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Hydroxyproline-rich glycoproteins in plant reproductive tissues: structure, functions and regulation

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Abstract. The plant reproductive process of pollination involves a series of interactions between the male gametophyte (the pollen grain or pollen tube) and extracellular matrix (ECM) molecules secreted by different cell types along the pollen tube growth pathway in the female organ, the pistil. These interactions are believed to signal and regulate the pollen tube growth process to effect successful delivery of the sperm cells to the ovules where fertilization takes place. Hydroxyproline-rich glycoproteins secreted by plant cells are believed to play a broad range of functions, ranging from providing structural integrity to mediating cell-cell interactions and communication. The pistil and pollen tube ECM is enriched in these highly glycosylated proteins. Our discussions here will focus on a number of these proteins for which most information has been available, from *Nicotiana tabacum*, its self-incompatible relative *N. alata*, and *Zea mays.* In addition, the regulation of the synthesis and glyco-modification of one of these proteins, TTS (transmitting tissue-specific) protein from *N. tabacum* will be discussed in the light of how differential glycosylation may be used to regulate molecular interactions within the ECM.

Key words. Arabinogalactan proteins; extensins; pollination; pollen tube elongation and guidance; nutrition and structural support.

Introduction

Plant ECM defines the continuum at the cell surface that spans the extracellular side of the cell membrane, the primary and secondary cell walls and the intercellular space between neighboring cells. It also marks the interface between the epidermal cells and the environment. The ECM is highly dynamic and plays a broad range of structural and biological roles in plant growth and development. It provides protection, structural support for cell shape and integrity, mediates cell-cell interactions and interactions between plant cells and their environment [1]. Embedded in a matrix built largely by cellulosic and pectate molecules [2] are a diverse set of cell wall proteins [3, 4]. The most extensively characterized among these cell wall components are a collection of proline-rich and hydroxyproline-rich glycoproteins [3, 4]. Classically, these proteins have been categorized into four major groups: the extensins, arabinogalactan proteins (AGPs), proline-rich proteins (PRPs) and solanaceous lectins (SLs). The traditional definitions for these classes of proteins were based on the presence of characteristic proline residue-containing motifs (e. g. repetitive Ser-Pro_4 for the extensins, multiple copies of Val-Tyr-Lys-Pro-Pro or its variants for the PRPs) [3, 4], reactivity towards diagnostic reagents (e. g. β-glucosyl Yariv reagent reactivity for AGPs) [5] or having a bipartite structure of a hydroxyproline-rich domain and a nonproline-rich chitin binding domain (for SL) [6]. However, the primary structures revealed for the large number of proline-rich ECM proteins thus far suggest that many of these proteins have structural features that are intermediate or composites of more than one of the four traditional classes, or have additional structural domains. For the proteins and their genes or complementary DNAs (cDNAs) we discuss here, we shall maintain their original nomenclature and classification, although some

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of these original assignments do not adequately accommodate all the structural and biochemical features for these proteins.

The expression of many proline-rich protein genes are highly regulated both spatially and temporally [3], implying considerable functional specificity for these proteins. Sexual development involves the differentiation of special organ, tissue and cell types [7–9] that together, architecturally and biochemically, contribute to optimum reproductive success. Reproductive organ-specific proline-rich proteins, their genes and cDNAs have been described in a large variety of plant species (e.g. [10–13]). Extensive biochemical, functional and regulatory analyses have been carried out for a number of these proteins from *Nicotiana tabacum*, *N. alata* and *Zea mays*. We shall focus our discussion on these proteins.

Plant reproduction and the ECM

The male and female gametophytes (the pollen grain/pollen tube and the embryo sac, respectively) develop within layers of protective and nutritive cells inside the anther (the male reproductive organ) and the pistil, respectively [7, 14] (fig. 1). Pollination, the process by which sperm cells are delivered from the pollen to the embryo sac, involves a series of extracellular interactions between the male gametophyte and a number of different pistil cell types (fig. 2) [15–18]. A mature pollen grain is either bicellular or tricellular, composed of a generative cell or two sperm cells, respectively, within a vegetative cell. *Nicotiana* pollen belongs to the bicellular type, whereas *Z. mays* pollen is tricellular [7]. Fertilization depends on further cellular activities in the mature pollen

