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A style-specific 120-kDa glycoprotein enters pollen tubes of *Nicotiana alata* in vivo

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Abstract Pistils of *Nicotiana alata* (Link et Otto) contain an abundant, style-specific glycoprotein (120 kDa) that is rich in hydroxyproline and has both extensin-like and arabinogalactan-protein-like carbohydrate substituents. An antibody specific for the protein backbone of the glycoprotein was used to localise the glycoprotein in both unpollinated and pollinated pistils. The glycoprotein is evenly distributed in the extracellular matrix of the style transmitting tract of unpollinated pistils and, despite the presence of extensin-like carbohydrate substituents, is not associated with the walls of the transmitting tract cells. In pollinated pistils the 120-kDa glycoprotein is concentrated in the extracellular matrix adjacent to pollen tubes, and is also present in the cytoplasm and the cell walls of pollen tubes. Pollen tubes grown in vitro do not contain the 120-kDa glycoprotein unless it is added to the growth medium, suggesting that the 120-kDa glycoprotein located in pistil-grown pollen tubes is derived from the extracellular matrix of the transmitting tract.

Key words Hydroxyproline-rich glycoprotein · Pistil · Pollen tubes · Self-incompatibility

Introduction

The role of the pistil is to provide an environment suitable for pollen germination and the growth of pollen tubes, and to guide compatible pollen tubes from the stigma surface to the ovules. In *Nicotiana alata* the extracellular matrix of the transmitting tissue is the medium through which pollen tubes grow, and thus is the site of interaction between the pollen tubes and the pistil. The extracellular matrix is rich in sugars and free amino acids (Tupy 1961; Konar and Liskens 1966), lipids (Konar and Liskens 1966; Cresti et al. 1986), proteins, glycoproteins and proteoglycans (Atkinson et al. 1994; Lind et al. 1994). Uptake of low molecular weight components into pollen tubes and their metabolism has been studied in detail (Labarca et al. 1970; Chen and Loewus 1977; Deshusses et al. 1981; Capkova et al. 1983; Schlüpmann et al. 1994). However, information on the interaction between pollen tubes and the macromolecules of the transmitting tract is more limited.

We have recently described an abundant 120-kDa hydroxyproline-rich glycoprotein that accumulates in the transmitting tract fluid of maturing *N. alata* pistils (Lind et al. 1994). The glycoprotein has carbohydrate substituents with features of both the arabinogalactan-proteins and the extensins. Specific antibodies raised to the deglycosylated molecule were used on immunoblots to show that the 120-kDa glycoprotein is a pistil-specific component, and that antigenically-related glycoproteins are present in the pistils of other solanaceous plants. In this paper we describe an immunolocalisation study which shows that the 120-kDa glycoprotein is present in the extracellular matrix of the transmitting tract and that the glycoprotein is also present in the cell wall and cytoplasm of pollen tubes growing through this tissue.

Materials and methods

Plant material

Nicotiana alata (Link et Otto) plants of self-incompatibility genotypes S_2S_2 and S_6S_6 were maintained under standard glasshouse conditions, as described previously (Anderson et al. 1989). Pistils were collected and were fixed for microscopy, extracted immediately, or stored at –70°C for later extraction. Mature, receptive stigmas covered in stigmatic exudate were pollinated manually using a wooden toothpick to transfer freshly collected pollen from fully opened anthers. The pollinations were performed in the morning.

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Relative growth rates of compatible and incompatible pollen tubes

Mature *N. alata* pistils (self-incompatible, genotype S_2S_2) were pollinated with S_6 or S_2 pollen and left on the plant for 2–12 h. The pollinated styles were stained with decolorised aniline blue (Maheswaran et al. 1986). The stained pistils were squashed between a coverslip and microscope slide and viewed under UV light. The position of the majority of pollen tube tips was marked on the slide and the distance from the stigma measured.

