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A Pollen Tube Growth Stimulatory Glycoprotein Is Deglycosylated by Pollen Tubes and Displays a Glycosylation Gradient in the Flower

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Summary

In plant sexual reproduction, pollen tubes elongate from the stigma, through the stylar transmitting tissue, to the ovary of the pistil to deliver the male gametes for fertilization. TTS protein is a tobacco transmitting tissue glycoprotein shown to attract pollen tubes and promote their growth. Here, we show TTS proteins adhere to the pollen tube surface and tips, suggesting that they may serve as adhesive substrates for pollen tube growth. TTS proteins are also incorporated into pollen tube walls and are deglycosylated by pollen tubes, suggesting that they may provide nutrients to this process. Within the transmitting tissue, TTS proteins display a gradient of increasing glycosylation from the stigmatic end to the ovarian end of the style, coincident with the direction of pollen tube growth. These results together suggest that the TTS protein-bound sugar gradient may contribute to guiding pollen tubes from the stigma to the ovary.

Introduction

Plant sexual reproduction depends on intimate interactions between pollen (the male gametophyte) and the pistil (the female reproductive organ). The pistil is an elongated structure composed of three major parts: an apical stigma that receives pollen, a basal ovary that contains the ovules, and a style that connects the stigma to the ovary. Pollen grains germinate on the stigmatic surface, each extruding a tube that elongates in the stigmatic and the stylar transmitting tissue extracellular matrix (ECM) and along the ovule surface until it enters an ovule and penetrates the embryo sac. The pollen tube tip bursts inside the embryo sac and releases the two sperm cells for fertilization (Knox, 1984).

Pollen tube elongation is a tip growth process and shares many properties characteristic of other tip growth processes, such as root hair elongation in plants, protonema extension in ferns, hyphal extension in fungi, and neural axon outgrowth in animals (Heath, 1990). However, pollen tube growth is unique in having the cell body restricted to the tip of the tube. The cytoplasm and nucleus of the pollen tube and the two sperm cells are blocked from the distal, nonliving region of the tube by the periodic depositions of callose (β-1,3-glucan) plugs behind this cytoplasmic zone as it moves forward (Heslop-Harrison, 1987; Steer and Steer, 1989). The extending tip of other tip growth cells is either cytoplasmically connected to the cell body or involves a dividing cell at the tip.

The elongation of pollen tubes in the ECM of pistil tissues is believed to be promoted and guided by pistil-derived nutrients and directional cues, since in vitro pollen tube growth is often slower and reaches shorter distances than the in vivo process and lacks a targeted directionality (Clarke et al., 1979; Heslop-Harrison, 1987; Mascarenhas, 1975, 1993; Lord and Sanders, 1992; Cheung, 1995). This is reminiscent of mammalian cell migration and axon outgrowth that occur in the ECM and are promoted and guided by molecules in the matrix or emanating from the targets (Hynes and Landers, 1992; Tessier-Lavigne and Placzek, 1991). Similar to the existence of multiple guidance mechanisms in mammalian cell migration systems, chemotropic, haptotropic (matrix adhesivity–driven), contact, and electrical guidance mechanisms have been proposed for the directional growth of pollen tubes in the pistil (Mascarenhas, 1975, 1993; Heslop-Harrison, 1987; Wang et al., 1989; Lord and Sanders, 1992). Pistil tissues and their extracts from a variety of plants have been shown to attract pollen tubes in vitro, suggesting the presence of chemotropic substances in these tissues (Rosen, 1961; Mascarenhas and Machlis, 1962a, 1962b; Reger et al., 1992a, 1992b). A gradient of increasing Ca^{2+} concentration has been observed in the pistil of Antirrhinum, and it was speculated to have a chemotropic role in pollen tube growth. Several Arabidopsis mutants defective in ovule development also show abnormality in the pollen tube growth direction within the ovary, indicating that the ovules play an important role in pollen tube guidance (Hulskamp et al., 1995). Mechanical (contact) guidance based on the architecture along the pollen tube growth pathway is believed to be important in some plant species, such as maize and pearl millet (Heslop-Harrison et al., 1985; Heslop-Harrison and Reger, 1988). Pollen tubes elongating in the lily style adhere to one another and to the epidermis of the transmitting tract, suggesting the presence of surface adhesive molecules (Jauh and Lord, 1995). The stylar transmitting tissue from a number of flowering plants has been shown to be capable of translocating inert latex beads in a manner similar to pollen tube growth (Sanders and Lord, 1989). These observations led to the suggestion that the stylar ECM provides surface adhesive substrates for pollen tube growth and that a matrix-driven guidance mechanism may operate to guide pollen tubes (Sanders and Lord, 1989; Lord and Sanders, 1992).

In plants with self-incompatible pollination systems, their pistil tissues are known to produce molecules important for pollen recognition to reject incompatible pollen or their tubes (Newbigin et al., 1993; Nasrallah et al., 1994; Li et al., 1994; Foote et al., 1994). One of these molecules, the S-RNase from Nicotiana alata, has been shown to be taken up by pollen tubes (Gray et al., 1991) and to hydrolyze pollen tube RNAs (McClure et al., 1990), indicating direct interactions between these pistil proteins and pollen tubes. In contrast, specific nutrient molecules, chemotropic agents, or surface adhesive molecules that may

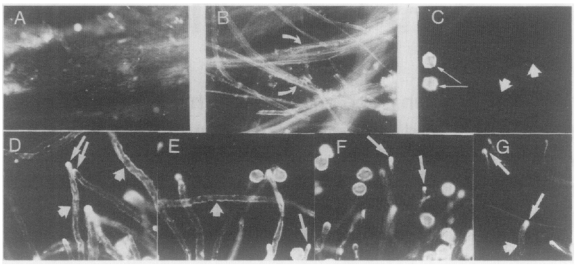

Figure 1. TTS Proteins Adhere to Pollen Tube Surface and Pollen Tube Tip

(A–B) PBS (pH 6) washed, longitudinally bisected unpollinated and pollinated styles were reacted with TTS antibodies, followed by FITC-labeled secondary antibodies. Unpollinated styles (A) showed low levels of autofluorescence from cell wall materials. In pollinated styles (B), pollen tubes were labeled (curve arrows), indicating the presence of TTS proteins on the pollen tube surface since antibodies have been shown not to penetrate pollen tube walls (Li et al., 1992).

