Pollen–Pistil Interactions in Nicotiana tabacum

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Pollination involves a series of interactions between the surface of the male gametophyte and the extracellular matrix of several distinct sporophytic tissues in the pistil. Pollen germinates on the stigmatic surface and develops a tube that elongates in the transmitting tract of the style to transport the male gametes to the embryo sac inside the ovary. Pollen tube growth is fuelled by cytosolic activities within the pollen, but female tissues facilitate this process to ensure that pollen tubes arrive at the ovary when the ovules are the most receptive for maximum reproductive success. Interactions between pollen and pistil factors, probably both physical and biochemical, must be transmitted to the pollen cytosol to elicit the appropriate pollen cellular activities. Our research focuses on identifying the molecules that are involved in these interactions, understanding their biochemical and cellular bases, transmittal of these interactive signals into the pollen cytosol and the pollen cellular responses. © 2000 Annals of Botany Company

Key words: Adhesion, AGPs, cytoskeleton, extracellular matrix, pistil, nutrition, pollen, pollen tube guidance, signal transduction, tobacco, vesicular transport.

INTRODUCTION

The diploid tissues in the anthers and pistils are believed to provide much of the nourishment and some of the recognition and signal molecules that are thought to be critical for effective pollination and fertilization events. Molecules on the pollen surface and the exine are largely derived from secretory cells in the anther tapetum (Bedinger, 1992; Murphy and Ross, 1998). On the other hand, at least some pollen coat components that are believed to play important roles in pollen–pistil interactions are gametophytically produced (e.g. Doughty et al., 1998). The male gametophyte also produces molecules that constitute the cytosolic machinery responsible for the pollen tube growth process (e.g. Kandasamy, McKinney and Meagher, 1999). Nicotiana tabacum pollen maturing in vitro from isolated unicellular microspores has been shown to be capable of accomplishing pollination and fertilization when applied to the female organ (Touraev et al., 1997). This suggests that in N. tabacum pollen germination and tube elongation in the presence of female tissues are probably adequately provided for by gametophytic functions; sporophytic activities that occur in normal pollen development most probably enhance the efficiency and accuracy of these functions. The pollen tube elongation pathway in the pistil, i.e. the extracellular matrix of the stigma, the stylar transmitting tissue and the placental and ovular surface, is filled with molecules secreted by the sporophytic tissues in the stigma and stylar transmitting tract (Cheung, 1996). The female gametophyte has also been shown to be crucial for the targeted entrance of pollen tubes into ovules (Hulskamp, Schneitz and Pruitt, 1995; Ray, Park and Ray, 1997; Higashiyama et al., 1998). Transport of the sperm cells to the egg cell and the polar nuclei of the central cell are believed to be supported by the cytoskeleton network produced by the female gametophyte (Huang and Russell, 1994). Thus, development of the male and female gametophytes and the pollination and fertilization processes rely significantly on sporophytic tissues as well as gametophytically-derived molecules.

The mature pollen grain is either tricellular (comprised of a vegetative cell and two sperm cells), or bicellular (comprised of a vegetative cell and a generative cell that undergoes mitosis during pollen tube elongation to produce two sperm cells). The function of the pollen is to transport the male germ cells to the female gametophyte, often over extremely long distances relative to the size of the pollen grain. Pollen tubes elongate by tip growth and the vegetative cell provides many of the activities needed for this process (Taylor and Hepler, 1997). Actomyosin-based cytoplasmic streaming delivers secretory vesicles to the pollen tube tip where copious amounts of cell wall materials are secreted to sustain the continuously advancing cell. Continuous deposition of callose plugs behind the extending pollen tube tip restricts the pollen cytosol to the most proximal segment of the pollen tube.

