short life cycle, small physical size, and small genome size have been described (for review, see Meyerowitz 1989). Moreover, each flower is self-pollinated, and under laboratory conditions pollen from one plant rarely lands on another. Thus, mutant plants defective in any essential aspect of the fertilization process produce no seeds. Seed pod formation also can be used to monitor fertility; after fertilization, conspicuous seed pods are formed by the expansion of the pistil contained within each *Arabidopsis* flower. The penetrance of a mutation can be assessed easily because the plants flower continuously for several months, yielding hundreds of flowers. In fertile plants, these flowers each produce thousands of pollen grains and as many as 40 seeds. Sterile mutations occur at a high frequency in *Arabidopsis*. Nearly 1% of mutagenized seed pools yield plants that are completely sterile (see Materials and methods). Although many of these mutations disrupt formation of the male and/or female

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gametes, approximately one-tenth result in structurally normal flowers that are nonetheless functionally defective.

Adhesion of pollen grains to the stigma surface is mediated in part by an extracellular pollen coat, or tryphine (Dickinson and Lewis 1973a), and the pollen then absorbs water rapidly from the stigma, resulting in germination and tube growth (Heslop-Harrison 1979; Dickinson and Elleman 1985). In some species, pollen obtains water from a damp matrix that coats the stigma surface. In plants with dry stigmas, such as *Arabidopsis*, the tryphine from the pollen grain spreads along the proteinaceous coating of the stigma (Elleman et al. 1992), and water is transferred through this matrix from channels in the stigma cell wall (Roberts et al. 1984). Only pollen that forms these contacts becomes hydrated, whereas nearby pollen grains, not in physical contact with the stigma surface, remain desiccated (Sarker et al. 1988). Pollen germination is a highly selective process; pollen generally fails to germinate on the stigma of a distantly related species (for review, Zenkteler 1990), and germination of self-pollen is inhibited in many plants, a process known as self-incompatibility (for review, see Nasrallah et al. 1991). In *Brassica* species (close relatives of *Arabidopsis*), discrimination against foreign pollen or self-pollen is achieved at the stigma surface, either through control of pollen hydration or through control of subsequent pollen tube growth (Roberts et al. 1980; Ferrari et al. 1983; Sarker et al. 1988). Thus, through cell–cell interactions that result in selective hydration, many plants promote germination of only compatible pollen grains on the stigma surface.

Not only is germination of inappropriate pollen blocked at the stigma surface, but in many species (including *Brassica*) stigma cells that contact incompatible pollen often synthesize callose, a β-1 $\rightarrow$ 3 glucan that is ordinarily absent from the stigma cell wall (Dickinson and Lewis 1973b; Heslop-Harrison 1975). Extracts from incompatible pollen grains can recapitulate this response when applied to stigma cells (Heslop-Harrison et al. 1974; Kerhoas et al. 1983); however, the molecules that stimulate callose synthesis have not been identified. Callose is a normal component of the pollen tube wall but also appears in many tissues after pathogen invasion or wounding.

In this study a male-sterile *Arabidopsis* mutation that eliminates most of the tryphine from the pollen surface is described. The mutant pollen is viable but no longer interacts properly with *Arabidopsis* stigmas; pollen germination fails as a result of restriction of pollen hydration, and callose forms in stigma cells that contact the mutant pollen. This is the first example of a mutation that removes the pollen coat, and it provides an opportunity to characterize tryphine components and to discern their role in cell signaling during fertilization.

Results

Pollen from the pop1 *mutant fails to germinate on the stigma surface*

A collection of 175 infertile *Arabidopsis* plants was obtained by screening 25,000 mutagenized (M2) seeds (see Materials and methods). The *pop1* mutant (defective in pollen–pistil interactions) is described here. This mutant was as healthy as wild-type plants but was completely infertile; no seeds were obtained from thousands of flowers. This mutation impairs only the male reproductive system. Both *pop1* flower buds and mature *pop1* flowers were fertilized successfully with wild-type pollen in 15/20 and 8/12 crosses, respectively, resulting in ~500 seeds. In contrast, application of mutant pollen to either wild-type or *pop1* flower buds yielded no seeds (0/20 and 0/13 crosses, respectively). *pop1*/+ heterozygotes are fertile and one-fourth of their progeny (320/1367 = 0.234) have a sterile phenotype. Thus, the *pop1* fertility defect results from a recessive mutation in a single genetic locus.

At the stigma surface (shown in Fig. 1A) germination of pollen results in the formation of a tube that serves to carry sperm cells to the ovules; one such tube, germinated in vitro, is shown in Figure 1C. These tubes contain high concentrations of callose, and this material can be visualized easily after staining with aniline blue and observing the resulting fluorescence (Fig. 1B; Eschrich and Currier 1964). Neither bright-field nor fluorescence microscopy detected pollen tubes on self-pollinated *pop1* stigmas (Figure 1D,E). Similarly, application of *pop1* pollen to wild-type pistils never resulted in visible pollen tubes. Thus, this early defect in pollen germination accounts for the observed male sterility in *pop1* plants.

The most striking phenotype associated with the *pop1* defect was the appearance of callose on the stigma surface. Stigmatic papillae in direct contact with *pop1* pollen (Fig. 1D,G), but not wild-type pollen (Fig. 1B,F) were highly fluorescent when stained with aniline blue, indicating that the mutant pollen stimulated an aberrant production of callose in stigma cells. The presence of callose was detectable within a few hours of pollination and persisted until the stigma degenerated 3–4 days later. This phenotype was observed in all sterile segregants from *pop1*/+ heterozygotes and, thus, is attributable to the *pop1* mutation. The presence of *pop1* pollen on mutant or wild-type stigmas consistently stimulated callose production in stigma cells, but this was not observed in control crosses, in which wild-type pollen was applied to *pop1* plants. Callose production did not occur when inviable wild-type pollen (treated with formaldehyde vapors) was applied to stigmas and, therefore, is not merely a consequence of a defect in pollen germination. Thus, *pop1* plants make pollen that fails to germinate on the stigma and induces callose formation within stigma cells, both of which are characteristic of incompatible pollination.

pop1 *pollen germinates in vitro*

Pollen from most plant species germinates efficiently and forms pollen tubes when incubated in a simple medium consisting of sucrose, calcium, and borate (Brewbaker and Kwack 1963). Surprisingly, when *pop1* pollen grains were incubated in this growth medium, germina-