(C–G) Pollen tubes were grown in vitro either in the absence (C) or in the presence (D–G) of added TTS proteins. (C) Control pollen tubes were washed with GM (pH 6) and reacted with TTS antibodies, followed by FITC-labeled secondary antibodies. (D and E) Pollen tubes grown in the presence of TTS proteins were washed with GM and then reacted with antibodies. (F and G) Pollen tubes grown in the presence of TTS proteins were sequentially washed with GM and TBS (pH 8) and then reacted with antibodies. Pollen grains appeared as bright spots (C–F; thin arrows in [C]) because of autofluorescence. Scale bar equals 20 μm.

contribute to compatible pollen tube growth have not been identified from the pistil of any plant.

Tobacco TTS proteins are stylar ECM glycoproteins shown to promote pollen tube growth in vitro and to attract pollen tubes in semi–in vivo pollen tube growth cultures (Cheung et al., 1995 [this issue of *Cell*]). Furthermore, the pollen tube growth rate is reduced in transgenic plants in which the level of TTS proteins is significantly lowered by antisense suppression or by sense cosuppression. Taken together, these results indicate that TTS proteins are important stylar components that support pollen tube growth. We show here that TTS proteins bind to the pollen tube surface and tip and that they are incorporated into the pollen tube wall. Furthermore, the sugar moieties on TTS proteins, which belong to the arabinogalactan protein (AGP) family (Showalter, 1993), are hydrolyzed by pollen tubes, suggesting that these glycoproteins may be a source of nutrients for pollen tube growth. The adhesiveness of TTS proteins to pollen tubes and the tendency of these proteins to polymerize into long-chain molecules (Cheung et al., 1995) suggest that they may function as adhesive substrate molecules for pollen tube growth. Within the style, TTS proteins display a gradient of increasing glycosylation level from the stigmatic end to the ovarian end of the transmitting tissue, the same direction as pollen tube growth. The potential significance of this gradient to directional pollen tube growth is discussed.

Results

TTS Proteins Adhere to Pollen Tubes and Are Incorporated into the Pollen Tube Wall In Vivo and In Vitro

TTS proteins are located in the transmitting tissue ECM (Wang et al., 1993) where pollen tubes elongate. Little TTS protein was detected in this matrix of the unpollinated styles after they had been washed in phosphate-buffered saline (PBS) (pH 6) and reacted with TTS antibodies and fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Figure 1A). When pollinated styles were similarly treated, substantial amounts of TTS proteins were de-

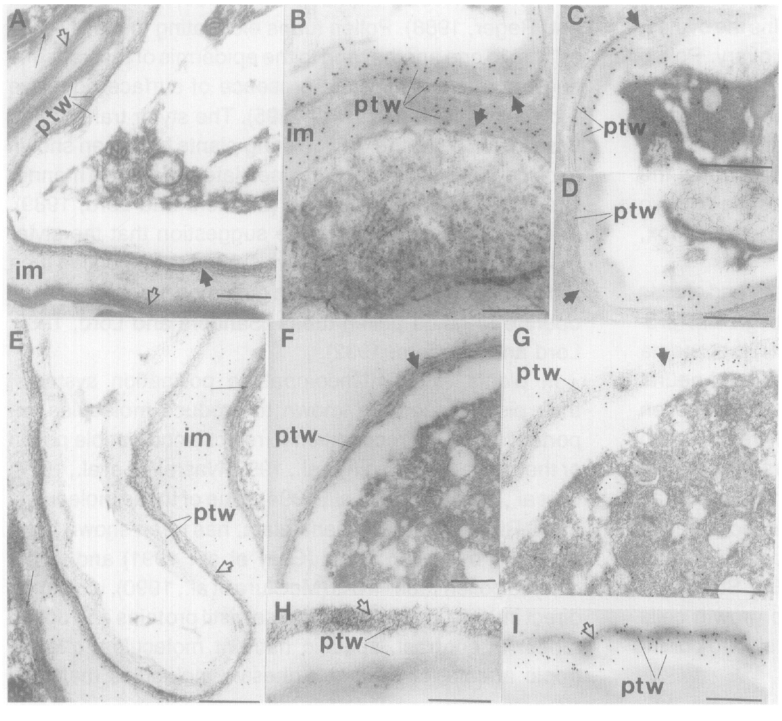

Figure 2. TTS Proteins Are Incorporated into the Pollen Tube Walls

(A–E) Ultrathin sections of in vivo pollen tubes from pollinated styles were treated with preimmune (A) or TTS antibodies (B–E), followed by colloidal gold-labeled secondary antibodies. (A) A section across the cytosolic region of a pollen tube (closed arrowhead) and the more distal and noncytosolic region of two pollen tubes (open arrowheads). (B) A section across the proximal cytosolic region of a pollen tube (bottom half) and the distal cytosolic region of another tube (top half). (C and D) Sections across the distal cytosolic region of two other pollen tubes. (E) A section across the noncytosolic region of a pollen tube.

(F–I) Ultrathin sections of in vitro pollen tubes grown without added TTS proteins (F and H) or in the presence of TTS proteins (G and I) were reacted with TTS antibodies and colloidal gold-labeled secondary antibodies. (F and G) Sections from the cytosolic region of pollen tubes. (H and I) Sections from the noncytosolic region of pollen tubes.

Abbreviations: im, transmitting tissue intercellular matrix; ptw, pollen tube walls. Thin arrows in (A) and (E) point to transmitting tissue cells. Scale bars equal 0.5 μm.