In the pistil, pollen tubes remain anchored to the stigmatic surface via a trail of spent pollen tube wall materials. In addition to polysaccharides, proteins are also secreted to the pollen cell surface-extracellular matrix continuum and provide for both the physical and chemical factors...
necessary for pollen tube growth. The in vivo pollen tube growth process is directionally predictable, from the stigma to the ovary. It is also extremely efficient [e.g. N. tabacum pollen tubes elongate at about 0.15 cm h⁻¹ to extend the approximately 4 to 4.5 cm between the stigma and the ovary (Wang, Wu and Cheung, 1996)]. Although pollen from many species may germinate and grow tubes in simple, chemically defined media, in vitro grown tubes neither attain the growth rates nor the distances reached by their in vivo counterparts. Presumably, in the in vivo process, pollen surface molecules interact with pistil factors that facilitate pollen tube growth towards the ovary. Molecules that play a role in linking the pistillate environment to the pollen cytosol are probably also present on the pollen surface to transduce the extracellular pollen-pistil interaction signals to elicit the appropriate pollen cellular responses.

Using N. tabacum as a model system, we study the molecular and biochemical bases that underlie compatible pollinations. N. tabacum flowers offer easy accessibility to reproductive organs for pollination and biochemical studies. Their pollen grains germinate and elongate efficiently in vitro, providing easy and reliable bioassay systems for factors that influence pollen tube growth. While inappropriate as a tool for classical genetic analysis, N. tabacum is highly amenable to transgenic and transient expression analyses. Transgenic manipulations of the male gametophyte and the female tissues make possible in vivo functional analyses. Our efforts focus on identifying pistil factors that influence and facilitate the pollination process, pollen molecules that interact with these pistil factors and those that transduce these pollen-pistil interactive signals and control pollen responses.

**PISTIL CONTRIBUTION TO POLLEN–PISTIL INTERACTIONS**

*Overview*

The extracellular environment along the pollen tube growth pathway in the pistil is enriched in polysaccharides, lipids and proteins, some of which can be elaborately glycosylated. Ablation of the stigmatic secretory zone by a cytotoxin in transgenic N. tabacum results in arrest of pollen tube elongation (Goldman, Goldberg and Mariani, 1994). Stigma exudates and lipids with compositions that mimic stigmatic exudate lipid constituents restore efficient germination and directional penetration of pollen tubes into the styles (Wolters-Arts, Lush and Mariani, 1998). Gradients of water and lipid molecules formed at the pollen surface and stigmatic interphase have been proposed to be necessary and adequate signals for guidance of emerging pollen tubes on the stigma to directionally penetrate the stigmatic surface.

In the solid style of N. tabacum, the transmitting tract (about 1–2 mm in the widest region) is filled with files of cells that secrete an extracellular matrix that is biochemically very enriched. However, this intercellular space is physically rather constrained for the numerous pollen tubes traversing it, which have diameters of about 10 μm at their tip (Fig. 1) (Bell and Hicks, 1976; Herrero and Dickinson, 1979; Wang et al., 1996). The architecture of the stylar transmitting tissue extracellular matrix is thus of a design that best accommodates the axially elongating tubes between the stigma and the ovary. On the other hand, there are also ample observations that suggest biochemical contributions by pistillate tissues to pollen germination and tube elongation.

The 4 to 4.5 cm-long N. tabacum style, three orders of magnitude longer than the diameter of its pollen grains, is by far the longest distance pollen tubes have to travel within the pistil. The extracellular matrix secreted by the transmitting tissue cells is apparently critical for female fertility since ablation of this tissue by a cytotoxin results in female sterility (Thorsness et al., 1991). Pollination on decapitated styles often results in failure of pollen grains to germinate. However, when decapitated styles and the deposited grains are covered with a drop of agarose, pollen germination and directional penetration of pollen tubes into the transmitting tissue occur efficiently (Fig. 2). A similar directional bias of stigmatic penetration is observed even when the protective agarose droplets are made of a pollen tube growth medium that, under in vitro conditions, supports pollen tube growth in random directions. Once germinated, pollen tubes in these decapitated styles also elongate at rates comparable to those in intact pistils. Apparently, the droplet of agarose protects the pistil tissue from dehydration and minimizes oxidative damage to the wound tissue, both of which inhibit pollen activities. These observations suggest that the stigmatic transmitting tissue presents highly favourable conditions for pollen germination and tube penetration. If lipid and water molecules are the sole factors needed for stigmatic germination of pollen grains, it should not be surprising that the biochemically enriched transmitting tissue exudates are also adequate to support similar activities. Depletion of cytosolic materials from the transmitting tissue cells after pollination (Fig. 1) and the observation of labelled pistil carbohydrate-containing materials being incorporated into elongating pollen tubes (Labarca and Loewus, 1973) are also suggestive of biochemical contributions from the stylar tissue in pollen tube growth.

