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Pistil Factors Controlling Pollination

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INTRODUCTION

The successful establishment of angiosperms on land is in part determined by their floral design. Because plants cannot move to find the ideal mate, they have developed a great variety of flowers to provide different mechanisms of pollen release, pollen transfer, and deposition of the pollen from the male to the female sexual organs, the anther and the pistil, respectively. Pollination ensures the maintenance of the species, but it is also a means to increase genetic diversity and, with it, the potential to adapt to new environments. The position and morphology of the anthers and the pistil have often coevolved with the mode of pollen dispersal and pollen receipt, aided either by wind or by animals. Nevertheless, pollination can fail at various points during these processes, causing the extinction of rare plants and lower crop yields (Wilcock and Neiland, 2002).

The pistil, the pollen-accepting organ that occupies the central position in a flower, is composed of one or more fused carpels that bear the ovules. Pistil development initiates with the formation of the carpel primordia, and the floral identity genes of class C, such as *AGAMOUS* in *Arabidopsis thaliana* and *PLENA* in *Antirrhinum majus*, dictate carpel identity (Ng and Yanofsky, 2000). Carpel fusion occurs very early in pistil development. Even in species with single pistils, fusion of the carpel margins is required to form a closed carpel. For instance, tobacco (*Nicotiana tabacum*) and Arabidopsis pistils are made from the fusion of two carpels, and that of the cultivated tomato is formed from five fused carpels (Gasser and Robinson, 1993). Close to the time of ovary closure, the carpel walls extend vertically to form one or more hollow cylinders, the styles. This process requires cell division at first and cell elongation later. The length, number, and structure of the styles are typical within each species and variable between them. Stylar extension facilitates pollen capture, and the wide variety of pistil morphologies reflects the different pollination mechanisms found among the angiosperms (Barrett et al., 2000). While the style is elongating, the inner tissues differentiate to form the specialized secretory zone of the stigma on the top of the style and the transmitting tissue within the hollow cylinder of the style. In some species, such as lily, the style remains hollow, with only one layer of secretory tissue lining the inner surface of the cylinder (Dickinson et al., 1982).

At flower maturity, when pollination takes place, the pistil is fully developed and composed of stigma, style, and ovary. Whether the pollen is transported by the wind or by animal pollinators, after landing on the stigma the pollen grain hydrates and germinates a tube. This tube then penetrates the specialized tissues of the pistil, growing into the stigma and the style to reach the ovules in the ovary. During this process, numerous cell–cell interaction events occur between the cells of the sporophyte (the pistil) and the male gametophyte (the pollen grain and the tube). Most of the existing knowledge regarding pollen–pistil interaction was gathered from the study of self-incompatibility in several species. Nevertheless, considerable data are now available on the prepollination and postpollination events and molecules that make the pistil ready to interact with the pollen after compatible pollinations (Lord and Russell, 2002). Some of these interactions are discussed in this review.

POLLEN–STIGMA INTERACTIONS

Stigma receptivity to pollen can persist from 1 h to several days in different species (Heslop-Harrison, 2000) and is influenced by several factors. Whether it is a dry stigma or a wet stigma, receptivity is defined as the ability to ''capture'' pollen by adhesion, to let it hydrate and consequently germinate a pollen tube. The appropriate stage of stigma development is crucial for receptivity. For example, on immature stigmas of pear flowers, mature pollen can adhere but do not hydrate and germinate. On a degenerating stigma, pollen can adhere, hydrate, and germinate, but pollen tube growth arrests abruptly (Sanzol et al., 2003). A cuticle of varying thickness is deposited on most stigmas above the epidermis, and compatible pollen deposited on a heavily cutinized surface never germinates (Heslop-Harrison, 2000). At the receptive stage, the cuticle breaks at some places, aided either by visiting insects or by increasing turgidity of the stigma, and the activity of some enzymes such as esterases increases in the stigma of several species (Dafni and Maues, 1998). The pollen of *Brassica napus* was shown to carry an active cutinase to break the cuticle of the papilla (Hiscock et al., 1994; Edlund et al., 2004).

The epidermis of the stigma differentiates to form the specialized papilla cells. Crucifers, such as *Brassica* species and Arabidopsis, have a dry stigma with many large papillae that interact directly with the pollen. Here, the pollen is accepted if

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compatible, or rejected if incompatible or from a foreign species, and adheres and hydrates. The best-characterized pollen–pistil interaction on a dry stigma is the self-incompatibility response in *Brassica*. Its components constitute the male determinant S-locus cystein rich protein, the S-locus glycoprotein female determinant secreted by the stigma into the cell wall, the S-locus receptor kinase located in the stigmatic plasma membrane, and its target arm-repeat-containing protein 1, also produced in the stigma (Nasrallah, 2000).