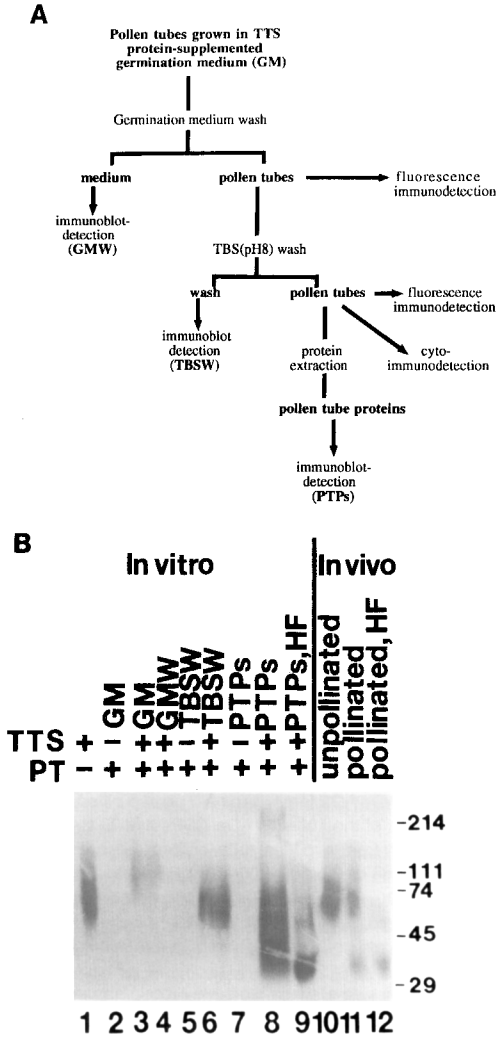

Figure 3. Immunoblot Analysis of In Vitro TTS Protein–Pollen Tube Interactions

(A) Flow chart for differential fractionation of TTS proteins from in vitro pollen tube growth cultures supplemented with these proteins. Pollen tubes were washed sequentially with sugar-depleted GM (pH 6) and with TBS (pH 8). Recovered GM, GM wash (GMW), TBS (pH 8) wash (TBSW), and pollen tube proteins from the washed pollen tubes (PTPs) were analyzed for the presence of TTS proteins by immunoblot assays. Pollen tubes after GM and TBS (pH 8) washes were also treated for fluorescence- and cytoimmunodetection of TTS proteins (see Figures 1C–1G and 2F–2I).

(B) Immunoblot showing that TTS proteins associate with and are deglycosylated by pollen tubes. Either no TTS protein (control) or TTS proteins were added to in vitro pollen tube cultures and incubated for 12 hr. These cultures were treated as outlined in (A) and described in the Experimental Procedures. Various protein fractions were electrophoresed by SDS–PAGE (12.5%) for immunoblot analysis. Lane 1, input TTS proteins; lane 2, GM from a control pollen tube culture; lane 3, GM from a pollen tube culture supplemented with TTS proteins; lane 4, GMW of pollen tubes grown in the presence of TTS proteins; lane 5, TBSW of control pollen tubes; lane 6, TBSW of pollen tubes grown in the presence of TTS proteins; lane 7, PTPs from control pollen tubes; lane 8, PTPs from tubes grown in the presence of TTS proteins; lane 9, sample in lane 8 after chemical deglycosylation; lane 10, unpollinated style proteins; lane 11, pollinated style proteins; lane 12, chemically deglycosylated style proteins. The unbound TTS proteins recovered in GM from pollen tube cultures grown in the presence of TTS proteins (lane 3) were usually at the highest molecular weight range of the input TTS proteins. After chemical deglycosylation, these residual TTS proteins left in the GM were detected by the TTS antibodies predominantly as a 65 kDa species; sometimes protein species at around 90 and 120 kDa were also detected (data not shown). These observations suggest that the TTS protein molecules left in GM were predominantly molecules that have irreversibly aggregated.

HF, hydrogen fluoride treated. Numbers on the side of this blot are protein apparent molecular weights in kilodaltons. The faint 65 kDa protein bands detected in the deglycosylated samples (lanes 9 and 12) represent the backbone polypeptides for dimeric TTS proteins that irreversibly self-aggregated.

tected along the surface of these in vivo grown pollen tubes (Figure 1B). Cytoimmunodetection analysis showed that TTS proteins were also present in the pollen tube wall, from the cytosolic tip region (Figures 2B–2D) to the noncytosolic region (Figure 2E). In contrast, *TTS* mRNAs (data not shown) and proteins have not been detected in in vitro grown pollen tubes by any of the detection methods employed (Figure 1C; Figures 2F and 2H; Figure 3B, lanes 2, 5, and 7). Therefore, the presence of TTS proteins on the pollen tube surface and in their walls indicates that they have been incorporated from the surrounding transmitting tissue ECM by pollen tubes.

The interactions between TTS proteins and pollen tubes were also examined in vitro. Pollen tubes grown in the presence of exogenously added TTS proteins were treated by different wash conditions, and the partition of TTS proteins into different fractions was determined immunologically (Figure 3A). After unbound TTS proteins have been removed from these pollen tubes by washing with germination medium (GM) (pH 6), significant amounts of TTS proteins were detected along the pollen tube surface and at the pollen tube tips (Figures 1D–1E). When similarly grown pollen tubes were washed sequentially with GM and Tris-buffered saline (TBS) (pH 8), the pollen tube surface–adhered TTS proteins were released, whereas the association of TTS proteins with pollen tube tips was resistant to these washes (Figures 1F and 1G). Cytoimmunodetection analysis showed that TTS proteins were also present in the walls along the entire length of the pollen tubes (Figures 2G and 2I). Since pollen tubes grown in the absence of TTS proteins did not show detectable reactions with the TTS antibodies (Figures 2F and 2H; Figure 3B, lanes 2, 5, and 7), these results show that exogenously added TTS proteins in in vitro pollen tube cultures adhered to pollen tubes and were incorporated into their walls.

TTS proteins recovered from the various washes and from washed pollen tubes (PTPs, Figure 3A) were examined on immunoblots (Figure 3B). Small amounts of TTS proteins remaining in the GM (Figure 3B, lane 3) represented excess proteins that had not adhered to the pollen tubes. Substantial amounts of pollen tube–associated TTS proteins that were not dislodged by GM washes (GMW, Figure 3A) were released from the pollen tubes by washing with TBS (pH 8) (TBSW, Figure 3A; Figure 3B, lane 6). These TTS proteins were those that presumably adhered to the pollen tube surface after pollen tubes had been washed by GM and that were released from the pollen tubes by TBS (pH 8) (Figures 1D–1G). About half of the TTS proteins originally added to the pollen tube growth