Immunogold labelling of stigma and style tissue

Stigma and style tissues (3 mm segments) were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, 60 mM PIPES/KOH (pH 7.2) at room temperature for 2 h and then at 4°C overnight. After fixation, the segments were washed in 60 mM PIPES/KOH (pH 7.2) and dehydrated for 3 h at room temperature in acidified dimethoxypropane (concentrated HCl:dimethoxypropane, 1:2 000 v/v). The dehydrated segments were embedded in LR Gold containing Benzil (London Resin) by polymerisation under a Phillips TUV 15-W UV lamp at a distance of 10 cm for 12 h. Immunogold labelling of ultra-thin sections of styles was performed as described in Anderson et al. (1987). Antibodies to the deglycosylated 120-kDa glycoprotein (Lind et al. 1994) were purified from rabbit serum by chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described by Harlow and Lane (1988), and incubated with the sections at a concentration of 2.5 or 5.6 µg IgG/ml. The specificity of labelling was tested by replacing the antibodies to the deglycosylated 120-kDa glycoprotein with antibodies (2.5 µg IgG/ml) purified from pre-immune serum using protein A-Sepharose, or by pre-incubating the antibodies (2.5 µg IgG/ml) with the deglycosylated 120-kDa glycoprotein (40 µg/ml) (Lind et al. 1994) for 30 min at room temperature.

In vitro culture of pollen tubes

N. alata pollen (S_2 genotype) was cultured according to the method of Read et al. (1992). Pollen was collected in the morning from flowers approximately 12–24 h after anthesis, suspended in the pollen growth medium by vortexing (0.5 mg pollen/ml), and placed in the wells of a sterile, 96-well (50 µl/well) tissue-culture tray (Sigma, St Louis, Mo.) or a 24-well (300 µl/well) tissue-culture tray (Sigma). The pollen tubes were grown in a moist atmosphere at 28°C. In some experiments the pollen tube medium was supplemented with 100 µg/ml pure 120-kDa glycoprotein (Lind et al. 1994) or 100 µg/ml bovine serum albumin (BSA, Sigma).

Immunogold labelling of in vitro-cultured pollen tubes

Pollen tubes grown in vitro as described above were lightly fixed by the addition of fixative solution (4% formaldehyde, 0.5% glutaraldehyde, 60 mM PIPES/KOH pH 7.2; 20 µl fixative/50 µl of culture volume) for 10 min. The pollen tubes were transferred to a silanised microfuge tube, pelleted (10 000 *g*, 5 s, room temperature), resuspended in fixative for 1 h at room temperature and then incubated overnight at 4°C. The pollen tubes were then washed three times in 60 mM PIPES/KOH, pH 7.2 (1 ml, 30 min) by gentle resuspension and centrifugation (10 000 *g*, 5 s, room temperature). After washing, each sample was rapidly resuspended in a gelatine solution (4% w/v gelatine in 60 mM PIPES/KOH pH 7.2 at 28°C; 100 µl of gelatine solution/10 µl packed pollen tubes). The gelatine was allowed to set at 4°C (10 min), and 2- to 3-mm pieces were dehydrated in a graded ethanol series before infiltration and embedding in LR Gold using the procedure described for the pistil segments.

Preparation of protein extracts and protein gel blot analysis

Proteins were extracted from pistils and analysed by SDS-PAGE and immunoblotting as described in Lind et al. (1994). Proteins were extracted from pollen tubes by grinding frozen tubes in pistil extraction buffer containing 1% (w/v) 3[(3cholamidopropyl)-dimethylammonio]-1-propane-sulphonate (CHAPS, Sigma). The pollen tubes were ground in buffer, refrozen in liquid nitrogen, and then re-ground and frozen three more times. The soluble proteins were recovered in the supernatant after centrifugation (10 000 *g*, 4°C, 5 min) and analysed by SDS-PAGE and immunoblotting as described in Lind et al. (1994).

Results

Localization of the 120-kDa glycoprotein in *N. alata* pistils

The antibodies to the 120-kDa glycoprotein bound to the extracellular matrix of styles, but not to the cytoplasm of transmitting tract cells or to vesicles or organelles within the transmitting tract cells (Fig. 1A). No antibody binding was detected in the transmitting tract cell walls (Fig. 1A,B), or between the cells where the walls were closely appressed and the cells connected by plasmodesmata (Fig. 1B). The antibodies did not bind to the cortical or epidermal cells or their cell walls (data not shown). When the primary antibodies were replaced with IgG purified from pre-immune rabbit serum, a very low level of background labelling was observed across the sections (Fig. 1C). Similarly, when the antibodies to the 120-kDa glycoprotein were pre-incubated with deglycosylated 120-kDa glycoprotein, binding of the antibody to the extracellular matrix was completely inhibited (data not shown).