However, little is known about the genes required for successful (compatible) pollen–stigma interaction in crucifers. No mutants have been identified to date with defects in papillar cell functions, although cell ablation experiments clearly demonstrated that papillae are required for pollination. Papillar cell ablation, in combination with differential display, also was used to identify genes expressed in the *Brassica napus* epidermis (Kang and Nasrallah, 2001). The results of this experiment led to the identification of the *PIS63* gene, which is homologous with an Arabidopsis gene of unknown function. By a transgenic approach, it was shown that a reduction of *PIS63* expression in the stigma correlates with reduced pollen germination and seed set, but pollen adhesion was not affected (Kang and Nasrallah, 2001). This finding, together with the identification of Arabidopsis pollen mutants affected in adhesion (Edlund et al., 2004), supports the idea that pollen adhesion on a dry stigma is a property of the pollen. The adhesion of pollen on wet stigma is facilitated by the presence of the exudate, which can be aqueous, as in lily, or lipidic, as in tobacco and petunia. In addition, proteins and sugars are present in both types of exudate.

Pollen hydration and tube germination can occur very fast or may take up to 1 h, depending on the degree of pollen desiccation at the time of anther dehiscence. Grass pollen, for example, is never fully dehydrated and is metabolically active when shed from the anthers. This makes it very vulnerable, but it germinates within minutes after landing on a stigma. By contrast, pollen of lily is very dehydrated when released, which enables it to survive under extreme environmental conditions, but it takes $\sim$1 h to germinate after hydration on the appropriate stigma (Heslop-Harrison, 2000). Hydration on the dry stigmas of the crucifers requires contact with the papillae and is aided by the pollen coat that flows from the exine to form a contact zone between the two (Elleman et al., 1992). In particular, lipids and oleosin-like proteins on the pollen coat were shown to be essential for hydration in Arabidopsis (Edlund et al., 2004), demonstrating that, as in pollen adhesion, hydration also is determined by the pollen. We found no oleosin-like proteins on the coat of tobacco pollen, suggesting that on this wet stigma hydration may be facilitated by other factors (Bots and Mariani, 2004).

In tobacco and other solanaceous species, the lipidic exudate is produced in the cells of the secretory zone of the stigma and is secreted at pistil maturity. Underneath the exudate, a thin layer of water (in the form of crystals) surrounds the cells of the secretory zone. After pollination, pollen grains sink through the exudate and establish direct contact with the stigma or with each other. Using cryo-scanning electron microscopy, it was evident that at the contact site between pollen and stigma, the water crystals gradually disappeared (Wolters-Arts et al., 2002). We produced transgenic tobacco plants in which the secretory zone of the stigma was ablated by tissue-specific expression of a Barnase ribonuclease. Pollen–pistil interaction is arrested in this plant, resulting in female sterility (Goldman et al., 1994). In particular, this stigmaless pistil does not produce exudate, and cryo-scanning electron microscopy images confirmed that no water crystals were present around the dead cells of the ablated stigma (Wolters-Arts et al., 2002). Yet, upon application of exogenous tobacco exudate or a mixture of triacylglycerides, pollen still could hydrate. It is not clear how the water passes through the dead cells of the stigmaless pistil to the pollen grain. However, contact alone is not sufficient for hydration, because even the exertion of a constant mechanical pressure on the pollen grains did not lead to hydration without exudate or lipids (Wolters-Arts et al., 2002). Recently, many water channel proteins, collectively named aquaporins, were described in plants; these indicate the existence of a very rapid and regulated water transport across biological membranes (Johanson et al., 2001). Some of these aquaporins or aquaporin-like genes are expressed in the pistil, among other plant tissues, of *Solanum chacoense* (O'Brien et al., 2002) and *Brassica* (Marin-Olivier et al., 2000; Dixit et al., 2001). Although it is attractive to speculate that they play a role in pollen hydration by the stigma, 35 putative aquaporin genes, classified on the basis of sequence identity, were found in the genome of Arabidopsis. With such high redundancy, it may be difficult to prove the function of aquaporins in the pollen–stigma interaction. We have produced transgenic tobacco plants in which a class of PIP2 aquaporins, expressed in flower organs, was silenced by RNA interference. We found no effects on pollen hydration, pollen tube growth, and seed set (M. Bots and C. Mariani, unpublished data).