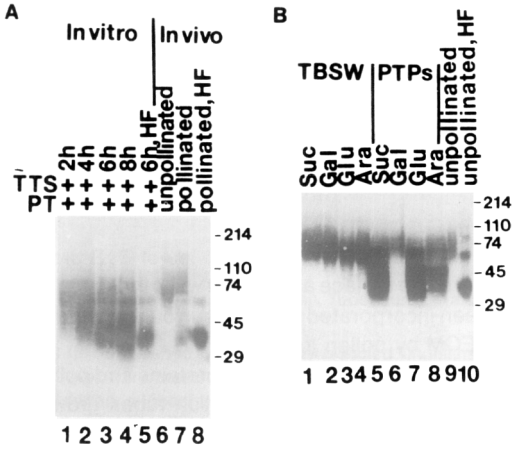

Figure 4. Characterization of the Pollen Tube–Mediated Deglycosylation of TTS Proteins

(A) An immunoblot showing that TTS proteins are progressively deglycosylated by pollen tubes. Proteins were electrophoresed on SDS–PAGE (15%). Lanes 1–4, PTPs from GM and TBS (pH 8) doubly washed pollen tubes that had been grown in the presence of TTS proteins for 2, 4, 6, and 8 hr, respectively. Lane 5 is the sample shown in lane 3 after chemical deglycosylation. Results were identical when samples in lanes 1, 2, and 4 were deglycosylated (data not shown). Lane 6, unpollinated style proteins; lane 7, pollinated style proteins; lane 8, chemically deglycosylated style proteins.

(B) Galactose inhibits pollen tube hydrolysis of TTS protein sugar moieties. Lanes 1–4, TBS (pH 8) washes (TBSW) of pollen tubes that had been grown in the presence of TTS proteins and sucrose, galactose, glucose, or arabinose, respectively, for 6 hr. PTPs from these TBS (pH 8) washed pollen tubes were respectively shown in lanes 5–8. Lane 9, unpollinated style proteins; lane 10, chemically deglycosylated stylar proteins. Proteins were electrophoresed on SDS–PAGE (15%).

HF, hydrogen fluoride treated. Numbers on the side of these blots are protein apparent molecular weights in kilodaltons. The faint 65, 90, and 120 kDa protein bands detected in the deglycosylated samples (lane 8 in [A], lane 10 in [B]) represent backbone polypeptides for irreversibly aggregated di-, tri-, and tetrameric TTS protein molecules.

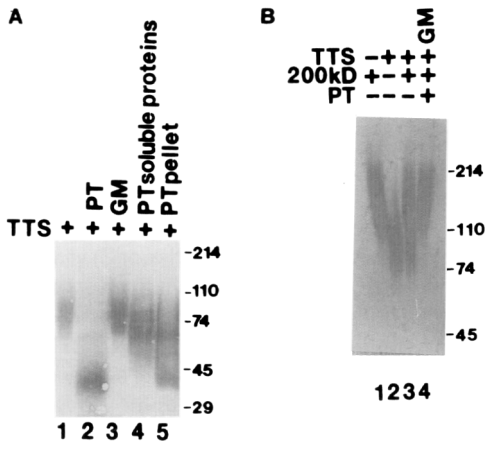

Figure 5. TTS Protein Deglycosylation Activity Is Tightly Bound to the Pollen Tubes

(A) An immunoblot showing that the TTS protein deglycosylation activity is tightly bound to the pollen tubes. Lane 1, input TTS proteins; lane 2, PTPs from pollen tubes that had been grown overnight in the presence of TTS proteins; lane 3, TTS proteins after overnight incubation with the GM recovered from a 6 hour pollen tube growth culture; lane 4, TTS proteins after overnight incubation with a soluble pollen tube protein extract; lane 5, TTS proteins after overnight incubation with a suspension of pollen tube membrane and wall-bound proteins. Proteins were electrophoresed by SDS–PAGE (12.5%).

(B) A protein blot stained by a Yariv β-glucosyl reagent that reacts specifically with AGPs (Yariv et al., 1967). Lane 1, 2 μg of partially purified 100–200 kDa AGP family; lane 2, 2 μg of purified TTS proteins; lane 3, 2 μg each of the 100–200 kDa AGPs and TTS proteins; lane 4, GM recovered from a pollen tube culture incubated with equal amounts (20 μg/ml) of the 100–200 kDa AGPs and TTS proteins for 12 hr. Only the 100–200 kDa AGPs were recovered in the GM. When only the partially purified 100–200 kDa AGPs were added to pollen tubes, they were also quantitatively recovered in the GM (data not shown).

Numbers on the side of these blots are protein apparent molecular weights in kilodaltons.

cultures was recovered in the pollen tube protein (PTP) fraction after pollen tubes had been washed sequentially by GM and TBS (pH 8) (Figure 3B, lane 8). These pollen tube–bound TTS proteins presumably were comprised of the tube tip–associated (Figures 1F and 1G) and the tube wall–associated TTS proteins (Figures 2G and 2I), since they also resisted TBS (pH 8) washes.

TTS Proteins Are Deglycosylated by In Vitro Grown Pollen Tubes

Immunoblot detection of TTS proteins in the PTP fraction (Figure 3A) showed significant reductions in their apparent molecular weights (Figure 3B, lane 8). The apparent molecular weights of these TTS protein molecules were reduced progressively with incubation time in the pollen tube growth cultures until the majority of them reached a minimum of about 30 kDa (Figure 4A, lanes 1–4), which is in the same range as the chemically deglycosylated native TTS proteins (Figures 3B, lane 12; Figure 4A, lane 8; Cheung et al., 1995) and as an underglycosylated 30 kDa TTS protein species (TTS-30) that accumulates in the pollinated style (Figure 3B, lane 11; Figure 4A, lane 7; Wang et al., 1993). When these tightly pollen tube–bound TTS proteins were chemically deglycosylated, their molecular weights were all reduced to 30 kDa (Figure 3B, lane 9; Figure 4A, lane 5). This implies that the molecular weight reduction in the tightly pollen tube–bound TTS protein molecules had resulted from the hydrolysis of their sugar moieties, while their polypeptide backbones remained intact. These results are consistent with the conclusion that in vitro grown pollen tubes deglycosylate TTS proteins that are tightly bound to them. They also suggest that the TTS-30 species that accumulate in the pollinated styles may also have arisen from pollen tube–mediated deglycosylation of TTS proteins in the transmitting tissue ECM.