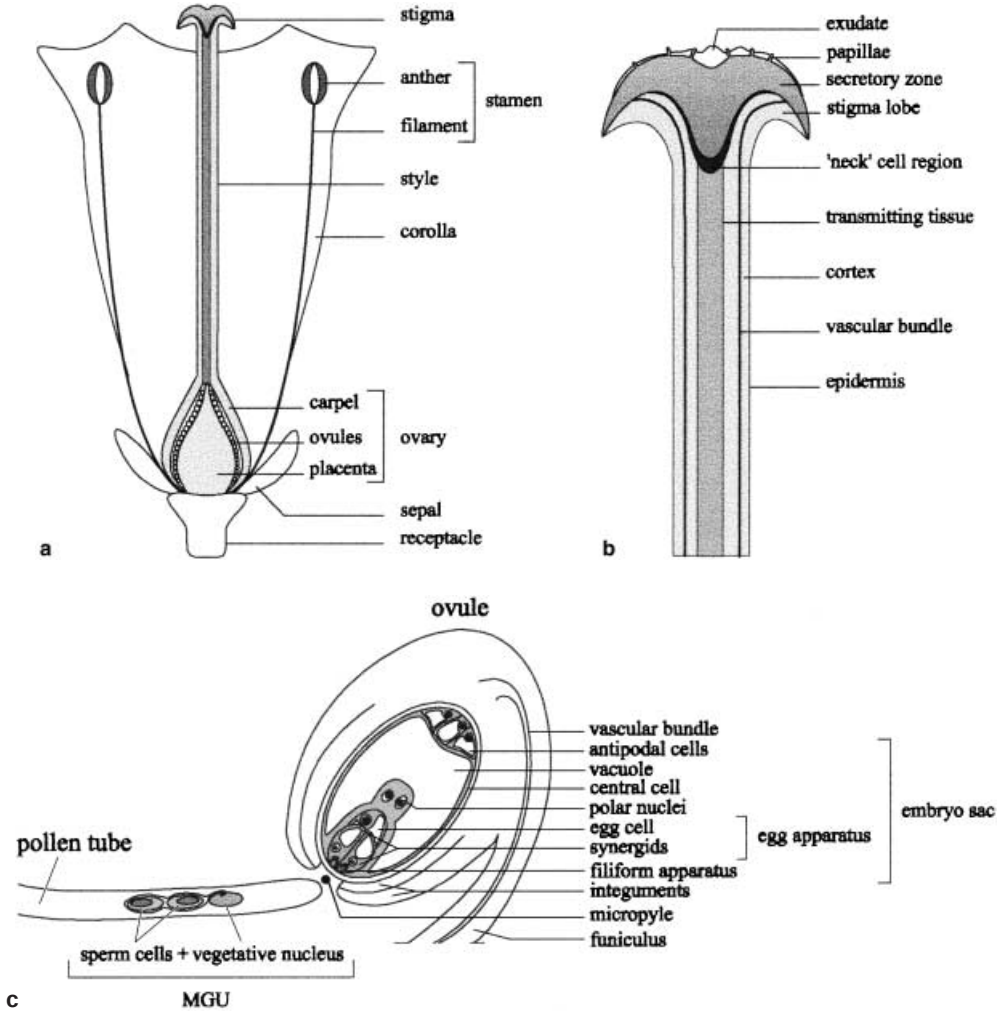

Figure 1. Schematic representations of the floral structure through which pollen tubes grow and enter the ovules. (*a*) Longitudinal view of a mature tobacco flower showing the different flower parts. The stigma, style and ovary make up the female reproductive organ, the pistil. Pollen grains develop within the anthers; each of the five anthers is subtended by a long filament. The anther and filament together make up the male reproductive organ, the stamen. (*b*) A longitudinal section through the stigma and style showing the different tissues, papillae, secretory zone, transmitting tissue, through which pollen tubes grow. (*c*) A longitudinal section through an ovule, showing the embryo sac, and an approaching pollen tube. The pollen tube needs to turn its growth trajectory in order to gain entrance into the embryo sac via the micropyle.

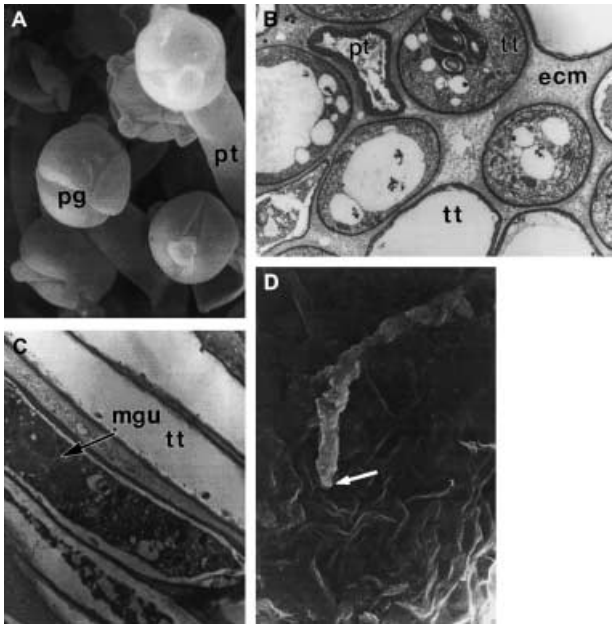

Figure 2. Scanning (*A,C*) and transmission (*B, C*) electron micrographs of various stages of tobacco pollen tube growth in the pistil. (*A*) Pollen grains (pg) and pollen tubes (pt) on the stigmatic surface. (*C*) A longitudinal section of a pollinated tobacco style. The cytosolic region of a pollen tube showing the presence of the male germ unit (mgu) (the vegetative nucleus and the two sperm cells) is seen traversing the extracellular matrix of transmitting tissue cells (tt); some of these cells have become highly vacuolated. (*B*) A section across a pollinated style. Pollen tubes are seen occupying the extracellular matrix (ecm) of transmitting tissue cells (tt). (*D*) A pollen tube (white arrow) entering an ovule via the micropyle. (Modified from [11]).

upon encountering the pistil. These activities result in the polarized outgrowth of a pollen tube from each grain (fig. 2) that grows with high efficiency (e.g. up to 1 cm an hour in *Z. mays*, and 1.5 mm an hour in *N. tabacum*) and over long distances within the pistil, often reaching distances thousands the length of the diameter of the pollen grains themselves [19, 20]. Pollen tubes ultimately reach the ovary and, in many species, turn from a basally oriented growth direction to enter the ovules (fig. 1c). The sperm cells are deposited inside the embryo sac within the ovules for fertilization.

Pollen grains from many species germinate and develop pollen tubes in simple, defined chemical media in vitro [21, 22]. However, few attain pollen tube elongation rates and accomplish the distances traversed as occurred in the in vivo process within the pistil. Physical and biochemical contributions from the female tissues are believed to play critical roles in vivo to ensure successful pollination [15, 17, 18, 23, 24]. The ECM of the stigma is secreted by the stigmatic papillae and the underlying secretory cell layers [25, 26]. Solid styles, such as those in *Nicotiana* and *Z. mays*, are filled with files of transmitting cells that secrete a relatively thick ECM [27–29] that allows passage of the pollen tubes. In the mature ovary, the ovules are covered with exudates presumably secreted by the placental epidermal cells and from within the ovules. Genetic ablation of stigmatic secretory cells [30] and transmitting tissue cells [31], and the failure to develop a proper embryo sac [32, 33] have been shown to result in female sterility or reduced female fertility. These genetics and reverse-genetics experiments lend further support for previous suggestions that the female tissues provide considerable support for the pollen tube growth process [15, 17, 18, 23, 24].