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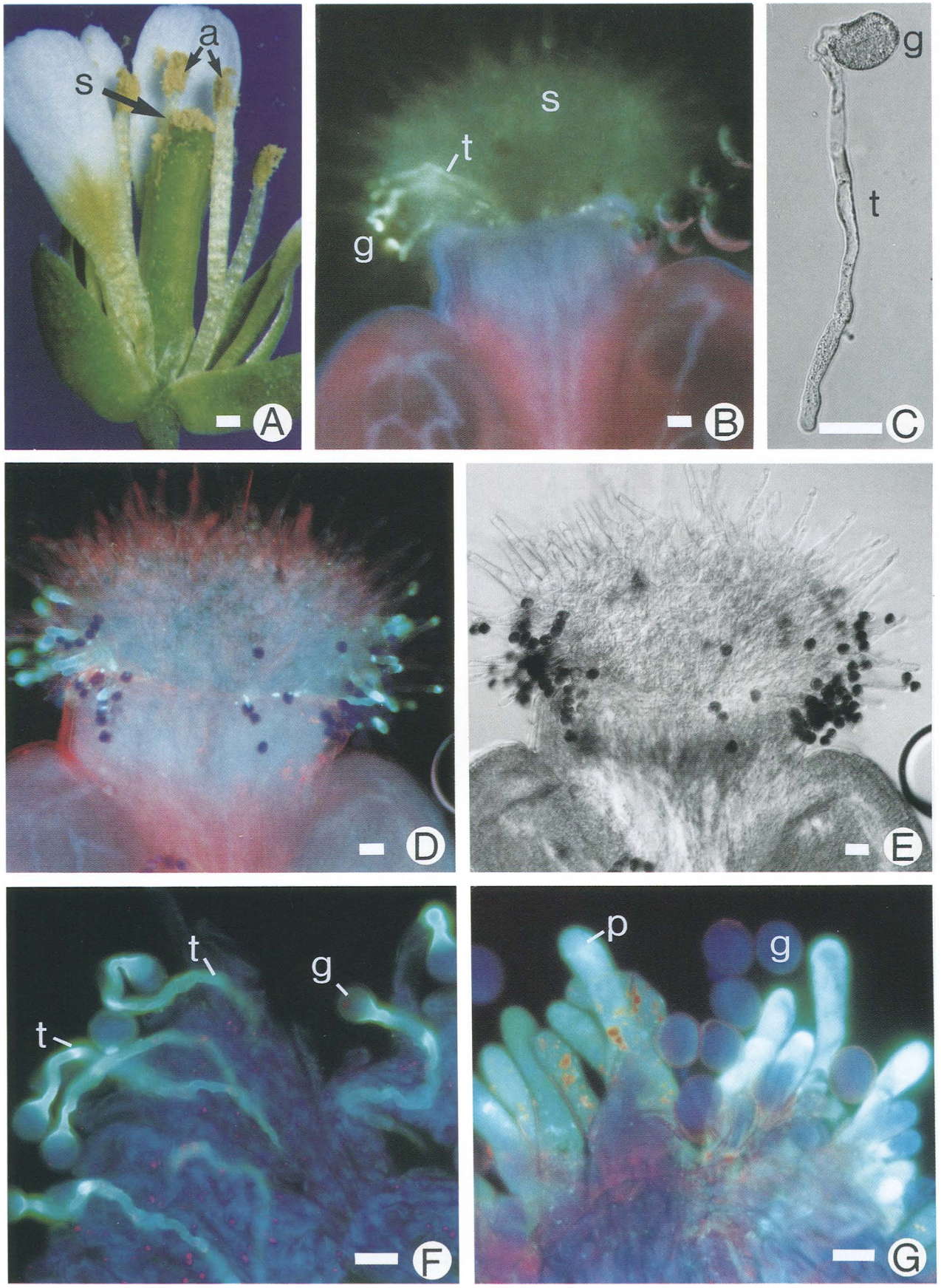

Figure 1. (*See facing page for legend.*)

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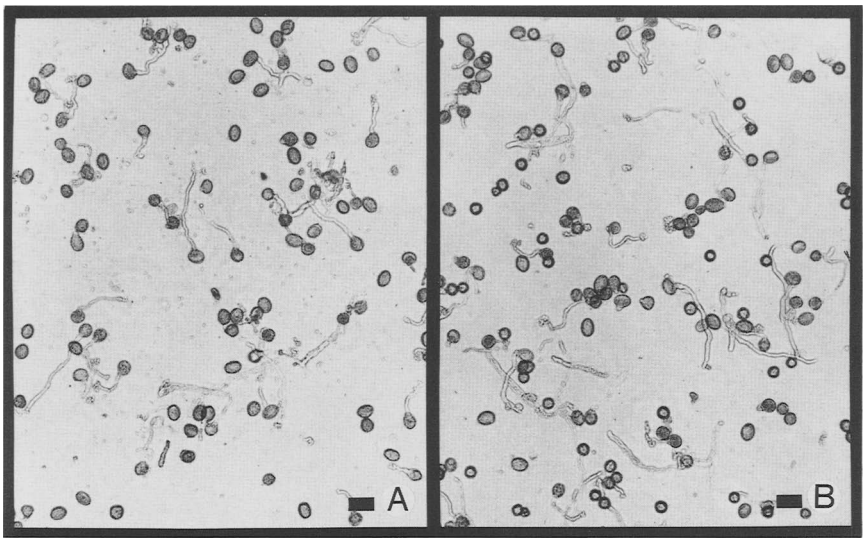

Figure 2. Germination of wild-type and *pop1* pollen in vitro. Pollen tubes were observed after wild-type (*A*) or *pop1* (*B*) pollen grains were incubated in pollen germination medium. Bars, 100 μm.

tion of the mutant pollen was indistinguishable from wild type (Fig. 2). Germination of *Arabidopsis* pollen is relatively poor in this medium; nonetheless, after a 16-hr incubation, 12% of wild-type pollen grains (118/1019) and 18% of *pop1* pollen grains (192/1064) germinated, and pollen tube lengths ranged from 0.1 to 0.5 mm. These results indicate that *pop1* pollen is viable and can synthesize, in vitro, all of the components necessary for pollen tube formation and growth.

pop1 *pollen germination is rescued in vivo by humidity or by cofertilization with* POP$^+$ *pollen*

Upon release from the anther, wild-type *Arabidopsis* pollen lands on the stigma surface and then begins to absorb water, becoming spherical rather than oval-shaped within a few minutes (a typical example is shown in Fig. 3A–E). A similar expansion of *pop1* pollen was not detected (Fig. 3F–J), even when observations were extended to several hours after pollination. Of the hundreds of pollen grains observed, >90% of the wild-type pollen was hydrated, whereas hydration of *pop1* pollen was never detected.