Pollens from many species, including N. tabacum, have been observed to make pronounced changes in their growth directions in order to gain access into the ovules (Fig. 3). Genetic analyses of female gametophytic mutants in Arabidopsis have provided genetic evidence of the essential role of this haploid chamber in guiding pollen tubes into the ovules (Hulskamp et al., 1995; Ray et al., 1997). These genetic analyses complemented bioassay studies in which pollen tubes were targeted to ovules and embryo sacs (Willems, Plyushech and Reinders, 1995; Higashiyama et al., 1998). In N. tabacum, the receptive ovules are covered with exudates. In the ovary, pollen tubes turn from their basally-oriented growth direction along the placental surface, sometimes by as much as 90°, to enter the ovules via the micropyle (Fig. 3). When ovules are presented to pollen tubes emerging from pollinated styles in semi-in vivo pollen tube growth assays, pollen tubes often show a preference for growing in the direction of these ovules.
**FIG. 2.** Pollination of decapitated *N. tabacum* pistils. Stigmatic tissues were excised, and pollen grains were deposited onto the cut surface (A) or were deposited on the cut surface and immediately covered with a drop of agarose made in pollen germination medium (B). Pistils were collected 24 h after pollination and processed for aniline blue staining to visualize the pollen tubes.

**FIG. 3.** A, A receptive *N. tabacum* ovule covered with exudates. B, A pollen tube (pt) dropping 90° to enter an ovule via its micropyle (m). C and D, Pollen tubes targeting ovules. Pollen tubes emerging from pollinated styles were cultured on an *in vitro* pollen tube growth medium. Ovules (20 h after anthesis) were presented to one side of the emerging pollen tube front. Micrographs were taken after 20 h of pollen tube growth in the presence of the ovules. Pollen tubes were stained with aniline blue.

sometimes turning from their original growth trajectories to gain access into the ovules (Fig. 3).

*Arabinogalactan-proteins (AGPs)* are the predominant glycoproteins along the pollen tube growth pathway

We have focused on examining the protein constituents along the pollen tube growth pathway and understanding their contribution to pollination. AGPs are a ubiquitous class of highly glycosylated (hydroxy)proline-rich glycoproteins in plants (Baldwin *et al.*, 1993; Nothnagel, 1997). They are among the most prevalent glycoproteins in the pistil extracellular matrices where pollen tubes elongate (Cheung and Wu, 1999). Their adhesive nature and highly glycosylated properties have led to speculation that AGPs...
participate in cell–cell adhesion and recognition or as nutrient resources for the pollination process (Lord and Sanders, 1992; Jauh et al., 1997).

AGPs are highly soluble and easily eluted from the extracellular matrix. Washing intact stigmas, transmitting tissue and placental tissue isolated from *N. tabacum* elutes an abundance of AGPs as revealed by their reactivity towards β-glucosyl Yariv reagent (Cheung and Wu, 1999; unpubl. res.), a reagent traditionally considered diagnostic for this class of proteins. Characterization of AGPs purified from the pistil and cDNAs corresponding to pistil proteins reveals the presence of several distinct AGPs along the *N. tabacum* pollen tube growth pathway. The deduced amino acid sequence of a *N. tabacum* cDNA (NtSg-AGP for *Nicotiana* stigma AGP) is almost identical to a characterized stigmatic AGP (RT35) from the self-incompatible *N. alata* (Du et al., 1996). It is most probable that NtSg-AGP represents the majority of the AGPs detected among stigmatic washes. The localization of NtSg-AGP on the stigma suggests a functional role in the early events in pollination.