Figure 2 illustrates that until the pollen tube enters an ovule, its entire journey is within the ECM of pistil tissues, sometimes also referred to as the intercellular matrix, never invading the protoplast of any cell. Thus studies on reproduction-related molecules have focussed on pistil ECM molecules deposited along the pollen tube growth pathway and on pollen surface molecules. The ECM along the pollen tube growth pathway is highly enriched in lipoidal and glycosylated compounds [27, 28], among which is an abundance of a large number of different hydroxyproline-rich glycoproteins, including AGPs [11] and extensin-like proteins (e.g. [10, 12, 34]). The pollen ECM also has a preponderance of hydroxyproline residues [35], most of which are likely to be contributed also by these two families of hydroxyproline-rich glycoproteins.

Hydroxyproline-rich glycoproteins in the stigma

Stigma provides the first contact between pollen and the female reproductive tissue (fig.1). Upon deposition of the pollen-receptive surface on the stigma, pollen grains hydrate and germinate to produce pollen tubes (fig. 2A), which elongate and penetrate the stigmatic tissues. Stigma exudates are enriched in lipids and glycosylated compounds [25, 26]. They are believed to provide recognition molecules, adhesives, nutrients, lubricants and directional guidance for the early phase of pollen tube development [24, 36–39]. Based on strong reactivities towards β-glucosyl Yariv reagent, stigmatic exudates from a large number of angiosperms have been shown to be enriched in AGPs [36, 40, 41].

AGPNa3 protein (also known as RT35 protein) from *N. alata* is the only stigmatic AGP that has been characterized thus far [42]. Like most AGPs, AGPNa3 protein is highly soluble, even in 95% $(NH_4)_2SO_4$, and is precipitated by Yariv β-glucosyl reagent. AGPNa3 messenger RNA (mRNA) accumulates almost exclusively in the stigma, and the protein was inferred from this data to be predominantly a stigma protein. The subcellular localization of AGPNa3 remains to be determined but is most likely extracellular.

The predominant monosaccharides in AGPNa3 protein are arabinose and galactose (45 and 52%, respectively),

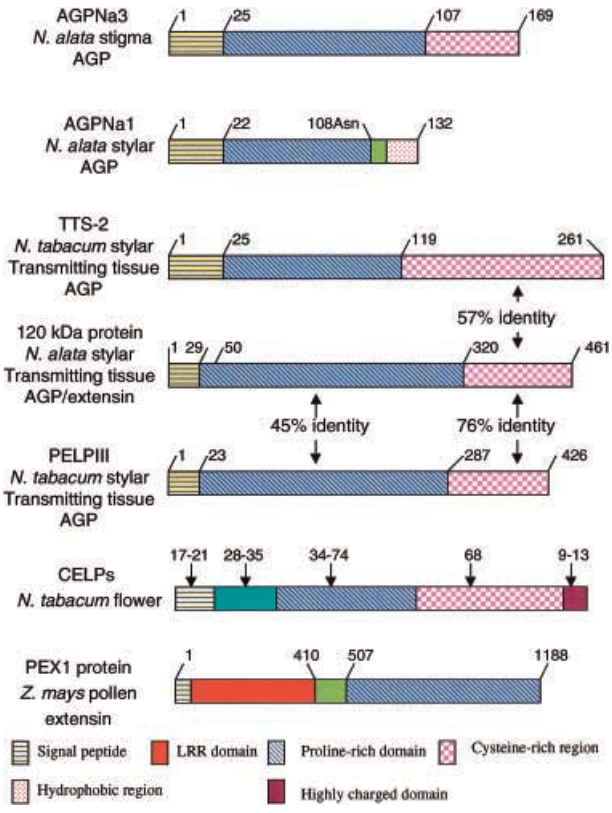

Figure 3. Primary structural features from deduced amino acid sequences of the reproductive tissue hydroxyproline-rich glycoproteins described in this paper. In AGPNa1 protein, residue 108Asn is the C-terminal of the mature protein. In TTS-2 protein, there are two predicted N-linked glycosylation sites in the cysteine-rich region (not shown), one or both of these are used. The number at the junction between the signal peptide and the proline-rich regions for each protein indicates the predicted or determined N-terminus of the mature proteins. In 120-kDa protein, amino acid residue 50 is the chemically determined N-terminus of the mature protein.

and they are primarily found as terminal arabinose furanoside (41%) and 1, 3, 6-linked galactoside pyranoside (41%). The nucleotide sequence of the AGPNa3 cDNA predicts a precursor polypeptide of 169 amino acids with a 25-amino-acids-long signal peptide. Data showing the apparent molecular weight of the native AGPNa3 protein and its protein-to-sugar ratio have not been reported. The putative mature polypeptide backbone for AGPNa3 has a proline-rich N-terminal domain and a C-terminal domain that contains eight cysteine residues (fig. 3). AGPNa3 mRNA has been shown to accumulate at the highest level in the mature pistils and decline thereafter to a very low level 5 days after pollination. Du et al. [42] suggested that AGPNa3 protein has a specific, yet to be determined, role in the pistil.

Hydroxyproline-rich glycoproteins in the style

After penetrating the ECM of stigmatic cells and cells of the underlying secretory layers, pollen tubes continue their journey in the transmitting tract (fig. 2B, C) and elongate directionally towards the ovary. Pollen tubes either traverse the ECM secreted by tightly packed transmitting tissue cells in the solid styles [27–29] or elongate along the surface of epidermal cells that line the transmitting canal of the hollow styles [43, 44]. Pollen tubes are believed to derive physical and biochemical support and directional cues from the stylar transmitting ECM [17, 18, 23, 24, 45–47]. Recent analyses of stylar proteins and stylar-expressed genes indicate that stylar glycoproteins, especially the hydroxyproline-rich glycoproteins, are predominant ECM constituents, and some may play important roles in pollen tube elongation [46–50]. For instance, the AGP-enriched lily stylar transmitting canal exudate has been shown to serve as an adhesive matrix and enhances pollen tube growth in vitro [48]. The adhesive activity was subsequently shown to be dependent on two different classes of molecules, a high molecular weight pectin species and a low molecular weight lipid-transfer protein-like species [49, 50].