This deficiency in *pop1* pollen hydration on the stigma, coupled with the growth of the mutant pollen in vitro, suggested that the *pop1* fertilization defect might be overcome by artificially hydrating the pollen grains. To test this possibility, *pop1* plants were shifted from standard growth conditions (50–70% relative humidity) to a high-humidity chamber (90% relative humidity). Under these humid growth conditions, the mutant plants became fully fertile. In Figure 4, *pop1* mutants are shown after growth in low humidity (Fig. 4A) and high humidity (Fig 4B); in the latter case, expanded seed pods indicate that fertility was restored. In addition, enclosing *pop1* flowers in a polyethylene bag for as few as 5 hr provided a sufficient increase in humidity to fully restore fertility. Sterility was also reversed when mature *pop1* pollen, produced under relatively arid conditions, was transferred to humid conditions (18/26 crosses), indicating that high humidity rescues *pop1* pollen germination rather than its development. In every case, the mutant plants became sterile when returned to a low-humidity environment (see Fig. 4B). Thus, the *pop1* mutation is conditional and completely reversible and represents the first conditional *Arabidopsis* mutation shown to affect pollen functions.

Although high humidity restores fertility to *pop1* plants, interactions between the mutant pollen and the stigma surface are nonetheless aberrant. Under humid conditions callose still forms and persists on stigmatic papillae that contact *pop1* pollen. In addition, whereas wild-type pollen tubes are intimately associated with stigma cells, growing between the cell wall and the plasma membrane of the papillae (Elleman et al. 1992), the *pop1* pollen tubes produced in humid conditions grew more randomly, often appearing to be independent of the papillae cells. Growth of *pop1* plants in a humid

Figure 1. Germination of wild-type and *pop1* pollen on the stigma surface. (*A*) Pollen is released from anthers onto the stigma surface. (*C*) Wild-type pollen tube after germination in aqueous growth medium. A fluorochrome in aniline blue preparations stains callose within pollen tubes; wild-type pollen produces tubes (*B*, *F*, turquoise staining), whereas no tubes are visible when *pop1* plants are self-pollinated (*D*, *E*). Within a few hours after pollination, callose forms on stigmatic papillae that contact *pop1* pollen (*D*, *E*, *G*) but not wild-type pollen (*B*, *F*); stigmas shown in these panels were fixed 1 day after pollination. Staining techniques result in hydration of both the mutant and wild-type pollen grains, and other fluorochromes in the aniline blue preparation result in faint, dark blue staining of pollen grains. (a) Anther; (g) pollen grain; (p) stigmatic papillae; (s) stigma and (t) pollen tube. Bars, 1 mm (*A*); 100 μm (*B*, *D*, *E*); 50 μm (*C*, *F*, *G*).

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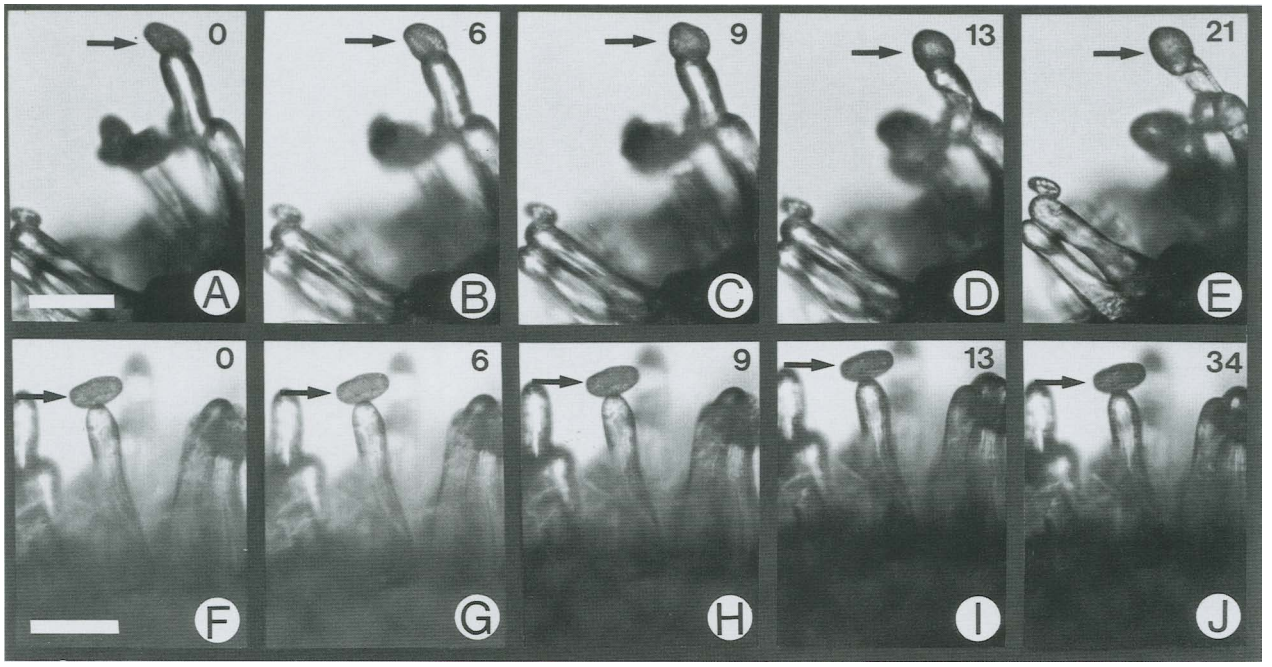

Figure 3. Hydration of wild-type, but not *pop1*, pollen occurs rapidly on the stigma surface. Wild-type pollen (arrows, *A–E*) expands within minutes when placed on a wild-type stigma surface. No change in pollen shape or size was observed in similar experiments with *pop1* pollen (*F–J*). Numbers (upper *right*) indicate time in minutes. The examples depicted here are representative of similar observations of >100 pollen grains. Bars, 100 μm.

environment therefore does not completely suppress all of the *pop1* defects but, instead, allows hydration of the mutant pollen presumably through simple absorption of water vapor.

The experiments described above suggested that the stigma surface discriminates among pollen grains, resulting in hydration of wild-type but not *pop1* pollen. In many species, hydration of foreign or incompatible pollen does not take place but can proceed if compatible mentor pollen is also applied to the stigma (Knox et al. 1987). Thus, to further characterize the requirements for hydration, *pop1* flower buds were pollinated (at 50–70% relative humidity) with a mixture of wild-type and *pop1* pollen, taking care to place the wild-type pollen near the

Figure 4. Humidity restores fertility in *pop1* plants. *pop1* plant grown in 50–70% relative humidity (*A*) or in 90% relative humidity (*B*). The arrow indicates the point at which the plant was returned to an arid environment. Expanded seed pods in *B* indicate that fertilization has occurred.