Abundant AGPs are also present on the *N. tabacum* placental surface (Cheung and Wu, 1999) where pollen tubes elongate after emergence from the style. Ovules are believed to emanate guidance molecules that direct pollen tube entrance. Ovule exudates have been shown to be enriched in glycosylated compounds (Chao, 1970; Fransen-Verheijen and Willemsen, 1993). Highly glycosylated molecules such as AGPs are attractive candidates as components of these exudates and play a role in pollen tube guidance to the ovules. Elucidating the cellular origin of these AGPs, their chemical properties and biological activities will be essential for assigning a functional role to these glycoproteins.

To date, two different AGPs have been characterized from *N. tabacum* styles: TTS (transmitting-tissue-specific) protein (Cheung et al., 1993, 1996; Wang, Wu and Cheung, 1993; Cheung, Wang and Wu, 1995; Wu, Wang and Cheung, 1995), and PELPIII (pistil extensin-like protein III) (Goldman et al., 1992; de Graaf, Knuiman and Mariani, 1998). In related species, the *N. alata* style expressed a glycosylphosphatidylinositol-anchored AGP (Youl, Bacic and Oxley, 1998) and the tomato style expresses an AGP with a hydrophobic C-terminal domain (Li and Showalter, 1996; Gao et al., 1999). These AGPs are believed to be anchored to the cell membrane, at least transiently, and play a signalling role. *N. tabacum* cDNAs corresponding to these two AGPs have been isolated (unpubl. res.) so functional analyses could be carried out in this plant system. Detailed analyses of how the *N. tabacum* stylar transmitting tissue AGPs interact with pollen tubes have been carried out only for TTS proteins (see below) and PELPIII (de Graaf et al., 1998). Whether individual stylar AGP performs unique or overlapping functions in the transmitting tissue will have to await a complete analysis of all AGPs in this tissue.

AGPs have been localized to pollen tubes (Li et al., 1992; Jauh and Lord, 1996; Ferguson et al., 1999) and pollen cDNA clones corresponding to putative pollen AGPs have also been reported (Gerster, Allard and Roberts, 1996). Li et al. (1992) observed that in elongating *N. tabacum* pollen tubes, AGPs are localized to the inner callosic walls except at the most apical region. These AGPs were deposited periodically and appeared as ring-like structures along the length of the tube and perpendicular to the growth direction. It was suggested that these ring-like structures might provide the tip-growing cells with mechanical support against external resistance and internal osmotic pressure. The identity of these AGPs and how they contribute to these properties remains to be determined. AGPs are also localized to the pollen grains and pollen tubes in a number of other plant species, although the details of deposition patterns differ. In lily pollen tubes, which have AGP deposition at their elongating tip, pollen tube elongation is arrested if AGPs are precipitated by β-glucosyl Yariv reagent. Appearance of grossly misshapen tips in these arrested tubes suggests a critical role for these glycoproteins in cell wall formation at the continuously advancing pollen tube tips (Roy et al., 1998).

### Other hydroxyproline-rich glycoproteins in the pistil

Although AGPs appear to be by far the most abundant glycoproteins in the extracellular matrix of the pollen tube growth pathway, other hydroxyproline-rich glycoproteins are also present. For instance, a 120 kDa glycoprotein with properties similar to both AGPs and extensins has also be characterized in the *N. alata* style (Lind et al., 1994). The 120 kDa glycoprotein shares significant homology with PELPIII (Goldman et al., 1992; Schultz et al., 1997), although not at the level usually seen between corresponding AGPs in *N. tabacum* and *N. alata* (e.g. Cheung et al., 1993; Chen, Mau and Clarke, 1993).

Anti-solannaceous lectins antibodies, which mainly recognize sugar moieties that are common to extensins and solannaceous lectin, detect high levels of a family of glycoproteins in the *N. tabacum* ovary placental exudates (unpubl. res.). Preliminary data suggest that these protein species, which are between the 25 and 45 kDa, correspond to a class of cysteine-rich extensin-like proteins (CELPs) whose mRNAs are detected along the surface cells in the placenta (Wu et al., 1993). CELP mRNAs also accumulate to high levels in the extracellular matrix secreted by one or two rows of cells that mark the boundary between the stylar cortical and transmitting tissue. Characterization of these proteins and analysis of their functions in pistils are underway.