AGPNa1 protein

AGPNa1 protein (also referred to as RT25 protein) is an AGP isolated from pulverized style or tissue of *N. alata* [51]. AGPNa1 protein appeared as a smear at molecular weights over 90 kDa. Ninety percent of its mass is contributed by carbohydrates, the most abundant of which are galactose and arabinose (60 and 30%, respectively). The amino acid sequence predicted from the AGPNa1 cDNA nucleotide sequence reveals a N-terminal proline-rich domain followed by a stretch of hydrophobic sequence at the C-terminal region (fig. 3). Mature AGPNa1 protein terminates without this hydrophobic C-terminal domain. Instead, a glycosylphosphatidylinositol (GPI) anchor is found attached to the C-terminal asparagine residue of this protein, 24 amino acid residues proximal to the predicted C terminus [52, 53]. GPI-anchored AGPs appear to be a common feature among some of these hydroxyproline-rich glycoproteins [54, 55] and may serve important functions at the interface of the ECM and the plasma membrane.

AGPNa1 mRNA is present in root, leaf, stem and all floral tissues, with root AGPNa1 mRNA level substantially higher than those observed in other tissue [51]. In situ localization of AGPNa1 mRNA or protein has not been reported, and no specific functional role has been proposed for AGPNa1 protein in the style or in other tissues. It was, however, suggested that presence of a GPI-anchor would help gain insights into its functions [52].

TTS protein, a pollen-tube-growth-promoting and -attracting protein

TTS (transmitting tissue-specific) protein is a *N. tabacum* stylar transmitting tissue AGP that has been studied extensively at the structural, functional and regulatory levels [46, 47, 56, 57]. Virtually identical proteins have also been characterized from the *N. sylvestris* [11] and *N. alata* [58, 59] styles. Two very similar genes, TTS-1 and TTS-2, encode the *N. tabacum* TTS protein. The predominant population of TTS protein molecules spans a molecular weight spectrum of between 45 and 105 kDa, whereas the deglycosylated backbone polypeptide has a molecular weight of about 28 kDa, approximating that predicted from the amino acid sequences deduced from their cDNAs. TTS protein has both N- and O-linked glyco-modifications. Seventy percent of their sugar residues are galactose, followed by arabinose. These protein molecules react efficiently with Yariv β-D-glucoside reagent (46) and JIM13, a monoclonal antibody against AGP. The deduced primary structure of TTS protein molecules has a proline-rich N-terminal domain and a six-cysteine residue C-terminal domain (fig. 3). Monomeric TTS glycoprotein molecules assume a spherical to ellipsoidal shape, reminiscent of the 'wattle blossom' model proposed for AGPs [41] and to another AGP purified from carrot [60]. TTS protein molecules tend to oligomerize (fig. 4), suggestive of a highly adhesive nature. Oligomerization has also been observed for a carrot AGP [60]. TTS polypeptide backbones are highly basic with pIs of about 10, whereas native TTS protein molecules have pIs ranging from 7.5 to ~9, with the higher molecular weight species having more acidic pIs [47]. Acidic sugar residues have been detected in purified TTS proteins. The declining pIs with increasing sugar contents in these glycoprotein molecules are consistent with acidification of the backbone polypeptide by sugar modifications on the N-terminal hydroxyproline-rich half of the molecules. Deglycosylation disrupts the ability of TTS protein molecules to assume these oligomeric states [unpublished observation]. It seems reasonable to speculate that native TTS protein molecules interact in vitro with each other in a head-to-tail fashion by charge-charge interactions between the N and C termini to assume the observed ordered configuration (fig. 4). In vivo, how TTS protein molecules interact with each other, with other transmitting tissue ECM molecules and pollen tube surface molecules (see below) remains to be determined.