Once pollen is hydrated and germination occurs, tube growth is directed within the stigma. In dry stigmas, tube growth occurs through the papilla cell wall, and pollen tube penetration is accompanied by cell wall expansion or loosening in the stigma (Elleman et al., 1992). Tube growth seems to be facilitated by wall-degrading enzymes produced either by the pollen itself or by the stigma. This is in agreement with the finding that group I allergens of grass pollen have expansin activity (Cosgrove et al., 1997) and that a polygalacturonase was localized in *Brassica* germinating pollen (Dearnaley and Daggard, 2001, and references therein). In tobacco, a species with a wet stigma and a solid style, pollen tubes grow through the intercellular spaces between the cells of the secretory zone, within the exudate produced by these cells. Interestingly, we recently discovered that tobacco exudate has cell wall–loosening activity (J. Nieuwland, J. Derksen, C. Hilbers, and C. Mariani, unpublished results). The pistil pollen allergen-like (PPAL) protein, one of the proteins secreted in the exudate, is highly similar to β-expansins (Pezzotti et al., 2002) and obviously was the most suitable candidate for a cell wall–loosening activity that could facilitate pollen tube growth in tobacco stigmas. However, we found that PPAL is not active in cell wall loosening in an in vitro assay using an expansometer constructed according to Cosgrove (1989). Moreover, we have determined that another protein is responsible for cell wall–loosening activity. This protein is a lipid transfer protein (LTP), the most abundant protein in the exudate of tobacco (J. Nieuwland J. Derksen, C. Hilbers, and C. Mariani, unpublished results). LTPs can bind acyl lipids in vitro (Kader,

1996) and constitute the largest group in the catalog of Arabidopsis genes involved in acyl lipid metabolism (Beisson et al., 2003). LTPs represent a group of proteins whose sequences are highly divergent and that may have different functions in vivo apart from binding lipids. Indeed, another function for LTP(-like) proteins is that of the SCA protein (stigma/stylar Cys-rich adhesin) secreted in the aqueous lily exudate (Lord, 2003). Lily has a hollow stigma and a style with a secretory epidermis that pollen tubes travel in to the ovary. Therefore, no tissue penetration is required for pollen tubes to grow from the stigma into the style. SCA, combined with another small stigma protein, chemocyanin, induces chemotropic activity on lily pollen tubes grown in vitro (S. Kim, J.C. Mollet, J. Dong, K. Zhang, S.Y. Park, and E.M. Lord, unpublished data). Pollen tubes reorient their growth toward a gradient of chemocyanin, and SCA potentiates this activity.

Using our stigmaless transgenic tobacco plants (Goldman et al., 1994), we studied pollen–pistil interaction in this system, hoping to dissect the components required. The ablated surface of this stigmaless pistil is dry, and when tobacco pollen is used for pollination, it fails to hydrate. If humidity is provided, pollen hydrates but only a few short pollen tubes germinate. This finding indicates that the defect is only on the stigma side and not in the pollen. The addition of germination medium containing boron, calcium, and sugar improved tube germination, but penetration of the pistil tissues failed. Surprisingly, when we applied exudate collected from wild-type pistils, pollen tubes germinated and grew directly in the pistil tissues (Goldman et al., 1994). Currently, we are investigating the function of the proteins secreted in the exudate, some of which (LTP and PPAL) have been discussed above.

One question that arose from the discovery that exudate is sufficient for pollen tube growth in the style was whether exudates secreted on the stigmas of other species could produce a similar effect (Wolters-Arts et al., 1998). Figure 1 shows that exudate of petunia is as good as that of tobacco in restoring pollen tube penetration, whereas that of lily only enables pollen hydration and germination. By comparing the types of exudate and by performing other experiments, we concluded that lipids might be important for directional pollen tube growth. One particular class of unsaturated triacylglycerides, applied on the ablated stigmas of transgenic tobacco, was revealed to be sufficient for this to occur (Figure 1) (Wolters-Arts et al., 1998). This conclusion excludes the possibility that the proteins of the exudate have a direct and decisive function in the pollen–pistil interaction, at least in tobacco. The cell wall–loosening activity of the tobacco LTP, for instance, may turn out to have only a fine-tuning activity on pollen tube growth in vivo, perhaps to speed up pollen tube growth or to make the pollen tube wall more permeable to larger pistil compounds. It also is possible that some functions may be accomplished in different ways and are redundant in the stigma, to ensure that pollen tube growth is carried on to deliver the sperm cells to the embryo sac.

Another function attributed to nonspecific LTPs and to other proteins secreted in the exudate is that of defense against pathogens (Garcia-Olmedo et al., 1995). Proteinase inhibitors (Miller et al., 2000), thaumatin-like proteins, and other