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mutant pollen grains. As expected, viable seeds were formed. Surprisingly, these seeds not only yielded fertile, *pop1*/+ plants, but also sterile, *pop1*/*pop1* plants, indicating that the wild-type pollen promoted *pop1* pollen germination. An equal mixture of the two types of pollen resulted in 4 homozygous mutant plants and 10 heterozygotes, suggesting that rescue of the mutant pollen was fairly efficient. Callose formation on the stigma was detected, but because *pop1* and wild-type pollen grains are indistinguishable in the light microscope, it was difficult to assess whether the callose on the stigma resulted from isolated clusters of *pop1* pollen or from mixtures of mutant and wild-type pollen. These results indicate that interaction between POP^+ pollen and the stigma surface can result in hydration of nearby pollen grains, including defective grains that, by themselves, might fail to send appropriate signals. Mechanistically, rescue of the *pop1* pollen might be achieved through diffusion of a wild-type pollen component. Alternatively, hydration of wild-type pollen in *Arabidopsis* might trigger hydration of nearby pollen grains, although this seems unlikely given the high specificity of pollen hydration in *Brassica* species. Other *Arabidopsis* mutants with defects in pollen hydration have been identified in another laboratory, and these mutants similarly can be rescued by wild-type mentor pollen (R.E. Pruitt, pers. comm.).

pop1 *mutants are deficient in wax production*

In addition to a defect in fertility, the original *pop1* isolate also had a striking defect in the production of waxes on the surface of stems and seed pods. Epicuticular wax is composed of long-chain lipid molecules and is visualized easily on the surface of wild-type *Arabidopsis* stems as a dull, glaucous coating. In contrast, stems from the *pop1* isolate were bright green and glossy in appearance (Fig. 5A), resembling wax-defective, or *eceriferum* (*cer*) mutants (Dellaert et al. 1979; Koornneef et al. 1989). Scanning electron microscopy confirmed that the *pop1* mutant (Fig. 5C) was completely devoid of the wax blooms present on wild-type stems (Fig. 5B; Koornneef et al. 1989). Crossing the *pop1* mutant to a wild-type plant showed that this *cer* phenotype, like sterility, was completely recessive. Moreover, the sterile and *cer* phenotypes were tightly linked. Self-crossing of a *pop1*/+ heterozygote yielded 1367 progeny, 320 of which were sterile and waxless, whereas the remainder were fertile and had a waxy cuticle. No recombination between the sterile and *cer* phenotypes was observed, indicating that these defects are tightly linked. Linkage of 0.073 map units (m.u.) ($<1/1367 \times 100\%$) indicates that mutations could be within ~15 kb, assuming that recombination frequencies are uniform throughout the genome, with a genome size of ~100,000 kb (Hwang et al. 1991) and genetic map of ~500 m.u. (Koornneef 1987), or ~200 kb/m.u. These results strongly support a model in which defects in wax production and in fertility might be the result of the mutation of a single gene.

To explore further the relationship between fertility and wax synthesis, other *cer* mutants were characterized. Mutations in only 3 of the 21 *cer* genes (*cer1*, *cer2*, and *cer6*) completely eliminate wax structures on stems (Koornneef et al. 1989) and thus resemble the *pop1* mutant. These mutants can be distinguished on the basis of their lipid content (Hannoufa et al. 1993). Chromatographic analysis of *pop1* stem extracts indicated that like plants with defects in the *cer2* or *cer6* genes, *pop1* mutants accumulate shorter lipids (24–27 carbons) but have low levels of the longest lipid chains (29–30 carbons; Table 1). In contrast, *cer1* plants accumulate 30-carbon alcohols and aldehydes but are deficient in 29-carbon lipids (Hannoufa et al. 1993). Thus, the phenotype of the *pop1* mutant, with respect to wax synthesis, closely resembles that of *cer2* or *cer6*, suggesting that these gene products might affect the same biochemical step in lipid biogenesis.

In their screen for wax-defective plants, Koornneef et al. (1989) discarded mutants with severe fertility defects;

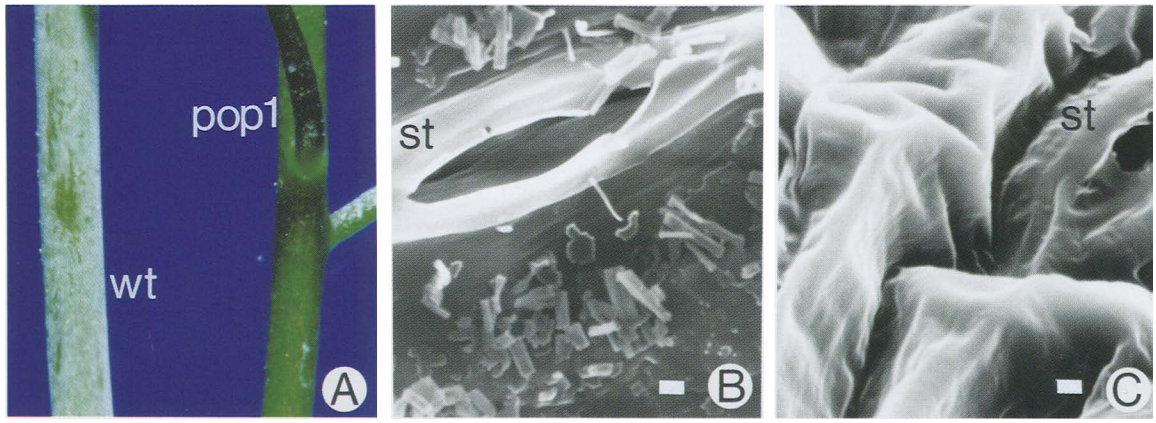

Figure 5. *pop1* mutants are defective in epicuticular wax production. *pop1* plants are deficient in the waxes that coat wild-type stems (*A*), and scanning electron microscopy confirms that plates of wax are present on wild-type stems (*B*) but are absent from *pop1* stems (*C*). As a size reference, two stomates (st) were included in the photographs. Bars, 1 μm.

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Table 1. *Epicuticular wax components from wild-type,* pop1, *and* cer *stems*

Number of carbon atoms	Wild type	*pop1*	*cer1*	*cer2*	*cer6*
24	0.6	22.5 (38)	1.5 (2.5)	7.3 (12)	7.5 (12)
26	4.6	61.5 (13)	9.0 (2)	57.1 (12)	40.5 (9)
27	0.3	2.8 (9)	0 (−)	0 (−)	3.9 (13)
28	11.5	5.1 (0.5)	8.6 (0.8)	26.5 (2)	29.1 (2)
29	64.3	1.7 (0.03)	12.0 (0.2)	1.0 (0.02)	17.2 (0.25)
30	17.5	3.5 (0.2)	49.7 (3)	1.0 (0.06)	<0.2 (<0.01)

The sum of alcohol, aldehyde, alkane, carboxylic acid, and ketone quantities is reported. Values represent percent of total lipid present in chloroform extracts from stems. Shorter chain lipids (14–22 carbons) and 25-carbon chains accounted for <2% of the total material and are not shown. Parentheses indicate fold-change from wild-type extracts.

nonetheless, most of the *cer6* (but not *cer2*) mutants that they recovered had reduced seed yields when grown in greenhouses. In contrast, in the low humidity chamber used here, both the *cer2* and *cer6* mutants were completely sterile. As with *pop1*, sterility was limited to the male reproductive system. Mutant pollen failed to fertilize wild-type flower buds (12 crosses each yielded no seeds), whereas wild-type pollen efficiently fertilized the mutants (8/11 *cer2* and 8/12 *cer6* crosses were successful). The reduced seed yields observed in *cer* mutants was previously attributed to excessive loss of water from the mutant plants (Dellaert et al. 1979; Koornneef et al. 1989); however, the crosses described above argue strongly that these mutants are capable of producing seeds if provided with the proper pollen.