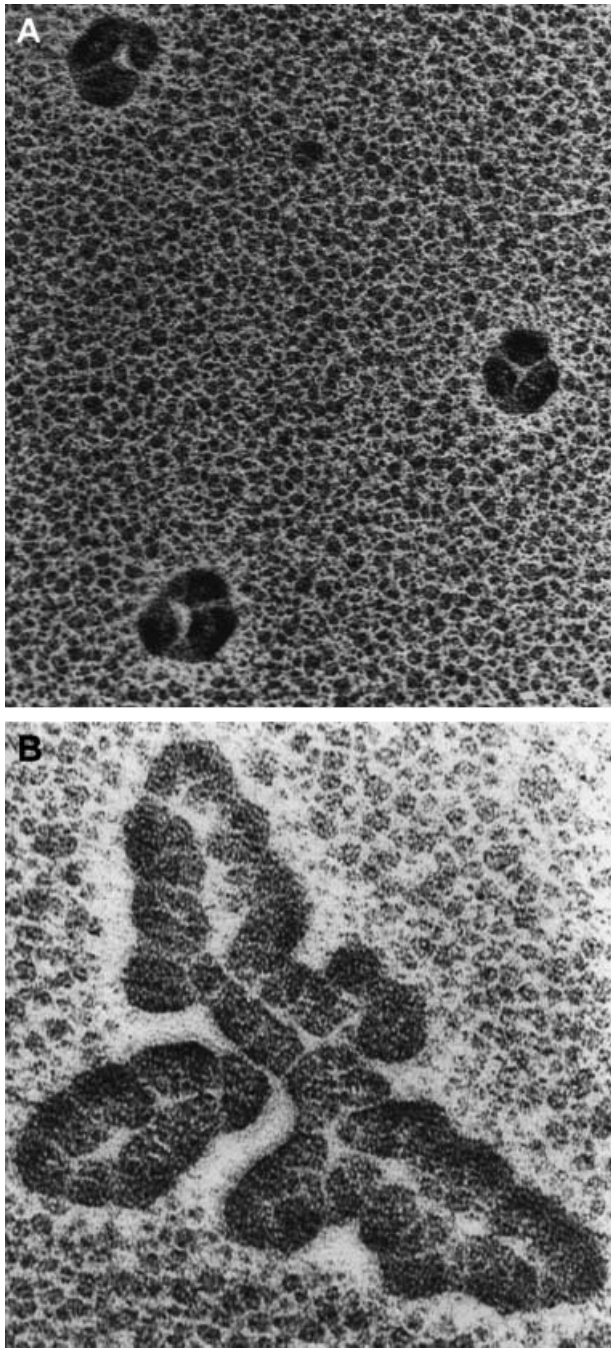

Figure 4. Transmission electron micrographs of trimeric (*A*) and multimeric (*B*) of rotary-shadowed TTS protein molecules. Each ellipsoidal unit is a TTS monomer. (Adapted from [46]).

TTS protein is located predominantly in the intercellular matrix of the stylar transmitting tissue where pollen tubes elongate [57]. Addition of TTS protein to in vitro pollen tube cultures results in an enhancement of the pollen tube elongation rate [46]. Transgenic plants in which the level of TTS proteins has been reduced to very low levels have reduced pollen tube growth rate and reduced female fertility. Furthermore, when provided at a distance, TTS protein attracts pollen tubes that have emerged from a pollinated style and are elongating in an in vitro medium. These observations together indicate that this stylar transmitting tissue ECM AGP has the ability to promote pollen tube growth both in vivo and in vitro and attract pollen tubes in vitro.

Analyses of pollen tubes elongating in the style and those growing in vitro in the presence of TTS protein showed

that both TTS protein and pollen tubes actively participate in a set of intimate interactions [47]. TTS protein molecules adhere to pollen tube tips and surface, and they are incorporated into the pollen tube walls. Pollen tubes deglycosylate TTS proteins by cell-wall-associated or membrane-bound enzymes, most probably galactosidases. The physical and biochemical interactions between TTS protein molecules and pollen tubes suggest a dual basis for the ability of TTS protein to promote pollen tube growth: by serving as nutrient resource and surface adhesive for elongating pollen tubes.

Interestingly, in vivo, the level of sugar modifications associated with TTS protein molecules increases from the apical end to the basal end of the style, in the same direction as pollen tube growth [47]. This, and the in vitro and in vivo properties of TTS protein, prompted the suggestion that this glycoprotein has the potential to behave as a chemotropic or haptotropic substance for pollen tube elongation in the tobacco style. The in vivo significance of this gradient remains to be determined. However, in the styles of the related *N. sylvestris* and *N. alata* styles, their respective TTS homologs also display a gradient of increasing glycosylation levels similar to that observed for the *N. tabacum* TTS protein [11, 59]. This conservation lends additional support to the notion that the gradient of increasing TTS protein glycosylation level may be of biological significance.

"120-kDa protein"

The so-called 120-kDa protein is an abundant *N. alata* stylar transmitting tissue ECM hydroxyproline-rich glycoprotein [61]. Its most predominant monosaccharides are arabinose and galactose (55 and 45%, respectively). The 120-kDa protein has both extensin and AGP properties: its pI (basic) and protein-to-carbohydrate ratio (65%: 35%) are similar to extensins, its high solubility in aqueous buffers is similar to AGPs, its sugar linkages have characteristics of both AGPs (1,3-linked galactose, 1,6-linked galactose and 1,3,6-linked galactose) and extensins (1,2-linked arabinose), it is recognized by antibodies to SL (which are reactive towards the sugar moieties on extensins and SL) and by an antibody active towards sugar residues on *N. alata* stylar AGPs. Nevertheless, the 120-kDa protein does not react with β-D-glucoside Yariv reagent, despite the presence of sugar modifications characteristics of AGPs.

The primary structure of the 120-kDa protein [62], which has been deduced from three overlapping cDNA fragments isolated from two different cDNA libraries and a primer extension reaction, has a proline-rich N-terminal domain and a C-terminal domain with six cysteine residues. It also suggests a precursor polypeptide of about 43 kDa (fig. 3). The deglycosylated backbone for the 120-kDa protein has an electrophoretic mobility on SDS-polyacrylamide gel electrophoresis (PAGE), suggesting a molecular weight of about 78 kDa [62]. Anomalies in mobility due to special structural parameters or yet to be determined processing of the precursor protein, in addition to cleavage of the N-terminal signal peptide, have been suggested to account for the differences of the observed and predicted molecular weights of the 120-kDa protein [62].

RNA expression analysis indicated that the 120-kDa protein mRNA accumulates in the style, starting at the mid-maturation stage. The level of this mRNA remains similar until floral maturation and declines thereafter [62]. The 120-kDa protein has been shown to be taken up into the cytosol of in vivo elongating pollen tubes [63], a unique characteristics among all the characterized stylar transmitting tissue ECM hydroxyproline-rich glycoproteins. Functions in defense, cell-cell communication, cell growth and development, maintaining structural integrity of the ECM and providing nutrient resources for pollen tubes have all been suggested as possible roles played by the 120-kDa protein.

PELP III

PELP (pistil extensin-like protein) III is a major *N. tabacum* stylar transmitting tissue ECM protein [34, 64] that shares the bipartite N-terminal proline-rich and C-terminal cysteine-rich structure and some homology with TTS and the 120-kDa proteins described above (fig. 3). However, antibodies that were produced against *Escherichia coli* expressed C-terminal domain of TTS protein and PELPIII do not cross-react with each other [unpublished observations]. Contrary to the 120-kDa protein but similar to TTS protein, PELPIII is recognized by Yariv β-D-glucosyl reagent [64], indicative of its AGP nature.