Growth rates of compatible and incompatible pollen tubes in *N. alata*

The in vivo growth rate of pollen tubes was measured to enable selection for microscopy of pistil sections containing pollen tube tips. Self-compatible (SC) pollen tubes had an average growth rate of 0.7 mm/h over the first 12 h of growth, whilst the incompatible (SI) pollen had an average growth rate of 0.4 mm/h over the same period (Fig. 2). The difference in growth rate between SI and SC pollen tubes was observed within 2 h of pollination; at this time the SC tubes had clearly penetrated the stigma whilst SI pollen tubes were restricted to the stigma surface. The incompatible pollen tubes, stained with decolourised aniline blue, appeared twisted and had thickened cell walls from the 4.5 h time point onwards. Despite their appearance, the incompatible tubes continued to grow, and few had swollen or burst tips after 12 h of growth.

Localization of the 120-kDa glycoprotein in compatible pollen tubes

When compatibly pollinated styles were immunolabelled with the antibody to the 120-kDa glycoprotein, the cyto-

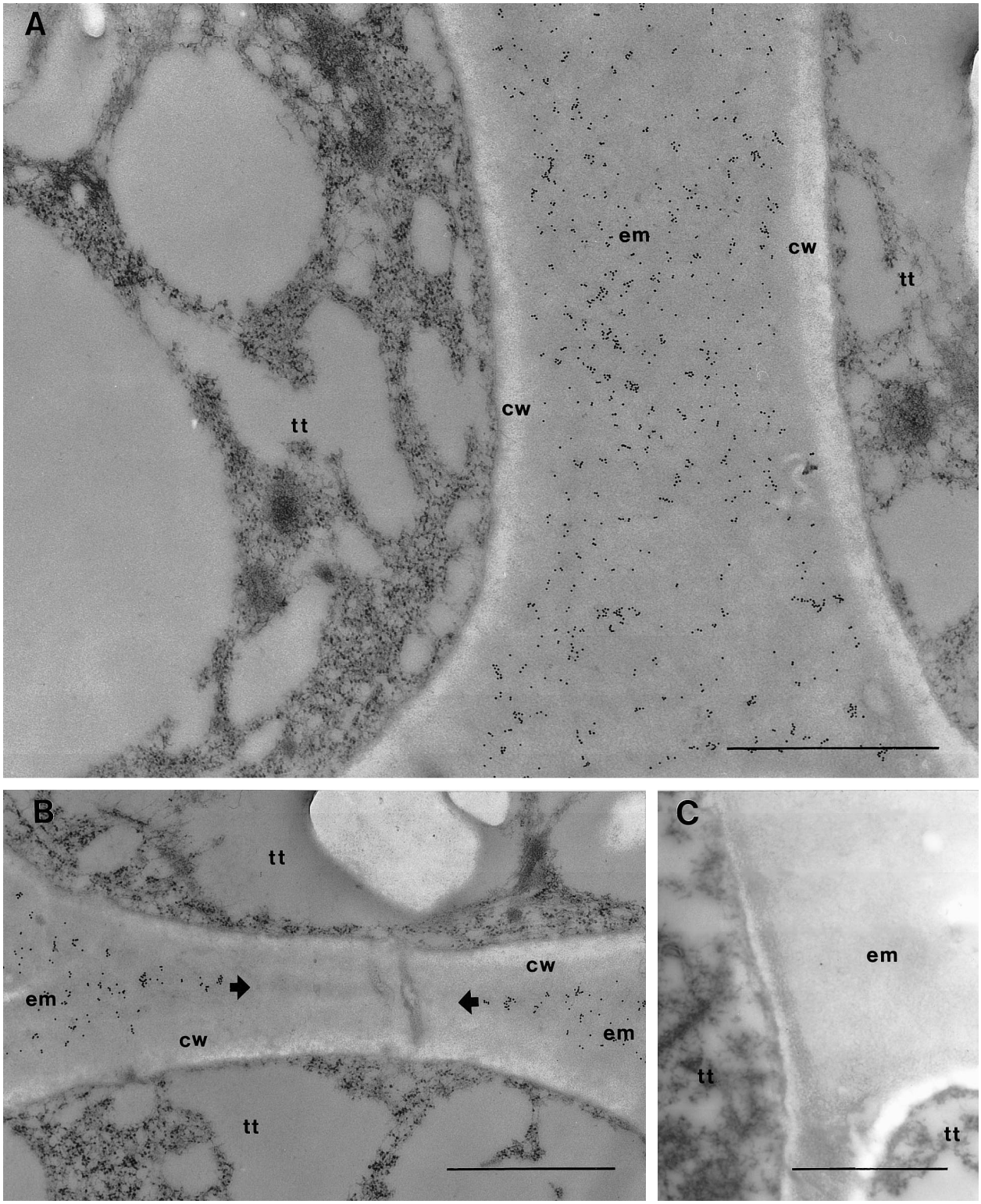


Fig. 1A–C Immunogold localization of the 120-kDa glycoprotein in the transmitting tract of unpollinated *N. alata* style.Transverse sections from style pieces (3–6 mm below the stigma surface) were immunolabelled with antibodies to the 120-kDa glycoprotein (**A,B**) or pre-immune antibodies (**C**), followed by protein A coupled to 10-nm gold. *Bar* 1 μm; **A** ×35 000, **B** ×27 000, **C** ×26 000. The antibodies to the 120-kDa glycoprotein bound to the extracellular matrix (*em*) of the style, but not to the cell walls (*cw*) or the cytoplasm of the transmitting tract cells (*tt*). The region between two closely appressed transmitting tract cells containing plasmodesmata (*arrows* in **B**) was also unlabelled. The pre-immune antibodies (**C**) did not bind to pistil sections