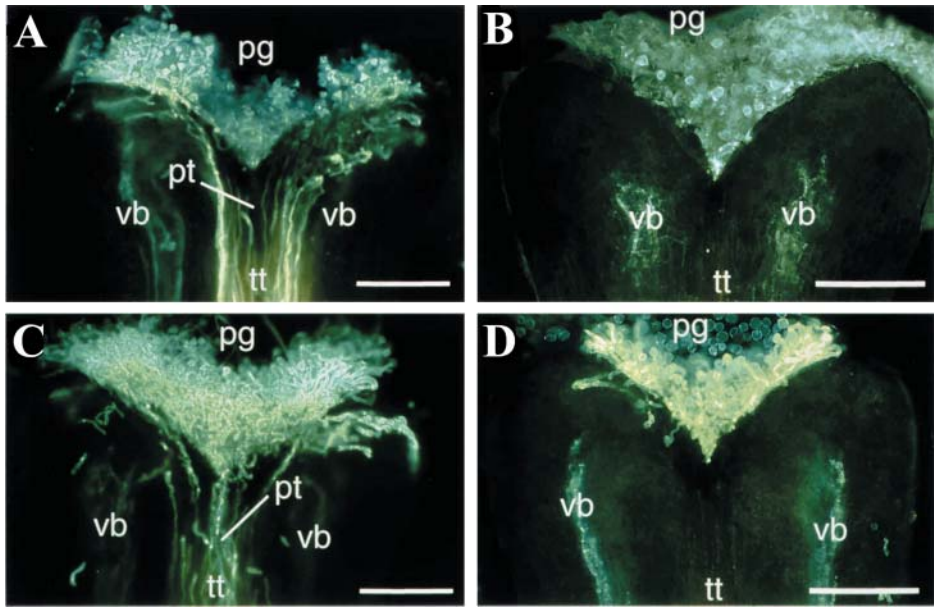

Figure 1. Fluorescence Micrographs of Longitudinal Sections of Stigmaless Pistils at 24 h after Pollination.

(A) After application of petunia exudate, pollen grains on the stigmaless surface become hydrated and germinate and the pollen tubes penetrate the stylar tissue.

(B) to **(D)** Effects of the application of lily exudate **(B)**, trilinolein **(C)**, and saturated triacylglycerides **(D)** are shown.

pg, pollen grain; pt, pollen tube; tt, transmitting tissue; vb, vascular bundle. Bars = 100 μm.

defense-related proteins (Kuboyama, 1998, and references therein) were shown to be expressed and to accumulate in the stigmas of species of the Solanaceae, possibly to prevent predator or pathogen attack. However, these functions are not related directly to pollination.

One conclusion that can be drawn regarding pollen–stigma interaction is that although pollination on dry stigmas and wet stigmas appears very different, there are similarities in the two systems. For example, lipids are required in crucifers and in solanaceous species for pollen hydration and tube penetration, and proteins such as expansins have been suggested to aid pollen tube growth in both plant families. The difference lies only in where these factors accumulate: the pollen coat in species with dry stigmas (crucifers) and the exudate in species with wet stigmas (Solanaceae).

POLLEN TUBE GROWTH IN THE STYLE AND THE OVARY

Based on morphological features, styles can be classified as open, closed, or semisolid. In open styles, characteristic of many monocotyledons, such as lily, pollen tubes grow in the canal filled with mucilage, along the transmitting tract epidermis. Most dicotyledons, such as Solanaceae species, have a closed style in which the pollen tubes grow through the transmitting tissue, a continuum with the stigma secretory zone. The cells of the transmitting tissue are connected in a file by plasmodesmata on their transverse walls, whereas their longitudinal walls are separated by the intercellular matrix (IM) that they secrete. Chemical analysis has shown that the IM contains free sugars, polysaccharides, free amino acids, proteins, glycoproteins, proteoglycans, and phenolic compounds, usually thought to function in pollen tube nutrition, recognition, and guidance (Cheung, 1996). In this matrix, in general, pollen tubes grow with considerable speed. Tobacco pollen tubes, for instance, take ~26 to 30 h to reach the ovary, at a distance of 4 cm from the stigma. This makes the speed of pollen tube growth ~1.7 mm/h. Pollen of many species can germinate and grow a tube in vitro in a medium that should contain at least calcium, boron, and an osmoticum. However, pollen tubes grown in vitro never reach the length they can attain when growing in the pistil (Lord, 2003). Nevertheless, the ease with which tube growth can be followed in vitro has been very valuable in the study of pollen tube cell biology (Hepler et al., 2001).

The discrepancy between in vitro and in vivo growth indicates a strong contribution of the sporophytic tissue of the pistil to pollen tube growth. A depletion in the amount of reserves within the transmitting tissue indicates that tube growth occurs at the expense of the stylar reserves (Herrero and Hormaza, 1996). The use of exogenous sugars by growing pollen tubes has been demonstrated for many species, and glycosylated proteins such as Hyp-rich glycoproteins (HRGPs) are ideal candidates to provide nutritional support to pollen tubes during growth. HRGPs are abundant in the pistils of several species, and many of them are arabinogalactan proteins (AGPs). Up to 90% of their mass can be contributed by carbohydrates linked by *O*-glycosylation to the Hyp and Ser residues of the protein backbone. AGPs in the pistil have been proposed to act as glue or lubricants and may guide pollen tubes to the ovary (Cheung and Wu, 1999).