After pollination, PELPIII is translocated from the transmitting tissue ECM directly into the walls of elongating *N. tabacum* pollen tubes [65–67]. This translocation appears to be extremely efficient, since by 40 h after pollination, PELPIII was no longer detectable in the transmitting tissue ECM. Instead, an abundance of PELPIII was detected in the callose wall and plugs of the pollen tubes, with the highest concentration of these proteins appressing the region next to the plasmalemma.

An extensive analysis of PELPIII homologs in various plant species has been carried out [66, 67]. High levels of PELPIII RNA are present in the styles of *N. alata*, *N. sylvestris* and *N. x sanderae*. Lower levels of PELPIII-related transcripts are also found in mature and nonpollinated stigma and styles of *N. rustica*, *N. paniculata* and *N. Brugmansia aurea*. No PELPIII-like mRNAs have been detected in the tissue of *N. trigonophylla*, *N. undulata*, *Salpiglossis sinuata*, *Petunia hybrida* and *Lilium longiflorum*. Interestingly, after interspecific crosses,

PELPIII from the pollinated *N. tabacum* transmitting tissue were also translocated to the pollen tube walls of *N. rustica*, *N. trigonophylla* and *P. hybrida*, species that normally do not accumulate extractable amounts of this protein.

The mechanism of PELPIII translocation into pollen tube walls and its biological significance remains to be determined. It has been speculated that PELPIII may be involved in the development of the specific characteristics of the semisolid transmitting tract and could play an additional, indirect role in pollen tube wall assembly by translocation into the callosic layer [67].

CELPs

Cysteine-rich extensin-like proteins (CELPs) are a class of *N. tabacum* flower predominant extensin-like hydroxyproline-rich glycoproteins [12, and unpublished results]. They are encoded by a complex family of more than 10 related genes [unpublished results]. The deduced amino acid sequences of five members of this protein family (fig. 3) show that they have two main structural domains. The N-terminal region is characterized by an extensin-like domain with multiple X-$(proline)_{3-7}$ motifs and X-proline doublets, where X can be serine, cysteine or tryptophan. This is followed by an eight-cysteine-residue domain (fig. 3). The C-terminal region of CELPs is a short, highly charged domain. The extensin-like domain in CELPs differs significantly in their lengths, probably the result of recombination-induced deletions and duplications within the proline-rich encoding region. The cysteine-rich region is highly conserved among all the characterized members of CELPs. The C-terminal region of the different CELPs differs in the number of charged amino acid residues. Of the two CELP genomic clones characterized, the region that encodes this highly charged domain is precisely separated from the rest of the coding region by an intron, suggesting that it may define a discrete functional region for CELPs.

In the pistil, CELP mRNAs predominantly accumulate in restricted cell layers that mark the boundary between the cortical and transmitting tissues in the style, and in the surface cell layers in the placenta of the ovary. These cellular regions delimit the path for the elongating pollen tubes, which do not invade the cortical tissues in the style or the placenta tissue in the ovary. The presence of a proline-rich and a cysteine-rich domain in CELPs is reminiscent of SL, which is believed to be important for cell-cell recognition [3]. The tantalizing possibility that CELPs may function as a lectin and interact with the traversing pollen tubes in the pistil remains to be explored.

Hyroxyproline-rich glycoproteins in the ovary

The last stage of pollen tube elongation is marked by the dramatic bent of the pollen tube tip from the basally oriented elongation direction, sometimes as much as 90°, in order to gain access into the ovules where the egg cells are located (figs. 1, 2). Directional cues originating from the ovules are believed to play an important role in this phenomenon [32, 33, 68, 69]. Carbohydrate-rich exudates cover the ovular micropyle [70, 71] through which a pollen tube gains access into the ovule (fig. 2D), and placental explants with attached ovules have been shown to attract pollen tubes [71, 72]. *Nicotiana* ovaries are enriched in AGPs [11, 73] and CELPs [unpublished results]. It would not be surprising if hydroxyproline-rich glycoproteins turn out to be part of the machinery contributing to the guided entrance of pollen tubes into the ovules.

Hydroxyproline-rich glycoproteins in the pollen

PEX

The best-characterized hydroxyproline-rich glycoprotein in pollen is the maize pollen-specific extensin (PEX) proteins [13, 74]. The deduced primary structure of the PEX proteins revealed two structural domains – a C-terminal extensin-like domain and N-terminal globular domain (fig. 3). The N-terminal domain was subsequently identified to be analogous to a leucine-rich-repeat (LRR), a structural domain believed to be involved in protein-protein interactions. The PEX polypeptide backbone is deduced to be around 120 kDa, but the native PEX protein molecules are highly glycosylated and have apparent molecular masses of around 300 kDa. They have been localized to the intine of mature pollen grains and to the callose wall of the pollen tubes [74]. Like extensins, PEX protein molecules are tightly associated with wall matrix, since treatment with high salt, chaotropic agents, reducing agents and SDS could not solubilize them from the pollen tubes. It has been suggested that PEX proteins may function in maintaining the structural integrity of the walls of the rapidly elongating maize pollen tubes, cell adhesion and cell-cell signaling. Homologs of PEX proteins have been identified in other Solanaceae, including *N. tabacum*, *Arabidopsis* [unpublished results] and tomato (GenBank).

AGPs

In many plant species, AGPs are prominently present on pollen tubes [e.g. 44, 75, 76]. Using monoclonal antibodies (Mac207 amd JIM8) against AGPs, Li et al. [76] showed that AGPs are present in the inner callosic walls along elongating *N. tabacum* pollen tubes, except at the pollen tube tip where continuous secretion of cell wall materials take place.

Contrary to the situation in *N. tabacum* pollen tube tip, the lily pollen tube tip is enriched in AGPs. Perturbation of the association of these proteins with the cell wall matrix by the action of Yariv β-D glucoside reagent resulted in arrest of pollen tube growth within 15 min of the treatment [72, 77]. The tip of these Yariv-treated lily pollen tubes enlarged abnormally, leading to highly deteriorated tip morphology. The enlarged tube tip morphology was largely due to the continued deposition of the newly secreted materials underneath the original cell wall of the tips that were no longer pushing forward. These observations suggest an important role for AGPs in the formation of new walls at the growing tips of in vitro grown lily pollen tubes.