### TTS Proteins Are Transmitting Tissue-Specific AGPs That Promote Pollen Tube Growth and Attract Pollen Tubes

TTS proteins are found in abundance in the transmitting tissue extracellular matrix of *N. tabacum* (Cheung et al., 1993; Wang et al., 1993). They are also present in a number of solannaceous plants, including *N. sylvestris* and *N. alata* (Cheung and Wu, 1999; Cheung et al., 2000; Wu et al., unpubl. res.). Molecules that cross hybridize with TTS cDNA cross-react with TTS antibodies have also been
peptide, the mature TTS protein backbone polypeptides are TTS gene. Amino acid sequences deduced from the TTS whereas its diploid progenitors genes (TTS-1 and TTS-2) in the amphidiploid and Wu, 1999; Cheung et al., 1996; Cheung et al., 2000) have a single TTS gene. Amino acid sequences deduced from the TTS cDNA clones predict that after cleavage of the signal peptide, the mature TTS protein backbone polypeptides are about 28 kDa. The polypeptide has an N-terminal proline-rich domain and a C-terminal domain that has a 6-cysteine residue-motif. Many proline residues in mature TTS proteins are hydroxylated and they are also glycosylated by N- and O-linked glycans (Wang et al., 1993). The predominant form of TTS protein isolated from stylar transmitting tissues is between 45 and 105 kDa when examined on SDS-PAGE. TTS proteins stimulate pollen tube growth in vivo (Cheung et al., 1995). In vivo, transgenic N. tabacum plants with their TTS mRNA levels reduced either by antisense suppression or sense-co-suppression showed reduced pollen tube growth rate, sometimes resulting in reduced female fertility (Cheung et al., 1995). Thus, in vivo, TTS proteins are important in maintaining a pollen tube growth rate to ensure delivery of male gametes to the ovary before stylar abscission or ovule senescence.

TTS proteins attract pollen tubes in vitro and display a gradient of increasing glycosylation levels in the same direction as pollen tube growth in vivo

When TTS proteins were presented in vitro to semi-in vivo grown pollen tubes from a distance, a gradient of TTS protein diffused from the protein source and pollen tubes were attracted towards regions of highest TTS protein concentration (Cheung et al., 1995). Whether this in vitro pollen tube attracting activity is based solely on the ability of these proteins to enhance growth, or on a combination of growth-stimulating and tropic effects on pollen tubes remains to be resolved. However, in vivo, a phenomenon associated with TTS proteins provokes tantalizing thoughts on whether these AGPs contribute to mechanisms that guide pollen tubes from the stigma to the ovary. Immunoblot analysis of TTS proteins from N. tabacum isolated from the stigmatic end, the middle stylar segment and the ovarian end revealed that these proteins are, on average, more highly glycosylated at the ovarian end than at the stigmatic end (Wu et al., 1995). The gradient of increasing TTS protein glycosylation level is coincident with the direction of pollen tube growth. Along with this TTS protein-bound increasing sugar gradient in the style is also an increasing acidity gradient since more highly glycosylated TTS proteins have more acidic pls (Wu et al., 1995).

Pollen tube–TTS protein interactions

Our ultimate aim is to understand the pollen–pistil interactions that underlie successful reproduction. The pollen tube responses to TTS proteins, increased growth rates and preferential growth along a gradient of TTS proteins diffusing from a source, appear to result from mutual contributions by pollen molecules and these pistil AGPs. In pollinated styles, TTS proteins in the transmitting tissue extracellular matrix become associated with the pollen tube surface as well as the callose wall of the pollen tubes. Analysis of pollen tubes grown in vitro in the presence of TTS proteins also showed that TTS proteins are associated with the pollen tube surface, its wall and the pollen tube tip (Wu et al., 1995). The pollen tube surface-associated TTS proteins can be collected at pH 8, close to the average pH of these proteins, whereas TTS proteins bound at the pollen tube tip and incorporated into the wall are resistant to this condition, suggesting different modes of pollen tube interactions with TTS proteins. Pollen tubes also actively participate in these interactions. In vitro grown pollen tubes deglycosylate TTS proteins by pollen tube-bound enzymes that are inhibited by galactose, the major sugar residues on TTS proteins (Wu et al., 1995). This, together with the observation that TTS proteins stimulate pollen tube growth, suggests that pollen tubes either incorporate the released carbohydrates into their own metabolism or receive them as signal molecules for the elongation process. In pollinated styles, small amounts of a low molecular weight form (around 30 kDa) of TTS proteins accumulate (Wang et al., 1993; Wu et al., 1995), suggesting that in vivo elongating pollen tubes also deglycosylate TTS proteins.