Addition of sucrose or glucose to the TTS protein–supplemented pollen tube cultures had no observable effect on the association of TTS proteins with pollen tubes or on their deglycosylation (Figure 4B, lanes 1, 3, 5, and 7). Addition of galactose, however, specifically inhibited their deglycosylation by pollen tubes (Figure 4B, lanes 2 and 6). Arabinose also reduced the level of TTS protein deglycosylation by pollen tubes (Figure 4B, lanes 4 and 8).

TTS Protein Deglycosylation Activity Is Tightly Associated with Pollen Tubes

The TTS protein deglycosylation activity is not secreted from the pollen tubes into the medium, since the GM recov-

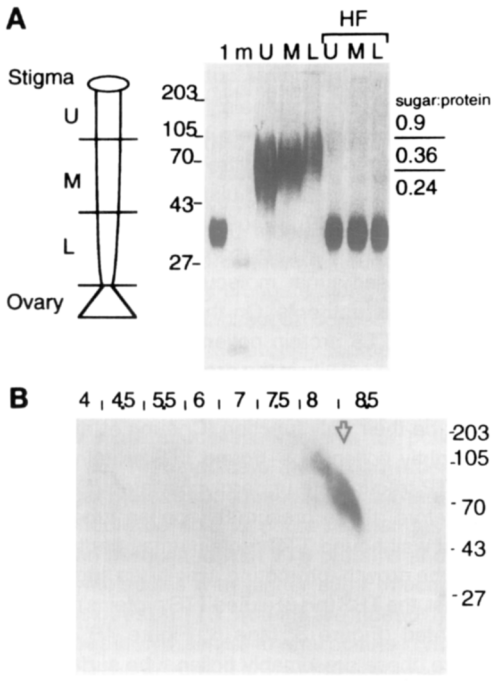

Figure 6. TTS Proteins Display a Gradient of Glycosylation Levels and Acidity in the Style

(A) An immunoblot of TTS proteins from the upper (U), middle (M), and lower (L) parts of the style. Lane 1, chemically deglycosylated total stylar proteins; lanes U, M, and L, proteins from the upper, middle, and lower, respectively, parts of the style; HF, hydrogen fluoride treated; lane m, prestained molecular weight markers. The numbers to the right of the blot indicate the sugar:protein ratio (w/w) for purified TTS proteins in the molecular weight ranges of above 80 kDa, between 60–80 kDa, and below 60 kDa. Equal amounts of style proteins (10 μg) were loaded in each lane. The quantities of TTS proteins in each of the three stylar segments were comparable as judged by the levels of the chemically deglycosylated proteins (lanes HF/U, M, and L). The TTS antibodies used against the polypeptide backbone of the C-terminal half of the proteins and were less reactive toward the highly glycosylated TTS proteins. The gradient of increasing glycosylation level of TTS proteins is qualitatively similar in developing, pre- and postpollinated styles.

Numbers to the left of this blot are apparent molecular weights in kilodaltons.

(B) An immunoblot showing TTS proteins increase in acidity with increasing apparent molecular weights. The pIs of TTS proteins range between 7.5–8.5. The pHs along the first dimensional isoelectrofocusing gel are indicated across the top of the picture. Arrow indicates the position of whale myoglobin (pI = 8.05). Numbers on the right indicate protein apparent molecular weights in kilodaltons.

ered from pollen tube cultures could not deglycosylate these molecules (Figure 5A, lane 3). Furthermore, soluble proteins isolated from pollen tubes showed only limited deglycosylation activity (Figure 5A, lane 4), whereas incubation of TTS proteins in the resuspended insoluble pollen tube tissue pellet reduced their molecular weights considerably (Figure 5A, lane 5). These results indicate that the TTS protein deglycosylation activities are tightly bound to pollen tubes.

Other major proteins present in the transmitting tissue ECM include a family of 100–200 kDa AGPs (Cheung et al., 1995; Figure 5B, lane 1). When these partially purified AGPs, or a mixture of these AGPs and TTS proteins, were added to pollen tube cultures, these 100–200 kDa AGPs were quantitatively recovered in the GM and their apparent molecular weights were not reduced by incubation with pollen tubes (Figure 5, lane 4). This indicates that not all tobacco transmitting tissue ECM glycoproteins interact with pollen tubes as TTS proteins do.

TTS Proteins Display a Gradient of Increasing Glycosylation Coincident with the Direction of Pollen Tube Growth

TTS proteins from the entire tobacco styles have apparent molecular weights ranging between 50–100 kDa on SDS–polyacrylamide gels (Wang et al., 1993; Cheung et al., 1995). Immunoblot analysis showed that TTS proteins isolated from the bottom third of the style have the highest average molecular weights, while those from the stigmatic third have the lowest average molecular weights (Figure 6A). After chemical deglycosylation, the TTS protein species from the three stylar segments detected by TTS antibodies are uniformly around 30 kDa. Moreover, the sugar: protein ratio (w/w) for TTS proteins with apparent molecular weights above 80 kDa is 0.9, and it declines to 0.36 and 0.24 for the TTS proteins between 60–80 kDa and those below 60 kDa, respectively. These results indicate that differences in apparent molecular weights of TTS proteins from the three stylar regions are due to the differences in their levels of glycosylation. Therefore, TTS proteins located closer to the stigmatic end of the style are on the average less glycosylated than those located closer to the ovary.

Superimposed on the gradient of increasing TTS sugar contents is an increasing TTS protein acidity gradient. While the predicted isoelectric point (pI) of the TTS polypeptide backbone is 9.9, immunoblots of two-dimensional SDS–polyacrylamide gel electrophoresis (SDS–PAGE) showed that TTS proteins have pIs ranging between 7.5–8.5, with the higher molecular weight TTS proteins having more acidic pIs (Figure 6B). Since TTS proteins have both neutral and acidic sugar components (Cheung et al., 1995), the higher molecular weight TTS proteins are increasingly acidified by sugar modifications. Thus, the increasing TTS protein glycosylation gradient also confers a continuously increasing acidity gradient from the stigma toward the ovary, in the same direction as pollen tube growth.