The best-characterized HRGPs that accumulate in the style are the tobacco transmitting tissue–specific (TTS) glycoproteins. The mature TTS proteins span a molecular mass range from 45 to 105 kD in which the backbone peptides are 28 kD, and the carbohydrate moieties consist mostly of galactose. TTS glycoproteins do react with Yariv reagent, an AGP binding β-glucosyl, but are classified as ''nonclassic'' AGPs (Cheung and Wu, 1999). A series of elegant experiments has shown several functions of TTS glycoproteins. They promote pollen tube elongation in vitro and in vivo (Cheung et al., 1995), they are deglycosylated by hydrolyzing enzymes, suggesting that they provide nutrients to pollen tubes (Wu et al., 1995), and they attract pollen tubes grown in a semi-in vivo culture system (Cheung et al., 1995). The carbohydrate moiety associated with the TTS protein is arranged to form a gradient of glycosylation, increasing toward the bottom end of the style. This gradient may have a chemotropic effect on growing pollen tubes (Wu et al., 1995). Recently, the *Nicotiana alata* homolog of tobacco TTS, NaTTS, also was shown to stimulate pollen tube growth in vitro and to attract pollen tubes in the semi-in vivo culture system (Wu et al., 2000).

Other HRGPs accumulate in the styles of *N. alata* and tobacco. They are very similar to TTS in the primary structure of their backbone but differ in the carbohydrate composition, which can explain the differences found in pollen tube growth assays. The galactose-rich style glycoprotein (GaRSGP) and the 120-kD glycoproteins both were isolated from *N. alata* (Lind et al., 1994; Sommer-Knudsen et al., 1996), and pistil-specific extensin-like protein (PELP) was isolated from tobacco (Bosch et al., 2001). These proteins do not form a glycosylation gradient, nor are they deglycosylated after pollination, and they do not attract pollen tubes in a semi-in vivo assay. Their localization pattern also is different from that of TTS. Whereas TTS is bound tightly to the IM, GaRSGP is localized to the cell wall of the transmitting tissue cells, and the 120-kD glycoprotein is in the IM but translocated through the pollen tube wall into the tube cytoplasm after pollination (Lind et al., 1996). Finally, PELPIII accumulates in the IM and also is translocated in the wall of the pollen tube; eventually, it ends up in the callose plugs, but it never enters the tube cell cytoplasm (de Graaf et al., 2003) (Figure 2). It is noteworthy that such large molecules can get across the pollen tube wall and in one case across the membrane. Probably the same or similar translocation mechanism, although not understood, is used to incorporate the large S-RNase determinant of the gametophytic self-incompatibility (SI) (Luu et al., 2000; Kao and Tsukamoto, 2004). This also is in agreement with the finding that in vitro S-RNases can form protein complexes with TTS and NaPELPIII glycoproteins (Cruz-Garcia et al., 2003). However, the function of the three HRGPs reported above is still unknown, although it is possible that they all contribute to creating an environment rich in nutrients and lubricants and physically favorable for pollen tube growth. The transition from tube growth in the stigma to growth in the style is characterized by a reduction of the space in which the tubes grow. Competition between pollen tubes increases during this transition, and only the fittest tubes reach the ovary. Therefore, it is important that the pistil provides an optimal environment to support pollen tube growth.

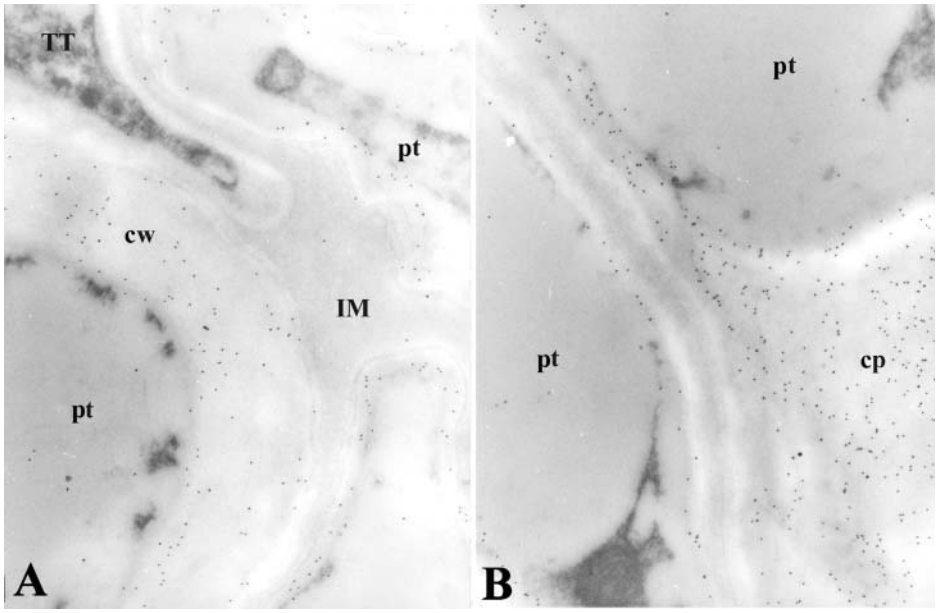

Figure 2. Localization of PELPIII in Style Sections Containing Pollen Tubes after 16 or 24 h of Pollination.