The in vivo association of stylar transmitting tissue extracellular matrix hydroxyproline-rich glycoproteins is not unique for TTS proteins. PELPIII from N. tabacum (de Graaf et al., 1998) and the 120 kDa protein from N. alata (Lind et al., 1996) have both been observed to be incorporated into the callose walls and cytoplasm, respectively, of elongating pollen tubes. How these interactions influence the pollen tube growth process remains to be demonstrated.

Glycosylation of TTS polypeptide backbones affects how these proteins interact with the extracellular matrix and is highly regulated

Glycosylation apparently acidifies the very basic TTS protein backbone polypeptides, rendering the more highly glycosylated proteins more acidic (Wu et al., 1995). Thus, in addition to quantitatively affecting the sugar contents of TTS proteins, glycosylation also alters the biochemical qualities of these proteins. How glycosylation affects the biochemical properties of these proteins is most dramatically revealed by differential extraction of TTS proteins from the transmitting tissue extracellular matrix (Cheung et al., 1999; Wu et al., unpubl. res.). Such studies showed that the predominant forms of these glycoproteins are loosely associated with the extracellular matrix and can be solubilized with salt free buffers. They are highly glycosylated, enriched in arabinogalactan moieties and span the
molecular weight spectrum of 50–105 kDa. A minor fraction of TTS proteins is tightly associated with the cell wall matrix and requires high salt conditions for solubilization. They are glycosylated to lesser extents, contain significantly lower levels of arabinogalactan moieties and span the molecular weight spectrum of 30–90 kDa. In in vitro pollen tube growth assays, chemically deglycosylated TTS proteins no longer stimulate pollen tube growth or attract pollen tubes in vitro (Cheung et al., 1995). Partially deglycosylated TTS protein purified from non-stylar tissues of transgenic N. tabacum that expresses a CaMV35S-TTS transgene (see below) also did not detectably stimulate pollen tube growth in vitro (unpubl. res.). These observations are consistent with the hypothesis that sugar moieties of these AGPs are essential for their biological activities and suggest that the less glycosylated and more tightly cell wall bound-TTS proteins are less active in promoting pollen tube growth. Similar differential extraction and characterization of the TTS proteins from N. alata indicate that a previously described TTS protein homologue, GaRSGP (galactose-rich styril glycoprotein) from N. alata (Sommer-Knudsen et al., 1996) is but the less arabinogalactanylated, more tightly cell wall-bound and less biologically active sub-population of total N. alata TTS proteins (Wu et al., unpubl. res.).

The differential glycosylation of one (in diploid Nicotiana species) or two (in amphidiploid N. tabacum) polypeptide backbones to produce the highly heterogeneous TTS proteins, respectively, suggest the existence of mechanisms that operate on the quantitative level for TTS protein glycosylation in the style. A stringent qualitative control at the tissue level is apparently also in place to ensure that full glycosylation of TTS proteins occurs only in the stylar transmitting tissue (Cheung et al., 1996, 2000). When TTS polypeptides are produced in all tissue types from a chimeric CaMV3S-TTS transgene in transformed N. tabacum plants, only the stylar transmitting tissue accumulates highly glycosylated TTS proteins. If TTS proteins indeed contribute to the directional elongation of pollen tubes in the style, the presence of highly glycosylated, active forms of these AGPs in the ovary may present confusing signals to the pollen tubes, which should be responding to ovular signals that guide them on a different directional course. Thus, the absence of TTS proteins and the activities to produce fully glycosylated TTS proteins in the ovary may be an important aspect of pollen tube guidance in the N. tabacum pistil.