Discussion

Although cellular interactions between pollen and pistil during compatible pollination have been thoroughly documented (Herrero and Dickinson, 1980; Knox, 1984; Kandasamy et al., 1994), little is known about the physical and biochemical interactions underlying successful pollination (Cheung, 1995). We show in an accompanying paper (Cheung et al., 1995) that a transmitting tissue–specific glycoprotein from tobacco, TTS protein, promotes pollen tube growth in vitro, attracts pollen tubes in semi–in vivo pollen tube growth cultures, and is important to pollen tube growth in vivo. This paper describes the molecular interactions between TTS proteins and pollen tubes. TTS proteins interact with pollen tubes physically through bind-

ing to their surface and tips and incorporating into their walls. They also interact with pollen tubes biochemically through their deglycosylation by pollen tube–bound enzymes.

TTS Protein-Pollen Tube Interactions

The pH-dependent release of TTS proteins from pollen tube surfaces (Figures 1D–1G) indicate that they adhere to pollen tubes through charge interactions. TTS proteins are basic molecules with pIs ranging between 7.5–8.5 (Figure 6B). At pH 6, the numerous positively charged lysine residues on TTS proteins may interact with the negatively charged pectins on the pollen tube surface (Li et al., 1994). These interactions would be weakened at pH 8, resulting in the release of these pollen tube surface–adhered TTS protein molecules (compare Figures 1D and 1E with 1F and 1G). The resistance of the pollen tube tip–associated TTS proteins to TBS (pH 8) washes suggests a different, and stronger pollen tube–TTS protein interaction than that on the pollen tube surface. Whether a specific receptor for TTS proteins at the tube tip is involved remains to be determined. The pollen tube tips continuously deposit cell membrane and wall materials at the extending apex. The strong binding of TTS proteins to pollen tube tips (Figures 1D–1G) favors the possibility that these proteins are incorporated into the pollen tube wall as wall materials are being deposited at the growth zone. Further experiments will be required to understand exactly how TTS proteins are incorporated into the pollen tube wall.

It is known that pollen tubes produce many hydrolases and cell wall metabolism–related proteins that may be required to penetrate pistil tissues and to support the metabolism of the tube growth process (Knox, 1984). Our results show that pollen tubes produce hydrolases that deglycosylate a predominant transmitting tissue glycoprotein (Figures 3 and 4). These deglycosylating enzymes are tightly bound to the pollen tubes (Figure 5A), perhaps to ensure that the released sugar molecules are readily available for pollen tube utilization. Whether the tightly pollen tube–bound TTS proteins are the only substrates for these enzymes remains to be determined. However, another major class of transmitting tissue AGPs, the 100–200 kDa AGPs, does not bind to pollen tubes with the same affinity as TTS proteins, and the sugar residues on these unbound AGPs are not noticeably hydrolyzed by the pollen tube–bound enzymes (Figure 5B). These results suggest that the pollen tube–binding property of TTS proteins may have special significance toward the function of these proteins (see below).

TTS proteins are synthesized and glycosylated in the transmitting tissue cells and secreted to the ECM where they interact with pollen tubes. Through these interactions, the glycomoieties on TTS proteins are made available to the pollen tubes, presumably for incorporation into their metabolism. These interactions between a pistil protein and the pollen tube exemplify a novel mechanism for sugar mobilization across cellular barriers.

The Basis of the TTS Protein Pollen Tube Growth-Stimulating Activity

Pollen tube growth requires continuous synthesis of cell wall and membrane materials. Therefore, the metabolic demands for this process are substantial. The deglycosylation of TTS proteins by in vitro grown pollen tubes (Figures 3 and 4) and the accumulation of underglycosylated TTS proteins (TTS-30) in pollinated styles (Wang et al., 1993; Figures 3 and 4) are consistent with the proposal that the released sugar molecules may be used by the pollen tubes as nutrients. On the other hand, the persistence of the TTS protein pollen tube growth–promoting and –attracting activity in the presence of free sugars suggests that supplying sugars to the growing pollen tubes may not be their only function (Cheung et al., 1995).

The tightly pollen tube–bound TTS proteins recovered in the PTP fraction are underglycosylated (Figures 3 and 4). Therefore, these presumably pollen tube tip–associated and wall-bound TTS proteins may contribute to the pollen tube growth–promoting activity via sugar donation. In contrast, the TBS (pH 8) eluted TTS proteins remain highly glycosylated (Figure 3, lane 6; Figure 4B, lanes 1–4). Therefore, these presumably pollen tube surface–adhered TTS proteins are not likely to contribute to the pollen tube growth–promoting activity by making available their sugar residues. Pollen tube growth has been considered analogous to cell migration in animals (Lord and Sanders, 1992), which depends on cell surface adhesive molecules in the ECM (Hynes and Landers, 1992). Sticky substances in the transmitting tissue ECM, such as AGPs, have been speculated to serve as surface adhesive substrates for this process. The tendency for TTS proteins to polymerize in vitro (Cheung et al., 1995) and their adhesion to pollen tube surfaces both in vivo and in vitro (Figure 1) suggest that these proteins may also act as cell surface adhesive substrates to facilitate pollen tube elongation.

It has been proposed that pollen tube growth may be accelerated by the synthesis of more extensible components and inhibited by the synthesis of more rigid components for the pollen tube wall (Rosen, 1961). Incorporation of TTS proteins into the pollen tube wall may influence the wall property such that pollen tube elongation is facilitated, although this possibility will need further investigation. If the ability of TTS proteins to associate with the pollen tube surface, tip, and wall and their pollen tube growth–promoting activity can be dissected on a structural–functional level, the functional contribution by each of these modes of interaction between TTS proteins and pollen tubes could be deciphered.

Contrary to other cell growth–promoting and cell-attracting proteins such as nerve growth factors (Gundersen and Barrett, 1979) and netrins (Serafini et al., 1994; Kennedy et al., 1994), which exist in miniscule amounts, TTS proteins are abundant in the transmitting tissue extracellular matrix. The interactions between pollen tubes and TTS proteins result in the physical transfer of TTS proteins from the transmitting tissue ECM to the tip, surface, and wall of pollen tubes as well as the biochemical alteration of the

properties of these proteins. Therefore, large amounts of TTS proteins are needed to support the long and numerous pollen tubes that elongate in the transmitting tissue.