PELPIII is distributed mainly over the pollen tube callose walls (cw), whereas the detection of PELPIII in the intercellular matrix (IM) is lost. This PELPIII distribution in the tube walls was found in transverse **(A)** and longitudinal **(B)** sections of pollinated style parts. cp, callose plug; pt, pollen tube; TT, transmitting tissue.

Besides nutrition, it is proposed that the style provides guidance cues to the pollen tubes (Lord, 2003). An opposite theory, however, says that chemical cues guide the tube only on the stigma and in the ovary, but that growth in the style is constrained by physical structures (Lush et al., 2000). Strong evidence for pollen tube guidance in the style, in addition to the chemotropic response to the glycosylation gradient of TTS, comes from the contact-stimulated guidance of the extracellular matrix in lily styles (Lord, 2003). SCA, an LTP-like protein, was isolated from the style of lily in combination with another stylar molecule, later identified as a low-esterified pectic polysaccharide (Mollet et al., 2000; Park et al., 2000). The SCA/pectin combination was identified using a functional adhesion assay in which an artificial stylar matrix was made by immobilizing crude stylar extract on a nitrocellulose membrane (Jauh et al., 1997). When germinated, lily pollen were incubated with the artificial matrix in ''in vitro'' germination medium and pollen tubes adhered and grew on the matrix as they do on the cells of the transmitting tract epidermis. Recently, it was demonstrated that a recombinant SCA protein produced in *Escherichia coli* is as functional in the adhesion assay as the native SCA (Park and Lord, 2003). The SCA and pectin bind each other and provide an adhesive matrix that acts as a contact-stimulated, or haptotactic, guidance mechanism for pollen tubes. It has been demonstrated that no chemotropic gradients exist in the lily style (Iwanami, 1959). When pollen is applied to an open style, pollen tubes grow toward both the stigma and the ovary.

A different mechanism of pollen tube guidance was discovered recently. In this case, a gradient of γ-aminobutyric acid (GABA) was detected in the Arabidopsis pistil (Palanivelu et al., 2003; Edlund et al., 2004). This important discovery was made while studying the function of the *pollen pistil 2* (*POP2*) gene of Arabidopsis, which was shown to encode a GABA transaminase. This finding also was in agreement with the finding that wild-type unpollinated pistils accumulate low levels of GABA in the stigma, an increasing amount in the style, and even more in the ovary locule, providing a gradient of GABA to the growing pollen tubes. In the *pop2* mutant, GABA is not degraded and accumulates at much higher levels than in the wild type, but it is distributed more equally through the whole pistil. Pollen tubes of the *pop2* mutant fail to grow in the mutant pistil, perhaps because they cannot degrade the excess GABA. By contrast, wild-type pollen tubes, which carry the functional allele of *POP2*, can grow in the presence of a higher concentration of GABA, creating a small gradient around the tip of the tubes. Further studies with pollen tube growth in vitro have shown that wild-type pollen tubes were stimulated to grow in the presence of 1 to 10 mM GABA, but higher levels were inhibitory (Palanivelu et al., 2003; Edlund et al., 2004). However, pollen tubes do not respond chemotropically to a GABA gradient in vitro, so other molecules must be involved in this system.

By far, the best signal transduction mechanism described in the pollen–pistil interaction is the sporophytic SI response in *Brassica*. However, new, very interesting signaling systems are emerging in which stylar molecules play a crucial role. McCormick and co-workers have demonstrated that the two tomato pollen-specific receptor-like kinases LePRK1 and LePRK2 coimmunoprecipitate from pollen and yeast when

expressed together. The 400-kD complex persists in pollen after pollen tube germination in vitro, but it dissociates if stylar extract is added to the germination medium. Dissociation of the complex is caused by the dephosphorylation of LePRK2. A model for signal transduction in vivo would predict that a small stylar ligand (the dissociation factor resides in a 3- to 10-kD protein fraction) binds to LePRK2 and dephosphorylates it, and the complex dissociates in the pollen tube (Wengier et al., 2003; McCormick, 2004). The significance of this signal route in pollen tube growth is still unknown.

Upon arrival at the placenta, the path for the growing pollen tube is much less defined. In some cases, pollen tubes wander around on the placenta surface covered by secretion produced and released by specialized structures, such as the obturators (Herrero, 2001). These structures appear as little bumps along the placenta, facing the ovule entrance, and seem to control pollen tube growth at that point. In some species, pollen tubes arrest if the obturator is not active in the production of secretion (Herrero, 2001). The final attraction of the pollen tube to the ovule was shown recently to depend largely on the female gametophyte. A discrete number of Arabidopsis mutants defective in embryo sac development or function also were found to be unable to guide, attract, and accept pollen tubes (Hulskamp et al., 1995; Ray et al., 1997; Shimizu and Okada, 2000; Huck et al., 2003; Skinner et al., 2004). Furthermore, an elegant study using laser cell ablation on ovules of *Torenia* has definitively shown that the cue for pollen attraction is a diffusible signal emitted by the synergid cells surrounding the egg cells (Higashiyama et al., 2001; Skinner et al., 2004).