Only a few putative AGP or AGP-like genes expressed in pollen have been reported thus far. Bcp1, a *Brassica* pollen-specific gene, has been shown to be essential for male fertility in *Arabidopsis thaliana* [78]. Two late pollen-expressed genes from *Brassica napus* (Sta39-3 and Sta39-4) [79], and a pollen-specific cDNA (PO2) and its corresponding genomic sequence from *Medicago sativa* L. (alfalfa) [80] encode proteins with deduced primary structures suggestive of their being AGPs.

Regulation of the biosynthesis of the stylar transmitting tissue TTS protein

Among the hydroxyproline-rich glycoproteins described here, TTS proteins provide an excellent example of how multiple regulatory mechanisms, from transcriptional to posttranslational, operate to ensure the accumulation of biologically active molecules in the appropriate temporal and spatial patterns [58].

Transcriptional regulation

TTS proteins are encoded by two comparably expressed genes, TTS-1 and TTS-2 [29, 56, 58]. Analysis of the expression pattern of chimeric TTS-1 5′ upstream regulatory region-GUS genes in transgenic N. tabacum plants showed that a region of ~600 bp upstream of the transcription start site is adequate to direct high levels of transmitting tissue-specific expression [58]. The TTS-1 promoter is activated relatively early in young pistils, and its activity increases with pistil development. The observed transcriptional activity of the TTS-1 promoter during pistil development paralleled closely the TTS mRNA and protein accumulation patterns [56, 57], indicating that transcriptional regulation plays an important role in the temporal and spatial accumulation of TTS mRNAs.

Pistil development has been shown to be under the regulation of a MADS box gene Agamous (in *Arabidopsis*) or its orthologs in a number of other plants [81–83]. Transgenic *N. tabacum* ectopically expressing NAG-1 (*Nicotiana* Agamous-1) from the CaMV35S promoter showed the transformation of sepals into pistil-like structures that can support pollen germination and tube growth [84]. TTS mRNAs are induced in these tissues [84], and the TTS-1 promoter is activated [58]. These observations indicate that the TTS-1 gene is under the control of developmental pathways regulated by NAG-1.

Posttranscriptional regulation

Pollination slightly stimulates the activity of the TTS-1 promoter, whereas RNA blot analysis showed that pollination results in a three- to fivefold increase in the level of TTS mRNA; concomitantly, the length of TTS mRNA became shorter [29, 57, 58]. Using a 3′ mRNA mapping technique, Wang et al. [29] showed that the poly(A) tail of TTS mRNA was reduced upon pollination for an average length of ~180 bases. The shortening of TTS mRNA was detectable in both the upper and lower halves of the styles between 3 and 6 h after pollination, when pollen tubes were elongating just past the stigmatic-transmitting tissue junction. This suggests a relatively fast-moving signal that travels ahead of the pollen tubes to induce the TTS-mRNA shortening process. Ethylene and okadaic acid were shown to be capable of inducing similar TTS mRNA-shortening in unpollinated styles, suggesting a signal transduction pathway involving this gaseous hormone and a phosphorylation cascade.

Pollination-induced TTS mRNA shortening appeared to be correlated with cell deterioration and death that occurred within the transmitting tissue after pollination [29]. Shortening of TTS mRNA preceded observable histological changes in the pollinated tissues. Pollination-induced transmitting tissue cell death suggests significant overall cellular deterioration and degradation among cellular biochemical components. mRNA shortening appears to be a common degradative event among transmitting tissue mRNAs since at least two additional mRNAs, MG15 and β-(1,3)-glucanase mRNAs, were also detectably shortened after pollination. However, in contrast to the pollination-induced enhancement of TTS mRNA levels, the levels of MG15 and β-(1,3)-glucanase mRNAs declined to a very low level by 48 h after pollination, consistent with the general observation that poly(A)-tail trimming often precedes mRNA degradation [85]. A threshold level of TTS protein is apparently necessary for optimum reproductive success, since pollen tube growth rate was reduced in transgenic *N. tabacum* plants in which the level of TTS protein was significantly reduced by antisense suppression or cosuppression, sometimes resulting in reduced female fertility [46]. The pollination-induced shortening of TTS mRNA poly(A)-tail suggests that TTS mRNA is sensitive to the deteriorating biochemical conditions within the pollinated transmitting tissue cells, like other cellular components [29]. On the

other hand, the pollination-enhanced TTS mRNA levels [57, 58] despite an overall RNA degradative environment [29] suggest that a need to ensure the presence of adequate amounts of TTS protein in the pollinated styles and mechanisms related to mRNA processing must have evolved to accomplished that.

Posttranslational regulation

On immunoblots, high molecular weight TTS protein species (50–105 kDa) are detectable only in the stylar transmitting tissue even when TTS mRNAs are expressed from a chimeric CaMV35S-TTS gene constitutively in transgenic *N. tabacum* plants [84]. These ectopically accumulating TTS mRNAs are translated; however, all the non-stylar transmitting tissue-produced TTS protein in these transgenic plants are underglycosylated and have molecular weights below 45 kDa. These protein molecules represented TTS polypeptides that had been N-glycosylated and O-glycosylated to extents significantly lower than the predominant TTS protein species found in the stylar transmitting tissue. These results suggest that activities to fully glycosylate TTS protein were either limiting or absent in all non-stylar transmitting tissue. It is probable that a glyco-linkage(s) in TTS protein requires an enzyme (or enzymes) uniquely active in the stylar transmitting tissue, thus precluding glycosylation of TTS polypeptides in other tissues. However, in the CaMV35S-NAG1 transgenic plants where TTS mRNA was induced in the carpelloid sepal tissues as a result of the NAG1 activated carpel developmental pathway, the carpelloid sepal-produced TTS polypeptides became fully glycosylated as in the wild-type stylar tissue [84]. These observations together indicate that a stringent regulatory mechanism is present to ensure only the transmitting tissue is uniquely capable of synthesizing highly glycosylated TTS proteins, which are active in their ability to interact with pollen tubes and promote their growth. It is particularly noteworthy that even ovary tissue lacks the ability to fully glycosylate TTS polypeptide backbones (even when they are synthesized from the constitutive CaMV35S-TTS transgene) [84]. If TTS protein indeed contributes to the directional elongation of pollen tubes in the style, 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 to a different directional course. Thus, the absence of TTS proteins and the activities to produce fully glycosylated TTS proteins in the *N. tabacum* ovary may be an important aspect of pollen tube guidance in their journey towards the ovules.