Possible Significance of a TTS Protein-Bound Sugar Gradient in the Transmitting Tissue in Pollen Tube Guidance

TTS proteins attract pollen tubes when presented at a distance in semi–in vivo cultures (Cheung et al., 1995). Pollen tubes presumably elongate along gradients of increasing TTS protein and sugar concentrations established by diffusion from the protein source. The ability of pollen tubes to deglycosylate TTS proteins (Figures 3–5) and the observation that chemically deglycosylated TTS proteins were not effective attractants for pollen tubes (Cheung et al., 1995) indicate that pollen tubes interact with the sugar moieties on these proteins. Therefore, pollen tubes should be able to perceive changes in sugar concentration associated with TTS proteins and grow toward those molecules with higher sugar modification. If the sugar residues on TTS proteins are indeed hydrolyzed by pollen tubes in vivo, similar to what occurs in vitro and as suggested by the accumulation of the underglycosylated TTS-30 species in pollinated styles (Wang et al., 1993), pollen tubes may sense the continuously increasing sugar level associated with individual TTS protein molecules presented at their tips as better sources of nutrients, and so respond by extending along the gradient of increasing TTS protein sugar modifications and, thus, toward the ovary. Although free sugars, polysaccharides (Knox, 1984), and other glycoproteins (e.g., Lind et al., 1994; Du et al., 1994) are abundant in the transmitting tissue, TTS proteins are the predominant pollen tube–binding glycoproteins (Figure 5B) and ensure their sugar moieties are in direct contact with the elongating tubes by adhering to their surface and tips, thus favoring their perception by and interactions with pollen tubes. This contrasts with free sugar molecules and glycoproteins that do not adhere to pollen tubes (Figure 5B). These glycomolecules only come into contact with pollen tubes by diffusion and so will not be as efficiently perceived or utilized by pollen tubes as the sugar moieties on TTS proteins are.

Direct evidence supporting a role in pollen tube guidance by varying the glycosylation level in TTS proteins remains to be obtained. Regardless of whether the gradient of increasing glycosylation associated with TTS proteins plays a role in pollen tube directionality, the properties associated with these proteins suggest that they are uniquely suited to support pollen tube growth. In microbial cell motility systems, cell attractants are often compounds that are central to the metabolism of the cells, e.g., sugars and sugar derivatives (Macnab, 1987; Devreotes and Zigmond, 1988). In mammalian cell motility systems, binding of matrix adhesive proteins to cell surfaces is important to cell migration (Hynes and Landers, 1992). TTS proteins combine these two features into the same molecule: they are cell surface adhesive proteins whose sugar residues are hydrolyzed, and presumably utilized by pollen tubes. If both of these properties indeed contribute to mediating the pollen tube–attracting activity of TTS proteins, these proteins may be considered to behave as matrix-bound adhesive (haptotropic) agents as well as chemotropic agents emanating from continuous intermediate target sites along the pollen tube growth pathways, keeping pollen tubes directed toward the ovary. The increase in acidity associated with increased TTS protein glycosylation (Figure 6B) may accentuate this gradient, or it may contribute to a guidance mechanism based on differential charge distribution. Furthermore, by maintaining differential levels of sugar modification, TTS proteins become the backbone of a gradient of sugar molecules. If a long range gradient of sugar molecules were indeed perceived as a directional cue for pollen tube growth, a protein-bound concentration gradient of low molecular weight sugar molecules should be more stable and, thus, more effective than a gradient of free sugar molecules. Moreover, the probable deglycosylation of TTS proteins by pollen tubes as they penetrate the transmitting tissue will also sharpen the glycosylation gradient locally. A self-sharpening gradient has also been proposed as a possible explanation for the chemoattraction of commissural axons by the floor plate–derived factor that it may be degraded or otherwise inactivated by neural tissues, creating a local concentration gradient (Placzek et al., 1990).

Gradients of morphogens are a hallmark in animal development (Jessel and Melton, 1992; Gurdon et al., 1994). Chemoattractants are important to microbial as well as animal cell motility systems and developmental pathways (Trinkaus, 1985; Macnab, 1987; Devreotes and Zigmond 1988; Tessier-Lavigne and Placzek, 1991), they are also believed to function in the directional growth of pollen tubes (Rosen, 1961; Mascarenhas and Machlis, 1962a, 1962b; Mascarenhas, 1975, 1993; Reger et al., 1992a, 1992b). The gradient of increasing TTS protein glycosylation coincident with the direction of pollen tube elongation is a unique protein-based sugar gradient observed in the female reproductive tissue. Understanding the regulation of TTS protein glycosylation and genetically altering the in vivo gradient of glycosylation level associated with these proteins should produce insights into whether this protein-bound sugar gradient provides directional guidance for pollen tubes through the transmitting tissue.

Experimental Procedures

TTS Protein Isolation

TTS proteins were purified from the styles of preanthesis Nicotiana tabacum (W38) flowers (Cheung et al., 1995).

Fluorescence Immunodetection of TTS Proteins on Pollen Tubes

To detect TTS proteins associated with pollen tubes in vivo, styles that had been hand-pollinated for 24 hr were bisected longitudinally. The exposed transmitting tissues were washed in 20 mM Na-phosphate (pH 6), 150 mM NaCl (PBS [pH 6], approximating the pH in the transmitting tissue) three times for 10 min each to elute free TTS proteins. They were fixed in 3:1 ethanol:glacial acetic acid for 30 min and washed twice in distilled water for 10 min each before

immunodetection. Unpollinated styles were treated similarly. Style tissues were blocked in TBS (pH 7.5) (20 mM Tris–HCl [pH 7.5], 150 mM NaCl), 20% calf serum for 30 min, reacted with preimmune or TTS antibodies (1:100) (Wang et al., 1993) for 4 hr, washed in TBS (pH 7.5), 0.05% Tween 20 (TBST) three times for 30 min each, treated with FITC-labeled secondary antibodies against rabbit IgG (1:100) for 1 hr, and washed three times in TBST before being squashed onto microscope slides for viewing by fluorescence microscopy.