Together, these data leave no doubt that the pistil produces molecules for nutrition and cues for pollen tube growth. However, many of these molecules seem to be redundant and present in the pistil before pollination occurs, implying that the pistil is preset to support pollen tube growth. This notion also is supported by the fact that, to date, only a few genes have been found to be induced strictly by pollination, such as 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (*ACS*) in orchids (Bui and O'Neill, 1998), although there is enough evidence for gene expression modulated by this event. Many nonprotein factors also are produced in the style and were implicated in pollen tube growth. However, these often are isolated examples in one particular species, which makes it difficult to discuss them all.

INTERSPECIES POLLEN REJECTION

To this point, only events occurring in self-pollinated species have been considered here. In nature, however, many different types of pollen are present simultaneously and may be transported by wind or insect on a pistil that is not of the same species. To prevent the ''wrong'' cross, many plants have developed barriers that operate in the pistil either before fertilization, inhibiting pollen tube growth, or after fertilization, causing abortion of the illegitimate embryo. The pistil, therefore, plays an important role in the prevention of gene flow between species and in the maintenance of the species. In a 2001 publication, de Nettancourt extensively examines the two mechanisms of intraspecies and interspecies barriers. Lewis and Crowe (1958) studied the compatibility relationship between different self-incompatible (SI) and self-compatible (SC) species of several angiosperm families. They observed that most of the crosses were compatible only in one direction, when the SC species was used as the female parent in the cross. By contrast, if the SI species was used as the female parent, the SI pistil would reject the SC pollen. These results gave origin to the SI $\times$ SC rule, and interspecies incompatibility was named ''unilateral incompatibility'' (UI).

To date, several examples of UI governed by the SI locus have been described, and the elegant work of Murfett et al. (1996) demonstrated the implication of S-RNases as barriers in some interspecies crosses within the genus *Nicotiana*. Furthermore, by genetic mapping of a quantitative trait locus associated with UI in *Lycopersicon*, Bernacchi and Tanksley (1997) showed that the SI locus was a major determinant in UI, although two additional quantitative trait loci were involved in enhancing UI. However, there are exceptions to the SI $\times$ SC rule (de Nettancourt, 2001). For example, interspecies barriers also act in crosses involving two SC species. Liedl et al. (1996) showed that in a cross of *Lycopersicon* species, the timing, location, and physiology of pollen tube arrest were distinct from those in self-incompatibility.

The barriers in interspecies crosses between SC species are mostly referred to as ''incongruity'' (Hogenboom, 1975), indicating the incompleteness of a relationship caused by evolutionary divergence of two species. Incongruity is an event determined by many genes unrelated to the SI locus. An interesting study of incongruity was performed by Kuboyama et al. (1994). They used the SC tobacco as the female parent in distinct crosses with three other SC species, *N. repanda*, *N. rustica*, and *N. trygonofilla*. Pollen tubes of these latter species arrested in three different places in the mature pistils of tobacco. Figure 3 shows pollen tubes of *N. rustica* arrested halfway in the style of tobacco at $\sim$20 h after pollination. Pollen tubes also curl and wind and in some cases bend back toward the stigma (A.M. Sanchez and C. Mariani, unpublished results). In the reciprocal cross, using *N. rustica* as the female parent, tobacco pollen tubes easily reach the ovary. Interestingly, when the three types of incongruous pollinations were performed on immature tobacco pistils, all pollen tubes reached the ovary (Kuboyama et al., 1994). This finding suggests that, if the arresting factor is determined by the pistil, it is most likely produced during pistil maturation. It could be argued that the arrest of pollen tubes is caused by the longer distance they have to grow in the tobacco pistil compared with their own pistil (i.e., 3.6-cm tobacco style versus 1-cm *N. rustica* style). We used *N. rustica* pollen on the pistil of *N. sylvestris* and the tubes grew 3.2 cm, which is approximately the length of the tobacco style (A.M. Sanchez and C. Mariani, unpublished results). This finding indicates that *N. rustica* pollen tubes are capable of growing longer than the length of their own styles. Other physiological factors have been ruled out (Sanchez, 2001), but the mechanism of pollen tube arrest in these incongruous crosses is not yet understood. In some cases, incongruous pollen tubes grow down to the ovary and even fertilize the embryo sac. This is the case when petunia pollen is used to pollinate a tobacco pistil. In this case, not only are seeds produced, although they abort, but also proteins of the tobacco