Crosses between the *cer2* mutant and *pop1* (in high humidity) yielded progeny that produced wax and were fully fertile. Similar crosses to the *cer6* mutant resulted in sterile progeny with a *cer* phenotype. In addition, the *cer6* and *pop1* defects are tightly linked; 23/23 self-progeny from a *cer6/pop1* heterozygote had a *cer* phenotype. Thus, *pop1* represents a new allele of the *CER6* gene; henceforth called *cer6-2*. Further examination of the *cer* mutants indicated that the pollen from *cer2* and *cer6-1* plants also failed to hydrate and thus germinate on the stigma surface but grew well in liquid medium. Consistent with previous observations (Koornneef et al. 1989), placing these *cer* mutants in a humid environment did not reverse the wax defect but completely restored fertility. The *cer2* and *cer6-1* mutants differed from *cer6-2* (*pop1*) plants in one respect; callose production within the stigmatic papillae was less dramatic. Thus, although *pop1* and *cer6-1* are allelic, the *pop1* mutation likely represents a more extreme allele.

Long-chain lipids are deficient in pop1 *pollen grains*

The striking similarities between the *cer2*, *cer6-1*, and *pop1* (*cer6-2*) mutants, including their defects in wax synthesis and pollen germination, suggested that lipid biogenesis might play a role in pollen–stigma communication. The lipid content of wild-type *Arabidopsis* pollen had not been characterized previously, and to test whether long-chain lipids were present, pollen lysates were prepared and extracted with chloroform, and the components in the organic phase were subsequently separated by gas chromatography. The chromatogram shown in Figure 6 indicated that wild-type pollen contains several long-chain lipid compounds. The identity of these molecules was confirmed by analysis of their of mass spectra, and their relative abundance is shown in Table 2.

To test whether the lipid content of *pop1* pollen might be aberrant, lysates were prepared in parallel and were compared to extracts from wild-type pollen. Although

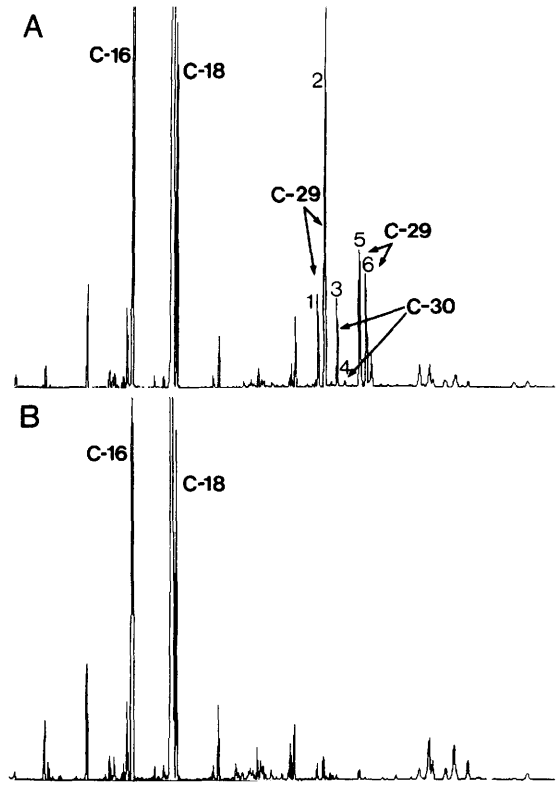

Figure 6. *pop1* pollen is deficient in long-chain lipids. Pollen lysates were extracted with chloroform and analyzed by gas chromatography followed by mass spectroscopy. The wild-type extract (*A*) contains 29- and 30-carbon lipids (identified in Table 2), whereas these compounds are virtually absent from the *pop1* extract (*B*).

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Table 2. *Lipid content in extracts from* pop1 *and wild-type pollen*

Peak number	Number of carbon atoms	Compound	Wild type	*pop1*	
	16	hexadecanoic acid	19	19	(1)
	18	octadecanoic acid	62	67	(1.1)
1	29	nonacosene	0.86	0.15	(0.17)
2	29	*n*-nonacosane	4.45	0.34	(0.08)
3	30	triacontene	0.92	0.05	(0.05)
4	30	*n*-triacontane	0.07	0.0	(–)
5	29	14- and 15-nonacosanol	2.04	0.19	(0.09)
6	29	15-nonacosanone	1.45	0.03	(0.02)

Peak numbers refer to those indicated in Fig. 6. Values represent percent of total lipids in pollen lysates. Identification of compounds was determined by analysis of their mass spectra, and quantitation is based on integration of total peak area from the ion chromatogram. Parentheses indicate fold-change from wild-type extracts.

the mutant pollen grains had normal levels of short-chain lipids (16 and 18 carbons), long-chain molecules were low in abundance (Fig. 6). Twenty-nine-carbon molecules (*n*-nonacosane, nonacosene, 14- and 15-nonacosanol, and 15-nonacosanone) were easily detected in the wild-type sample, but only a small fraction of these lipids (2–17% of wild-type levels) was found in the *pop1* extract, and 30-carbon molecules were present at <5% of the wild-type levels (Table 2). These results are completely consistent with the analysis of epicuticular waxes, indicating that *pop1* mutants extend few lipid chains to a length of 29-carbon atoms.

Pollen grains have an inner cell wall composed of pectin and cellulose (intine), an external wall formed by a complex polymer known as sporopollenin (exine), and a layer of tryphine, composed of lipid and protein molecules, embedded in the exine surface (for review, see Dobson 1989). The function of tryphine (also known as pollenkitt) is not fully understood; it may protect pollen from damage, attract insect pollinators, or promote adhesion of pollen to the stigma surface. Moreover, tryphine may play a direct role in cell signaling, because material extracted from the pollen surface stimulates callose production when applied to plants (Heslop-Harrison et al. 1974; Kerhoas et al. 1983).