Glycosylation as a mechanism to modulate the biochemical and biological properties of glycoproteins

The predominant amounts of *N. tabacum* TTS and its homolog from *N. alata* NaTTS proteins are between 50 and 105 kDa. They are loosely associated with the transmitting tissue cell wall matrix and are released from it under a broad range of buffer conditions (fig. 5) [46, 57–59]. A careful analysis of the extractability of TTS and NaTTS proteins from the transmitting tissue ECM revealed that the predominant amounts of these proteins are solubilized from the ECM by low salt buffers. High salt conditions are needed to extract the remaining and significantly smaller amounts of TTS and NaTTS proteins (fig. 5) [58, 59]. Protein blot analysis revealed that the low-salt-extractable, predominant population of TTS and NaTTS proteins span a molecular weight spectrum of 50–110 kDa, and are highly reactive towards the AGP diagnostic molecules, the β-glucosyl Yariv reagent and the monoclonal antibody JIM13 [58, 59]. On the other hand, the majority of the high salt-extractable TTS and NaTTS proteins span a lower molecular weight spectrum of between 30 and 95/100 kDa range. They are also less reactive towards the two AGP diagnostic reagents relative to their more highly glycosylated counterparts [58, 59]. The requirement of high-ionic-

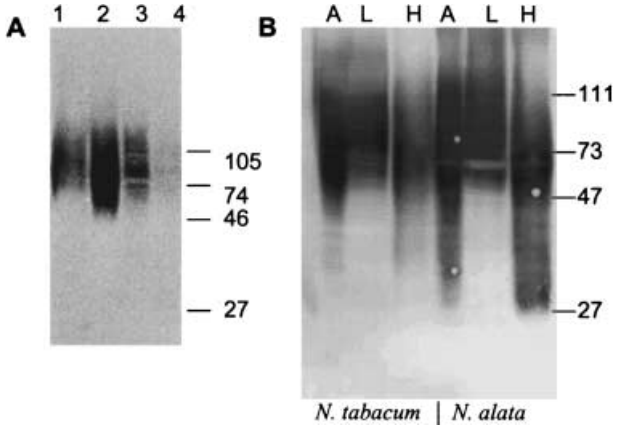

Figure 5. Immunoblots for TTS proteins isolated by different buffer conditions. (*A*) Protein samples are: lane 1, total proteins extracted from pulverized stylar tissues by boiling in an SDS-containing protein extraction buffer; lane 2, proteins eluted from isolated transmitting tissue by an isotonic buffer conditioned not to rupture transmitting tissue cells; lane 3, proteins extracted from pulverized transmitting tissue after it had been washed by the isotonic buffer to obtain the sample shown in lane 2; lane 4, total protein extracted from pulverized pistil tissues from which the transmitting tissues had been remove (thus containing almost exclusively epidermal, cortical and vascular tissues). (Adopted from [57]). (*B*) Lanes A, stylar transmitting tissue ECM proteins isolated by a mildly acidic, no salt wash (84 mM citric acid, pH 3); lanes L, proteins isolated by a low salt wash (100 mM NaCl, 20 mM Tris-HCl, pH 7.5; lanes H, proteins isolated by a high salt wash of previously low-salt-washed tissue (400 mM NaCl, 20 mM Tris-HCl, pH 7.5). All buffers contained 2 mM $Na_2S_2O_4$. Buffers that differed slightly from those described here yielded similar results [59]. (Adapted from [69]).

strength buffers to solubilize the less glycosylated TTS and NaTTS proteins compared with the relative ease with which highly glycosylated forms of these proteins are solubilized suggests that the level of glycosylation affects how these AGPs interact with the cell wall matrix. Glycosylation acidifies the TTS and NaTTS polypeptide from a pI of about 10 to pIs ranging between 7.5 and 9 [47, 59]. These properties together indicate that the less glycosylated, more basic TTS and NaTTS protein molecules associate more tightly with the ECM than the more highly glycosylated, relatively more acidic molecules. These findings indicate that a previously characterized *N. alata* analog of TTS protein, GaRSGP (which was purified from a high-salt-buffer extraction of stylar tissues that had been prewashed in a low-salt buffer) [86], was comparable to the high-salt-extractable, less-glycosylated, more highly cell wall matrix-bound and less biologically active species that constitute a minor portion of total NaTTS proteins.

Carbohydrate modifications are known to play key roles in modulating recognition and interactions between biological molecules. Glycoproteins such as the TTS protein family are synthesized from either a single or two highly homologous genes. The broad range of glyco-modifications observed for TTS and NaTTS proteins underscores the complexity of the biochemical and biological properties of these glycoproteins, allowing tremendous versatility in their interactions with other molecules. For proteins such as CELPs [12], the considerable complexity that already exists in their backbone polypeptides would only be compounded by differences in their glycosylation properties. The possibility for these glycoproteins to participate in biological processes in diverse ways is immense. In the reproductive processes of pollination and fertilization, the use of glycoproteins as agents that mediate the interactions between the male and female cells seems most expedient for a system that requires discrimination between compatibility and incompatibility. Functional analyses for more of the reproductive tissue hydroxyproline-rich glycoproteins will be needed to better understand how these ECM molecules participate in the pollen-pistil interactive processes to effect reproductive success.

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