plasm of the pollen tubes was labelled as densely as the extracellular matrix of the transmitting tract (Fig. 3). Several pollen tubes were examined and, in general, the antibody binding was localized in regions of the cytoplasm that were rich in secretory vesicles (200–300 nm diameter) that were fibrillar and had an appearance typical of P-particles (Fig. 3A,B,D). No binding was detected in the cytoplasm of the transmitting tract cells, on the section of pollen tube (pt2, Fig. 3A) that contained no cytoplasmic organelles, nor on pollen tube sections that were devoid of P-particles (Fig. 3C). The pollen tube profile lacking cytoplasmic organelles (pt2, Fig. 3A) appears to have a smaller diameter than the other pollen tube in the section (pt1). This appearance could result from oblique sectioning close to the cell wall or sectioning through a collapsed region of the tube distal to the tip. Whereas the 120-kDa glycoprotein was evenly distributed over the extracellular matrix in unpollinated styles (Fig. 1), it was often concentrated adjacent to the pollen tube surface in pollinated styles. This was most apparent when the pollen tubes and transmitting tract cells were in close proximity (Fig. 3D, arrowheads). Sections incubated with antibodies from the pre-immune serum showed no labelling of the pollen tubes or the extracellular matrix (Fig. 3E, P-particles arrowed).

Significant immunolabelling of the cytoplasm was detected in the longitudinal section through the apical/subapical zone of the pollen tube shown in Fig. 4. This pollen tube was sectioned very close to the tip. The proximity to the tip is reflected in the thin and ruffled appearance of the cell wall at the apex, the even, rounded-shape pollen tube, and the appearance of the electron-lucent callose layer about 5 μm distal to the tube tip. The tip of the pollen tube appeared intact and was not deformed.