To discern whether tryphine structure was altered in *cer2*, *cer6-1*, or *pop1* (*cer6-2*) mutants, flowers from each of the mutant plants were fixed and prepared for electron microscopy, using osmium tetroxide, a lipophilic stain. This analysis indicated a dramatic defect in *pop1* pollen grains. As shown in Figure 7, tryphine was apparent on the surface of wild-type pollen grains (Fig. 7A) but was virtually absent from the *pop1* pollen (Fig. 7B). Exine structure appeared normal both in thin sections (Fig. 7B) and in scanning electron micrographs (Fig. 7C–D). Pollen from *cer2* and *cer6-1* mutants showed a more subtle defect. Although tryphine was present, the number and size of the lipid droplets within the tryphine were significantly reduced (Fig. 7E–F), providing further evidence that the *cer6-1* allele is less extreme than *pop1* (*cer6-2*). Each of these *cer* mutants has defects in long-chain lipid biosynthesis, and the observation that tryphine structure is also aberrant strongly implicates long-chain lipids in the formation of structurally and functionally normal pollen.

In wild-type pollen grains, tryphine is deposited soon after meiosis by diploid tissue in the anther (tapetum) and becomes compact during anther dehydration (Dickinson and Lewis 1973a). Surprisingly, tryphine was observed on the surface of *pop1* pollen grains early in their development, although this material apparently lacked lipid droplets (Fig. 7G–H). As the mutant pollen developed, progressively less tryphine was detected until none remained at maturity. These results suggest the *pop1* mutation alters the stability of tryphine or its ability to remain bound to the pollen surface.

Discussion

The *pop1* (*cer6-2*) mutation results in pollen that behaves, in every respect, as if it no longer sends appropriate signals to the *Arabidopsis* stigma surface. At low humidity, hydration, and consequently germination, of the mutant pollen does not occur on the stigma. Moreover, the presence of callose within stigmatic papillae indicates that interactions with *pop1* pollen are aberrant. These results argue strongly that this mutation disrupts pollen–stigma communication.

In vitro germination substantiates that *pop1* pollen is viable. Moreover, the in vivo fertility defect is reversed when *pop1* pollen is mixed with wild-type pollen grains or when the mutant plants are shifted to a humid environment. These results indicate that, once hydrated, growth of *pop1* pollen is normal. Unlike the previously isolated temperature-sensitive defects in *Arabidopsis* flower development (Bowman et al. 1991), *pop1* represents a conditional, male-sterile mutation that specifically influences pollen function.

A deficiency in wax on the stems of *pop1* plants provided the first indication that long-chain lipid biosynthesis might be required for pollen function. Five wax-defective, *cer* mutations were shown previously to affect seed yields, but this defect was attributed to increased transpiration from the mutant plants (Dellaert et al. 1979; Koornneef et al. 1989). Under the growth conditions used here, two of these *cer* mutants (*cer2* and *cer6-*

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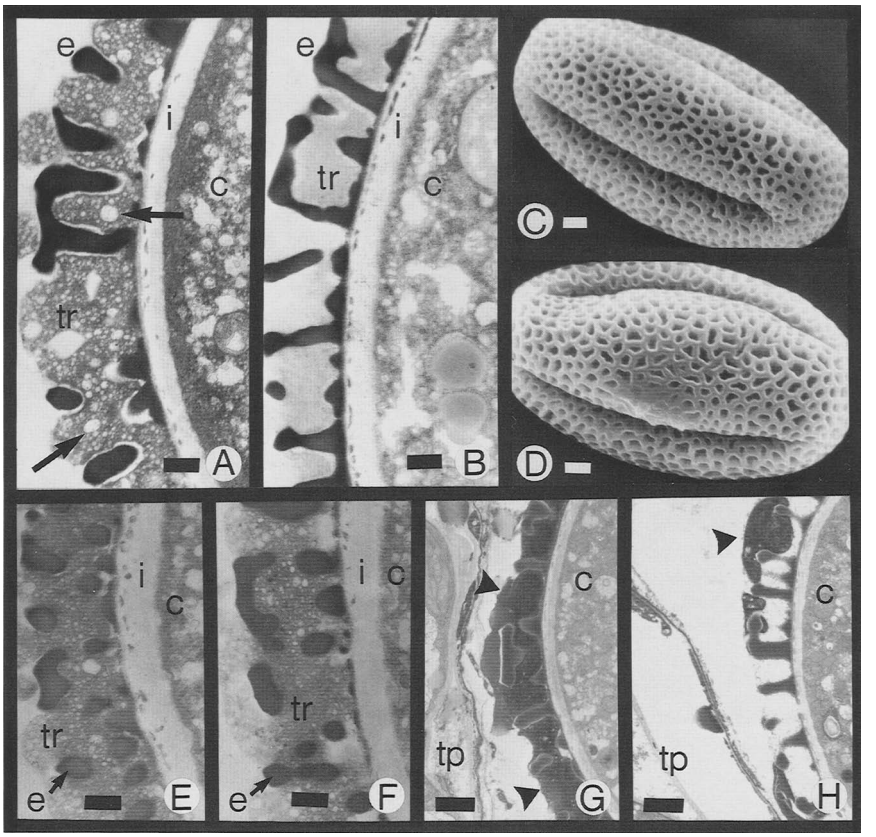

Figure 7. Ultrastructural analysis of the pollen surface. Transmission electron microscopy was used to visualize wall structure in wild-type (*A*), *pop1* (*B*), *cer2* (*E*), and *cer6* (*F*) pollens. Osmium tetroxide was used to stain lipids, and proteins were stained with uranyl acetate and lead citrate. Tryphine (tr) is notably absent from the surface of *pop1* pollen (*B*), whereas the size and abundance of tryphine lipid droplets (arrows in *A*) are reduced in *cer2* and *cer6* mutants. Exine structure, visualized by scanning electron microscopy, was identical in wild-type (*C*) and *pop1* pollen (*D*). Early in *pop1* pollen development, some aberrant tryphine (arrowheads) is present (*G*), which begins to disappear as the pollen matures (*H*) and the tapetum (tp) degenerates. (e) External cell wall (exine); (i) internal cell wall (intine); (c) cytoplasm; (tr) tryphine; (tp) tapetum. Bars, 0.5 μm (*A,B,E,F*); 2 μm (*C,D*); and 1 μm (*G,H*).

1) were shown to be male-sterile, producing pollen that has *pop1*-like defects and fails to germinate on the stigma surface. The phenotype of the *pop1* mutant differed from these *cer* mutants in one respect; only *pop1* pollen stimulated a dramatic production of callose within stigma cells. Complementation tests and linkage analysis indicated that *pop1* is a more deleterious allele of the *CER6* gene (*cer6-2*); *CER6* was mapped to chromosome 1 previously (Koornneef et al. 1989). Thus, alterations in either the *CER2* or *CER6* genes impair both wax synthesis and fertility, strongly indicating a role for long-chain lipids in the development of functional pollen.