TTS protein and directional pollen tube growth

Presence of TTS protein-bound gradients in the same direction as pollen tube growth in the pistil, and the observations that TTS proteins stimulate pollen tube growth, attract pollen tubes, bind to the pollen tube surface and tip, and that pollen tubes deglycosylate TTS proteins (Cheung et al., 1995; Wu et al., 1995), all suggest features reminiscent of both matrix-anchored guidance molecules and chemotropic molecules (Hynes and Landers, 1992; Wu et al., 1995). Any consideration of the potential that the TTS protein-bound sugar and acidity gradients have on directional pollen tube elongation in the style should note that these proteins are synthesized and modified in situ throughout the stylar transmitting tissue. Therefore, the gradient is not one that emanates from the locale of the pollen tube target, the ovary, which does not actually accumulate detectable levels of TTS proteins. Contrary to free sugars and other glycoproteins in the transmitting tissue that do not associate with pollen tubes (e.g. see Wu et al., 1995), TTS protein-bound sugar moieties are in direct contact with the elongating pollen tubes since these proteins adhere to the pollen tubes. This should favour the perception of the TTS protein-bound sugar molecules by the pollen tubes. On its journey from the stigma to the ovary, each pollen tube should encounter TTS protein molecules with increasing sugar contents every step of the way. Furthermore, the deglycosylation of TTS proteins by pollen tubes should produce a self-sharpening effect locally on the TTS protein-bound sugar gradient. Although transgenic plants with highly reduced levels of TTS proteins have reduced pollen tube growth rates (Wu et al., 1995), whether the TTS protein-bound sugar gradient is indeed critical to directional pollen tube elongation in the style remains to be determined. However, the phenomenon displayed by TTS proteins in N. tabacum is conserved in N. sylvestris (Cheung and Wu, 1999) and N. alata (Wu et al., unpubl. res.), indicating commonality in this in-situ produced protein-bound sugar gradient.

POLLEN CONTRIBUTION TO POLLEN-PISTIL INTERACTIONS

Molecules must be present on the pollen surface to interact with pistil components, e.g. TTS proteins, PELPHI and other factors that influence pollen tube growth, and to transduce these pollen-pistil interactive signals to the pollen cytosol to elicit the necessary cellular activities. To study the pollen contribution to pollen-pistil interactions, we focused on N. tabacum pollen surface protein molecules and molecules that span the pollen extracellular matrix-plasmalemma continuum with the potential to transduce pollen-pistil interactive signals to the pollen cytosol.

A cysteine-rich pollen surface molecule Nip-CysR

Among the cDNAs that we have characterized is a cDNA (NtP-CysR, for Nicotiana pollen-cysteine rich) that corresponds to a secreted protein precursor of 63 amino acids. The predicted mature protein shows a high homology with a protein designated as a pollen allergen from olive (Fig. 4). CtP-CysR mRNA is expressed almost exclusively in developing anthers, reaches the highest level in mature anthers and is highly abundant in pollen. A family of Brassica gametophytically-produced pollen coat proteins that have been shown to interact with stigmatic proteins, including the self-incompatibility response-related SLG proteins, are low molecular weight proteins with eight conserved cysteine residues (Doughty et al., 1993; Hiscock et al., 1995; Doughty et al., 1998). It is possible that these tobacco pollen surface cysteine-rich molecules also interact with pistil factors. In this context, it should be noted that a
in vesicular transport must respond to the pistil environment to orchestrate the cellular activities most appropriate for the different phases of the pollen tube elongation process. In addition to actin, molecules that interact with actin to regulate its polymerization and depolymerization, e.g. villins (Yokota, Takahara and Shimmen, 1998; Vidali et al., 1999) and actin-depolymerizing factors (Lopez et al., 1996), play important roles in maintaining the proper dynamics of the actin cytoskeleton to keep up with the pollen tube growth process. Vesicles containing cargoes of various cell wall polysaccharides and proteins are transported along actin cables, they bud and fuse between different secretory compartments and the plasmalemma to deposit the continuously synthesized wall around the advancing pollen tube tip. Retrograde transport of vesicles is also believed to play an important part of retrieving membrane components and protein molecules to replenish the pollen tube tip. A large battery of small GTPases (rab G-proteins) regulates vesicular transport in yeast and mammalian cells (Zerial and Huber, 1995). We have isolated cDNAs for several pollen rab G-proteins that, if analogous to their mammalian or yeast counterparts, regulate different parts of the vesicular transport pathway. Pollen-expressed green fluorescent protein-labelled cytoskeletal proteins and rab G-proteins are currently being used to examine how the various cytosolic activities that underlie the pollen tube elongation process respond to pistil factors.