To detect association of TTS proteins with pollen tubes in vitro, pollen grains were inoculated at 10^4 grains/ml of GM (pH 6) (Cheung et al., 1995) supplemented with 2% sucrose and grown for 3 hr at 28°C. Pollen tubes were separated from ungerminated grains by filtering through 80 μm nylon filters and rinsed extensively with the sugar-free GM. All subsequent steps were carried out on these nylon sieves in petri dishes. TTS proteins (20 μg/ml) were added to these pollen tubes in the sugar-free GM and were incubated at room temperature for another 2 hr. The tubes were either washed with the sugar-depleted GM or sequentially with this medium and TBS (pH 8). Control pollen tubes grown in the absence of TTS proteins were washed with GM before immunodetection assays. These pollen tubes were fixed and used in immunodetection of TTS proteins as described above. All washes were performed by passing 25 ml of wash solutions over the nylon sieves on a Buchner funnel. Pollen tubes were viewed by fluorescence microscopy.

Cytoimmunodetection of TTS Proteins

For in vivo grown pollen tubes, pollinated styles were cut into 2 mm segments and fixed in 2% glutaraldehyde, 1% paraformaldehyde, 2% sucrose, 0.05% $CaCl_2 \cdot 2H_2O$, 0.1 M PIPES (pH 7), 0.1% picric acid, 0.1% Tween 20 for 4 hr, dehydrated, and embedded in LRWhite (Sommerville and Scheer, 1987). In vitro pollen tubes were grown as described above for fluorescence immunodetection. After washing with TBS (pH 8), they were fixed and embedded as described above for the in vivo style segments. Ultrathin sections from these samples were blocked in TBST, 3% BSA for 15 min, incubated with TTS or preimmune antiserum (1:25 dilution in TBST, 3% BSA) at room temperature for 2 hr, and washed extensively with TBST, 1% BSA. The grids were then incubated with colloidal gold (15 nm)-labeled secondary antibodies against rabbit IgG (at 1:50 dilution in TBST, 3% BSA) for 1 hr at room temperature. They were then washed in TBST, 1% BSA and in distilled H_2O. Grids were stained by uranyl acetate and lead citrate before viewing. The sections were viewed on a Zeiss-10 electron microscope.

Immunoblot Analysis of TTS Protein–Pollen Tube Interactions In Vitro

Pollen grains (10^4/ml, unless otherwise stated) were germinated and grown for 3 hr in pollen GM (pH 6) (Cheung et al., 1995) supplemented with 2% sucrose. Pollen tubes were separated from ungerminated grains by filtering through 80 μm nylon filters and rinsed with the sugar-free GM. The pollen tubes were returned to the sugar-free GM in microfuge tubes. Either no TTS protein (control) or 20 μg/ml TTS proteins were added to the pollen tube growth cultures, and they were maintained at normal pollen tube growth conditions for specified periods of time. After growth, these pollen tube cultures were treated as outlined in Figure 3A. Pollen tubes were collected by centrifuging at 3 krpm for 3 min. The GM was recovered and saved for analysis (unbound TTS proteins). The pollen tubes were washed twice by 1.5 ml of GM each. The pollen tubes were then washed again in 1.5 ml of TBS (pH 8) and centrifuged, and the suprenatants were collected for analysis (pollen tube surface–bound TTS proteins). These pollen tubes were washed twice more in TBS (pH 8). The pollen tubes were then resuspended in 200 μl of protein extraction buffer (100 mM Tris–HCl [pH 6.8], 50 mM NaCl, 2% SDS, 10 mM β-mercaptoethanol) and boiled for 10 min. The extract was centrifuged, and the suprenatants were analyzed (tightly pollen tube–bound TTS proteins). The recovered GM, the first GM wash (1.5 ml) (GMW), and the first TBS wash (1.5 ml) (TBSW) were acetone precipitated, and the precipitated proteins were resuspended in 100 μl of protein loading buffer. These proteins (10 μl) and 20 μl of the PTPs were used in protein gel blot analysis. Immunoblots were carried out using TTS antibodies followed by alkaline phosphatase–labeled secondary antibodies against rabbit IgG (Wang et al., 1993).

To examine the effect of sugars on TTS protein–pollen tube interactions, TTS proteins (20 μg/ml) and 20 mg/ml of sucrose, galactose, glucose, or arabinose were added to pollen tube growth cultures (10^4 grains/ml) and incubated for 6 hr. The TBSW and PTP samples were analyzed as described above.

To examine the interaction between other stylar AGPs and pollen tubes, 20 μg/ml each of TTS proteins and of a partially purified 100–200 kDa stylar AGPs (Cheung et al., 1995) were added to the pollen tube growth cultures (2×10^4 grains/ml) for 12 hr. The GM from these cultures was recovered, electrophoresed on SDS–PAGE, and electroblotted. The protein blot was stained with a β-glucosyl Yariv dye (Biosupplies, Australia) (Cheung et al., 1995).

To determine the localization of TTS protein deglycosylation activity, the GM was recovered from a 6 hr pollen tube growth culture (inoculated at 10^4 grains/ml in the absence of TTS proteins). The remaining pollen tubes were pulverized in liquid nitrogen. Soluble pollen tube proteins were extracted by TBS (pH 7.5), 1 mM PMSF. The pollen tube soluble protein fraction was separated from the pollen tube tissue pellet that should retain all the membrane- and wall-bound proteins. TTS proteins (20 μg/ml) were incubated overnight in the recovered GM, the pollen tube soluble protein fraction, or in the pollen tube tissue pellet resuspended in TBS (pH 7.5), 0.1% Triton X-100. TTS proteins in each of the reactions were detected by immunoblot analysis after SDS–PAGE.

Immunoblot Analysis of Stylar TTS Proteins

Isolation of total stylar proteins from pollinated and unpollinated styles and immunoblot analysis of TTS proteins were carried out as described (Wang et al., 1993). Proteins were also isolated separately from the upper, middle, and lower thirds of the styles. Two-dimensional SDS–PAGE (12.5%) for highly basic proteins (O'Farrel et al., 1977) was carried out with 100 μg of transmitting tissue proteins eluted by the TTS protein isolation buffer (Cheung et al., 1995).

Sugar:Protein Determination for TTS Proteins

Purified TTS proteins were separated on 12.5% SDS–PAGE. TTS proteins with apparent molecular weights above 80 kDa, between 60–80 kDa, and below 60 kDa were excised from the gel and electroeluted and dialyzed extensively against distilled water. Total sugar and protein concentrations were determined spectrophotometrically (York et al., 1985).

Chemical Deglycosylation of Proteins

Hydrogen fluoride treatment of proteins was carried out as described (Van Holst and Varner, 1984).

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