Wild-type, but not *pop1*, pollen was found to contain significant quantities of long-chain lipid molecules. As in maize (Bianchi et al. 1990), these lipids are predominantly alkanes, secondary alcohols, and ketones, rather than the aldehydes and primary alcohols found on the surface of stems (Hannoufa et al. 1993). Most likely, these long-chain pollen lipids reside within the tryphine that coats the surface of pollen grains. No tryphine remains on the surface of *pop1* pollen grains at maturity; less severe tryphine alterations were also detected in *cer2* and *cer6-1* pollen. These observations thus provide direct genetic evidence that, at low humidity, tryphine is required for pollen germination on the stigma and indicate that pollen–stigma signaling is mediated, at least in part, through tryphine components. After pollen contacts the stigma, some of the tryphine spreads along the proteinaceous coating of the stigma surface, establishing a region of continuity (Elleman et al. 1992). Long-chain lipids might be required to mobilize tryphine components, to signal stigma cells directly, or to provide a matrix for assembly of other essential tryphine molecules.

The results described above suggest a general model for pollen hydration in *Arabidopsis* (Fig. 8). First, compatible wild-type pollen must signal the stigma surface, resulting in the transfer of water through channels in the stigma cell wall (Fig. 8A). In *cer2*, *cer6-1*, and *pop1* (*cer6-2*) mutants, alterations in tryphine structure disrupt this communication, consequently eliminating pollen hydration. It is conceivable that the loss of tryphine might affect hydration indirectly, resulting in highly desiccated pollen grains that require more than normal amounts of water to become fully hydrated. The failure to observe any decrease in the diameter of the mutant pollen relative to wild type, or, at low humidity, to detect even partial hydration of the mutant pollen by stigma cells (Fig. 3) argues against this interpretation. More likely, tryphine is required directly to establish communication between pollen and the stigma surface.

The tryphine defect in *pop1* plants is clearly more extreme than that observed in *cer2* and *cer6-1* mutants. Presumably, the surface of *pop1* pollen is so aberrant that it strongly induces callose production on the stigma, a response that was less dramatic after pollination with

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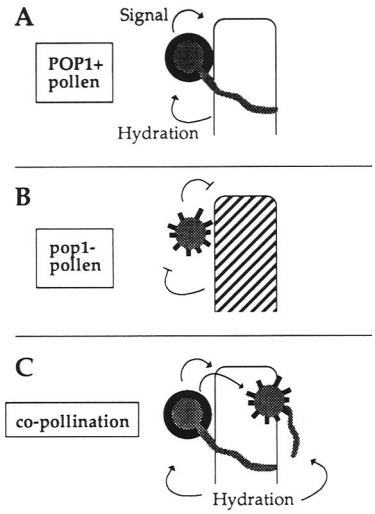

Figure 8. Interaction of pollen with the stigma surface. Wild-type pollen (*A*) signals the stigma surface, resulting in hydration. Defective signaling occurs with *cer2*, *cer6-1*, or *pop1* (*cer6-2*) pollens (as a result of tryphine defects), resulting in no pollen hydration (*B*). *pop1* pollen also stimulates callose production within stigma cells (shading). Copollination with wild-type and *pop1* pollen results in germination of both pollen grains, suggesting that the pollen signal diffuses (*C*).

cer2 or *cer6-1* pollen (Fig. 8B). Although the presence of callose in stigma cells is correlated with incompatible pollination (especially in *Brassica*), it is not produced in every case, and its role therefore remains unclear. Callose may serve as a general defense against pathogens, and only those pollen grains that differ significantly from compatible pollen might stimulate callose production in the stigma. High humidity rescues *pop1* sterility and presumably bypasses potential callose barriers by allowing pollen tube growth that is independent of a close association with the papillae cells.

Finally, copollination experiments suggest that the pollen signal can diffuse and, thus, promote the growth of nearby pollen grains (Fig. 8C). This could be accomplished through spreading of wild-type tryphine to the mutant pollen grains. Although molecules that mediate the pollen signal have not been identified, recent experiments indicate that low levels of flavonoids are required for pollen germination in maize, petunia, and tobacco, suggesting a role for these compounds in pollen–pistil signaling (Mo et al. 1992; Taylor and Jorgensen 1992; Ylstra et al. 1992). *pop1* and other mutants with similar phenotypes provide an opportunity for testing the effects of such molecules, or other plant factors in vivo; any substance that signals the stigma and, hence, stimulates pollen hydration should promote pollen germination and thus seed formation.

The germination of *pop1* pollen in humid conditions not only supports the idea that the mutant pollen, if hydrated, is fully functional, but also allows propagation of *pop1* plants. This conditional, male-sterile mutant should greatly facilitate construction of *Arabidopsis* strains. Fertilization of the sterile plants with *POP1*$^+$ pollen will result in both heterozygous hybrids and *pop1* homozygotes; heterozygotes can be identified subsequently on the basis of epicuticular wax production, a phenotype that is apparent at an early stage. In crop plants, much effort has focused on identifying male-sterile mutations that allow breeders to control hybrid production. In addition, Mariani et al. (1990) genetically engineered male sterility by expressing a cytotoxic ribonuclease gene within the tapetum, completely eliminating pollen production. Propagation of these natural and engineered male-sterile mutants requires either manual pollination (which is tedious and expensive on a large scale) or restoration of fertility, either through a compensating mutation or expression of a ribonuclease inhibitor (Mariani et al. 1992). By using the same tissue-specific promoter, expression of antisense *CER2* or *CER6* messages, solely within the tapetum, should be possible. This alteration presumably would result in conditional male sterility without eliminating the waxy coating on stems and would thus greatly facilitate the propagation of any plant that requires these genes, or their homologs, for pollen function.

The similarities between the *pop1* mutation and the self-incompatibility (SI) studied in many *Brassica* species is striking. Both regulate pollen germination at the stigma surface, often through control of pollen hydration. In addition, there are many examples of callose production in stigma cells that directly contact incompatible, self-pollen. High humidity results in efficient germination of *pop1* pollen and can sometimes rescue self-incompatible pollen, although in the latter case, pollen tube growth is eventually arrested. Finally, copollination with compatible mentor pollen can overcome SI (Knox et al. 1987; Sarla 1988), resembling the effect of wild-type pollen on *pop1* pollen germination. SI responses differ from *pop1* pollinations in one respect: Immature flower buds from self-incompatible plants often can be self-pollinated, but neither mature nor immature *Arabidopsis* stigmas promote germination of *pop1* pollen. Nonetheless, SI could serve as a model for many aspects of *pop1* pollen rejection in *Arabidopsis*. Recently, a multigene family involved in pollen–stigma interactions in *Brassica* was identified (for review, Nasrallah et al. 1991). These highly polymorphic genes encode glycoproteins that are present at high levels in the stigmatic papillae. Genes similar in sequence are present in *Arabidopsis*, and at least one of these, *AtS1*, is expressed in flower buds (Dwyer et al. 1992). The *AtS1* gene product thus potentially functions at the stigma surface and might regulate interaction between pollen and the stigmatic papillae.