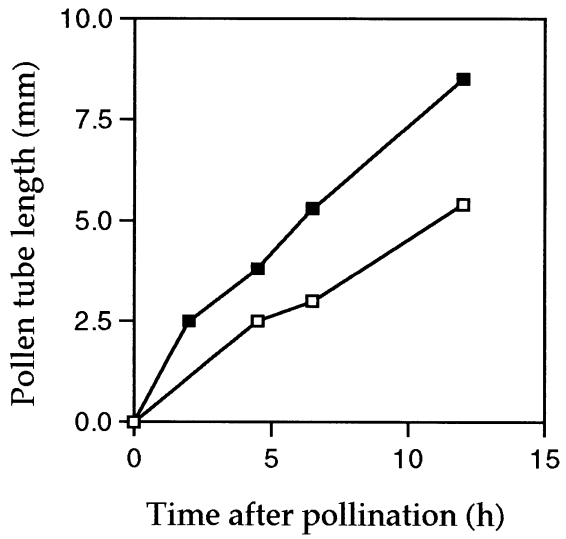


Fig. 2 Growth curve for compatible and incompatible *N. alata* pollen tubes grown in vivo. A comparison of the growth rate of compatible (S_6-genotype —■—) and incompatible (S_2 genotype —□—) pollen tubes in pistils of S_2S_2 genotype during the first 12 h after pollination. Each data point is the mean of at least three independent pollinations

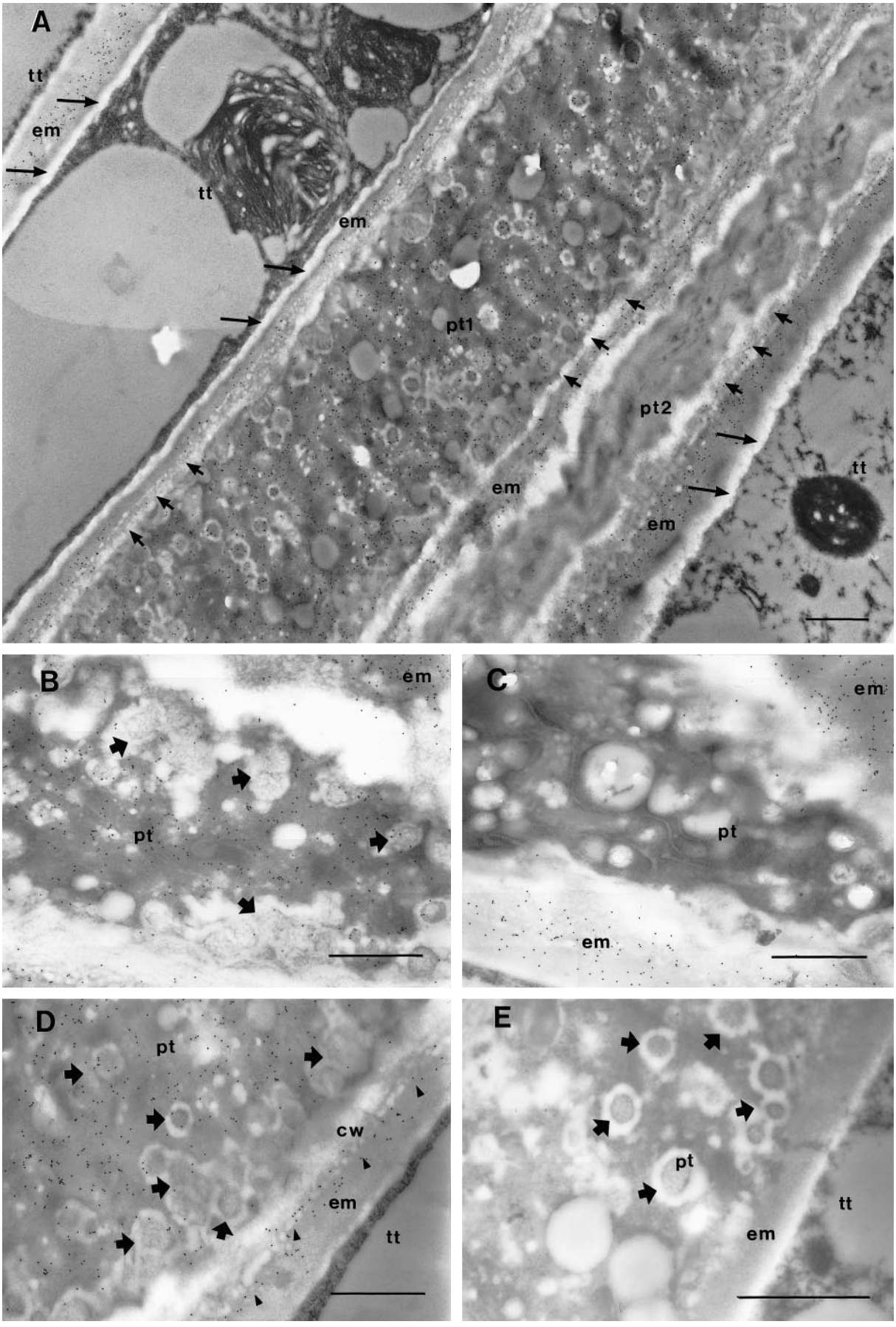
The extracellular matrix surrounding the pollen tube tip was labelled heavily with the antibody to the 120-kDa glycoprotein. The cytoplasm 1–2 μm behind the tip, which is rich in very small vesicles, was not heavily labelled, but there was significant labelling of the cytoplasm 5 μm back from the apex of the tube (Fig. 4).