To address the question of how extracellular pollen–pistil interactive signals are mediated to the pollen cytosol, we have isolated pollen cDNAs corresponding to candidate surface molecules that may link the extracellular milieu to the pollen cytosol. These include a number of putative receptor kinases, leucine-rich repeat containing extensin-like proteins and glycosylphosphatidylinositol-anchored proteins (unpubl. res.). The challenge will be to determine how these molecules contribute to the pollination process both individually and together.

PERSPECTIVES
Pollen–pistil interactions have been studied at different levels in a large variety of plant species. Diversity among angiosperms in their floral structures must be accompanied by the evolution of reproductive strategies that best benefit individual species. While universality of pollen–pistil interactive mechanisms may be an unlikely scenario, studies using a few model systems such as the Nicotiana species, Brassica, lily, maize and Arabidopsis should provide the framework from which we may attempt to interpret observations in other systems. On the other hand, extrapolation from one plant system to another should be applied with caution. The conservation of cellular activities that have thus far been observed in pollen from a large variety of species suggests that pollen is extremely well-designed to deliver the male gametes. The diversity of pistil structure and biochemical compositions suggests diverse mechanisms to ensure timely delivery of sperm cells to the embryo sac. Even within a single plant species, it would probably be naïve to assume that nature would allow the

FIG. 4. A, Amino acid sequence alignment of NtP-CysR with the olive pollen allergen Ole-e6. Amino acid identities are indicated by vertical bars and similarities by a plus sign. The conserved cysteine residues are bold and underlined. The N-terminal extension in NtP-CysR represents a putative signal peptide. (GENBANK accession # is BankIt308910 AF213464) B, RNA blot analysis of NtP-CysR mRNA showing tissue specificity (top) and floral developmental expression (bottom). Abbreviations: P. tube, 6 h pollen tubes; stamen 2 cm, 4 cm, stamens from 2 cm and 4 cm buds, respectively; 5 mm bud, microspore mother cell stage; 8 mm bud, tetrad stage; 2 cm bud, individual microspores; 4 cm bud, binucleate pollen grains; H. pollen, pollen hydrated for 15 min.

A

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B

Signal transduction and cytoskeletal molecules for pollen tube growth

Pollen tip growth requires highly active and polarized secretion at the tube apex. These activities are largely supported by actomyosin-based cytoplasmic streaming patterned like a reverse fountain (Taylor and Hepler, 1997). The cytoskeletal elements and molecules involved

number of the N. tabacum pistil extracellular matrix proteins, e.g. TTS proteins, PELPIII, CELPs and FST (Gu et al., 1992) have either a 6-cysteine or a 8-cysteine residue motif. It would be interesting to see if these cysteine motifs are functionally related to each other.

Signal transduction and cytoskeletal molecules for pollen tube growth

Pollen tip growth requires highly active and polarized secretion at the tube apex. These activities are largely supported by actomyosin-based cytoplasmic streaming patterned like a reverse fountain (Taylor and Hepler, 1997). The cytoskeletal elements and molecules involved
perpetuation of a species to be controlled by any single mechanism of pollen–pistil interaction. Progress in understanding reproductive strategies will be more expediently made if observations are objectively evaluated and constructively used in the formulation of testable models.

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