Clearly, the *CER2* and *CER6* genes are required both for stem wax formation and for pollen function. At the biochemical level, these gene products presumably mediate the addition of 2-carbon acetyl groups to long-chain fatty acids. In *Arabidopsis* seeds, fatty acid elongation is regulated by several genes (Lemieux et al. 1990), and in leaf tissue, long-chain fatty acids apparently are synthesized by an enzyme complex (Bessoule et al. 1989). The

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phenotypic differences between the *cer6-1* and *pop1* (*cer6-2*) mutant strains indicate that the *pop1* allele is more severe, perhaps representing a null mutation. In addition to *CER2* and *CER6* mutations, alterations in three other genes required for wax synthesis, *CER3*, *CER8*, and *CER10*, result in low seed yields (Dellaert et al. 1979; Koornneef et al. 1989), although none of these alleles are known to be null or to specifically affect pollen function. The common feature of all of the partially sterile *cer* mutants is a reduction in 29-carbon alkane, alcohol, or ketone levels (Hannoufa et al. 1993), indicating that these long-chain lipid molecules are necessary for pollen–stigma interactions, either for directly signaling the stigma or for stabilizing other essential molecules. Further characterization of the *CER* gene products, including identification of their biochemical functions, should elucidate the role of lipids in pollen–stigma communication, not only in *Arabidopsis*, but in other angiosperms as well.

Materials and methods

Genetic techniques

Strains were Landsberg *erecta*; *cer* mutants were obtained from the *Arabidopsis* Stock Center (Columbus, OH). For crosses, anthers were removed from flowers before pollen maturation (dehiscence), and immature stigmas were dusted with pollen immediately or 2–3 days later (mature). All *pop1* analyses were on segregants from a strain twice backcrossed to wild-type. Growth conditions were 24-hr fluorescent lighting [85 micro-Einsteins per square meter ($\mu E/m^2$)] at 18–22°C and 50–70% relative humidity (ambient conditions) or 90% relative humidity (Conviron chamber). Mutants were obtained by immersing seeds in 4 mM ethyl nitrosourea for 24 hr at room temperature. Mutagenized (M1) seeds (60,000) were grown, and plants generated from the resulting self-seeds (M2) were screened for sterile mutations at a density of ~500 seeds/ft^2. Reciprocal outcrosses indicated ~40% of the mutants were male-sterile (most of which produced no pollen) and 10% were female-sterile. Homozygous *pop1* seeds are strain WNN6.

Microscopy

Callose staining (Eschrich and Currier 1964) was performed on flowers fixed for 1 hr in chloroform/ethanol/acetic acid [(vol/vol) 6 : 3 : 1], incubated for 10 min in 4 M NaOH, then neutralized in 50 mM phosphate buffer (pH 7.5), and stained with aniline blue at 50 μg/ml in 50 mM phosphate buffer (pH 7.5). Fluorescence was observed on a Zeiss Axiophot microscope with a Zeiss 365G filter. Pollen hydration was assessed by light microscopy (Zeiss Axiophot); before hydration, *Arabidopsis* pollen has three conspicuous furrows and is oval-shaped. Hydration of wild-type pollen occurs within minutes of pollination, resulting in a twofold increase in pollen diameter, disappearance of the furrows, and formation of spherical pollen grains. For scanning electron microscopy, fresh stems and flowers were collected, placed on stubs coated with double-stick tape, and gold-coated to 8 nm with a Polaron E5400 sputter coater. Observations were with a Philips SEM505 microscope. For transmission electron microscopy, mature anthers and developing flower buds were fixed in 3% glutaraldehyde (EM Sciences, Ft. Washington, PA), 100 mM phosphate buffer (pH 7) for 5 hr at 20°C, then overnight at 4°C. Subsequently, tissues were fixed in 2% osmium tetroxide (EM Sciences), 100 mM phosphate buffer (pH 7) for 2 hr at 20°C, washed in several changes of boiled, distilled water, and then incubated in 1% uranyl acetate for 30 min. Dehydration was in a graded ethanol series (30–100%), and embedding was performed as described previously (Oliveira et al. 1983), except *n*-octenyl succinic anhydride was substituted for hexenyl succinic anhydride. The resin was polymerized in aluminum weighing dishes for 2 days at 60°C. Thin sections measuring 60–90 nm (silver–gold interference color) were prepared with a Reichert–Jung Ultracut E microtome, and observations were on a Philips TEM410 microscope.

Pollen preparation and extraction of lipids and waxes from pollen and stems

Pollen was removed from mature flowers by suspending them in pollen germination medium (17% sucrose, 2 mM $CaCl_2$, 1.65 mM H_3BO_3 at pH 7), mixing vigorously, and then withdrawing the pollen suspension with a pipette. Purified pollen was then collected by centrifugation in a microcentrifuge (5000*g*). For germination assays pollen was suspended in germination medium at 100 grains/μl and incubated in microtiter wells for 12 hr at 20°C. Pollen lipid analysis (Fig. 6) was performed on 5 mg of purified pollen (from ~1500 flowers), and unambiguous identification of long-chain lipid compounds (Table 2) required 15 mg of purified pollen. Pollen pellets were suspended in 40 μl of ice-cold lysis buffer (50 mM Tris-HCl, 5% glycerol, 100 mM KCl, 10 mM EGTA at pH 8.0) containing protease inhibitors (5 μg/ml of antipain, leupeptin, and pepstatin, 5 mM ε-amino-*n*-caproic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 10 μM *p*-aminobenzamidine). The suspension was Dounce homogenized (on ice) vigorously for 4 min in a microcentrifuge tube, and the cell lysate was suspended in 1 ml of lysis buffer with protease inhibitors and transferred to a 15-ml Corex tube containing 2 ml of chloroform and 2 ml of methanol. The extract was mixed, and the phases were separated in a tabletop clinical centrifuge. The chloroform phase was subsequently extracted once with water and was then collected and evaporated under nitrogen. Chromatographic analysis was performed as described previously (Hannoufa et al. 1993) by silylation with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). The presence of long-chain fatty alcohols was detected as fatty acids after treatment with 1 N HCl in methanol (Hannoufa et al. 1993); these long-chain fatty alcohols were present in trace amounts. The quantitations were based on an internal standard of nonadecane. Analysis of epicuticular wax was performed similarly, except wax was collected by immersing 1 gram of stems into chloroform for 20 sec.

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