In contrast to sections taken close to the pollen tube tip, sections further back from the tip contained electron-opaque fibrillar material in the inner wall layer that labelled heavily with the antibody (Fig. 5A,B). Large vesicle-like structures in the pollen tube cytoplasm were also heavily labelled. The vesicle-like structures appeared to be membrane-bound, and contained a mixture of components with different electron opacities (Fig. 5B). In this region of the pollen tube, the cytoplasm stained darkly and contained large lipid droplets and small vesicles but few P-particles.

Localization of the 120-kDa glycoprotein in incompatible pollen tubes

The cytoplasm of incompatible pollen tubes was also labelled with the antibody to the 120-kDa glycoprotein. The pollen tube shown in Fig. 6 contained P-particles as well as the concentric endoplasmic reticulum characteristic of incompatible pollen tubes (de Nettancourt et al. 1973). The incompatible tube in Fig. 6B was allowed to grow for 24 h before fixation and sectioning. The pollen tube, which is sectioned transversely, contains P-parti-

Fig. 3A–E Immunogold localization of the 120-kDa glycoprotein in a compatibly pollinated *N. alata* pistil. Longitudinal sections from pistils (genotype S_2S_2) pollinated with compatible pollen 7 h prior to fixation and immunolabelling with antibodies to the 120-kDa glycoprotein (**A–D**) or the pre-immune antibodies (**E**), followed by protein A coupled to 10-nm gold. *Bar* 1 μm; **A** ×12 000, **B–D** ×17 000, **E** ×24 000. The section shown in **A** contains three transmitting tract cells (*tt*) and two pollen tubes (*pt*). One of the pollen tubes (*pt1*) has cytoplasm that is densely packed with large vesicles (200–300 nm). The other pollen tube (*pt2*) contains no cytoplasmic organelles. The cell walls of the transmitting tract cells are electron-lucent (*long arrows*) and have clear boundaries. The pollen tubes have walls composed of an electron lucent inner layer of callose and an outer layer composed mostly of arabinan that does not have a clear boundary (*short