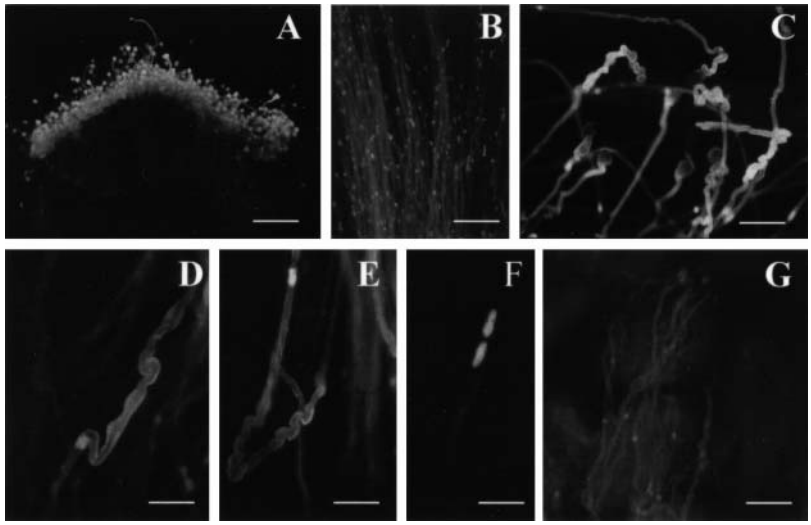

Figure 3. *N. rustica* and Tobacco Are Incongruous.

(A) and **(B)** *N. rustica* pollen grains germinate normally on the tobacco stigma **(A)**, and initial pollen tube growth is normal **(B)**.
(C) and **(D)** At half the length of the tobacco style, *N. rustica* pollen tubes curl and wind, and no further growth is observed.
(E) Sometimes pollen tubes can be seen growing backward.
(F) Division of the *N. rustica* generative cells inside the tobacco style takes place at ~6 h after pollination.
(G) Tobacco pollen tubes arrive easily at the ovary of *N. rustica*.
In **(A)** and **(B)**, bars = 250 μm; in **(C)** to **(G)**, bars = 25 μm.

transmitting tissue are translocated in the growing incongruous pollen tube, indicating an intimate pollen–pistil interaction typical of congruous pollinations (B. de Graaf, B. Knuiman, E. Pierson, G. van de Weerden, and C. Mariani, unpublished results).

Using a molecular approach, we set out to compare transcript profiles after congruous and incongruous pollinations on immature and mature tobacco pistils. Neither differential display reverse transcriptase–mediated PCR nor cDNA amplified fragment length polymorphism revealed transcripts specifically expressed in incongruous pollinations. Putative differentially expressed transcripts appeared to correlate with the developmental stage of the pistil and with the nature of the pollen used (A.M. Sanchez and R. Feron, unpublished results). If new transcripts are not detectable or are not made in incongruous pollinations, it still can be the case that well-characterized genes expressed in unpollinated and pollinated pistils play a role in incongruity. In most species, pollination is accompanied by an increase of ethylene production in the pistil and by the expression of genes that encode enzymes necessary for the hormone biosynthesis (O'Neill, 1997; Bui and O'Neill, 1998). Therefore, we followed the expression of *ACS* and ACC oxidase (*ACO*) after congruous and incongruous pollinations in tobacco. Their expression indeed correlated very well with the growth of different pollen tube types. The level of *ACS* expression was higher in self-pollinated tobacco pistils and lowest in the same pistils when pollinated with *N. maritima*, whose pollen tubes arrest around the stigma. Similar results were obtained for the expression of *ACO* (Sanchez and Mariani, 2002). In conclusion, the expression of these genes in the pistil of tobacco is modulated differently depending on the type of pollen used and possibly on the length the respective pollen tubes achieve. Because of the paucity of experiments performed on incongruity, it is difficult to conclude which factors other than the SI locus contribute to the arrest of foreign pollen tubes in a pistil.

CONCLUDING REMARKS

There is no doubt that the pistil plays a key role in plant reproduction and that it has multiple functions: it can accept or reject the proper pollen, it sustains pollen tube growth, and it produces and protects the female gametophyte in the ovary. The pistil is essential for flowering plant reproduction, even when asexual mechanisms of reproduction are available in some cases (Bicknell and Koltunow, 2004). The pistil must maintain the right balance between two important functions: on the one hand, it is a guarantee that pollen carries the sperm cells to the embryo sac, but on the other hand, it should select the fittest pollen tube to ensure the production of vigorous progeny. Therefore, it should not be surprising that many checkpoints exist in pollen tube growth in the pistil that are regulated by finely tuned mechanisms. Examples of these mechanisms are the formation of gradients in the style to attract pollen tubes, such as the gradient of glycosylation of TTS and the gradient of GABA, and the production of adhesion molecules that may function in a similar manner. Also, the redundancy of some proteins, such as HRGPs, probably is a safeguard to provide a good growth environment for pollen tubes. The fact that many genes are expressed in the pistil at some level before pollination takes place also suggests that the pistil is poised to accept pollen (or reject it if it is incompatible or incongruous) when pollination commences.

Although this may seem an anthropomorphic view of pollination, the pistil surely guarantees the continuation of plant life.

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