arrows*). The antibodies to the 120-kDa glycoprotein bound heavily in the extracellular matrix (*em*) of the transmitting tract and in the cytoplasm of the pollen tube (*pt1*). **B** Section through a pollen tube containing cytoplasm rich in vesicles containing fibrillar contents (P-particles, *arrows*). The antibodies to the 120-kDa glycoprotein bound to the cytoplasm and to a lesser extent to the cell walls of the pollen tube. **C** Section through a region of the pollen tube with vesicles that had a different appearance from the P-particles. The antibodies to the 120-kDa glycoprotein bound to the extracellular matrix of the transmitting tract but did not bind to the pollen tube cytoplasm. **D** Section through a pollen tube (*pt*) adjacent to a transmitting tract cell (*tt*). The 120-kDa glycoprotein in the extracellular matrix (*em, arrowheads*) is concentrated next to the cell wall (*cw*) of the pollen tube. The cytoplasm of the pollen tube is rich in P-particles (*arrows*) and is labelled heavily with the antibody. The transmitting tract cell is not labelled. **E** Pre-immune antibodies on a section similar to **D** did not bind to the extracellular matrix (*em*) or to the cytoplasm of the pollen tube that was rich in P-particles (*arrows*)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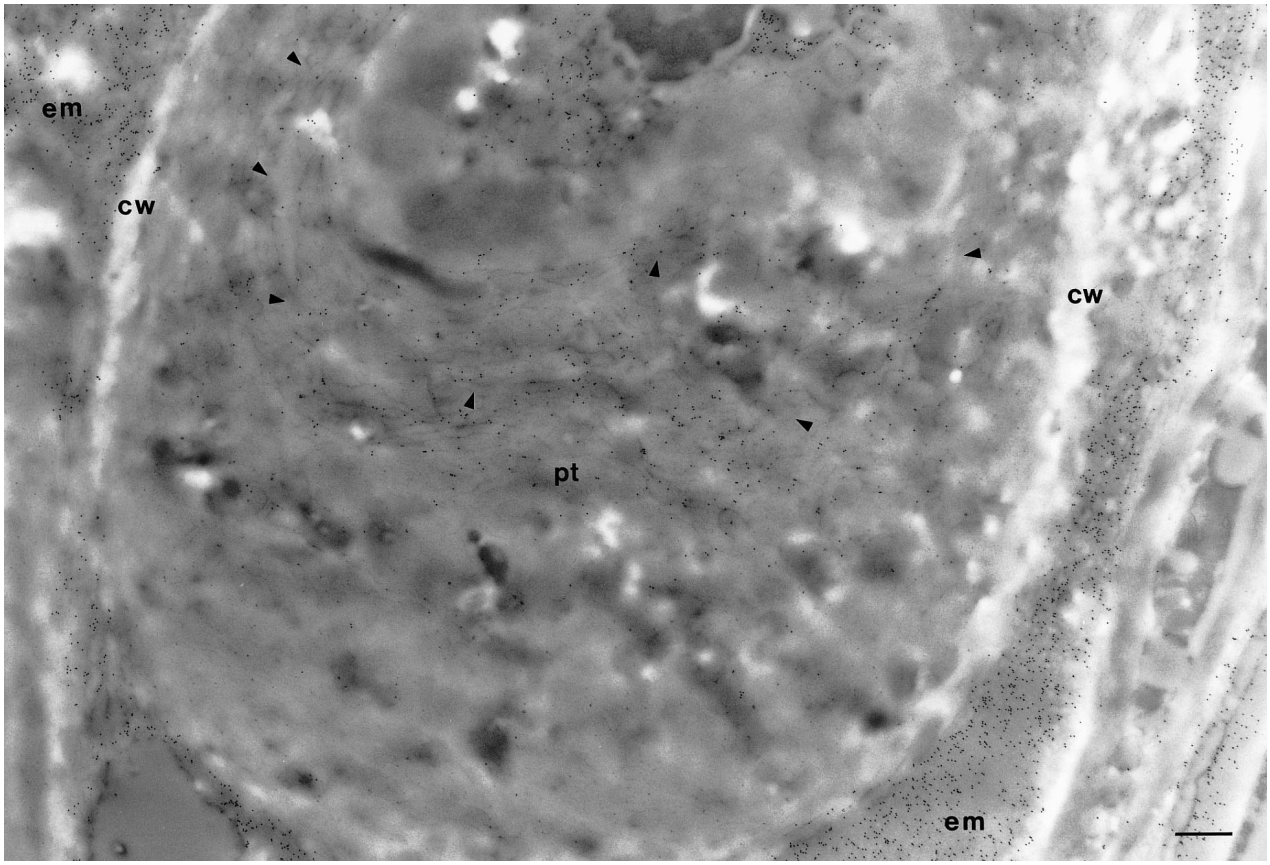


Fig. 4 Immunogold localization of the 120-kDa glycoprotein in the tip of a compatible pollen tube. The pollen tube (S_6-genotype) was immunolabelled with antibodies to the 120-kDa glycoprotein followed by protein A coupled to 10-nm gold. *Bar* 1 μm; ×7 500. The extracellular matrix (*em*) and the pollen tube cytoplasm approximately 5 μm distal to the tube apex(*arrowheads*) were labelled with the antibodies, whilst the cytoplasm in the apical region and the pollen tube walls (*cw*) were unlabelled

cles, a significant fibrillar component in its cell wall, and is relatively heavily labelled with antibodies to the 120-kDa glycoprotein. In some instances the gold particles are associated with the fibrillar component of P-particles.

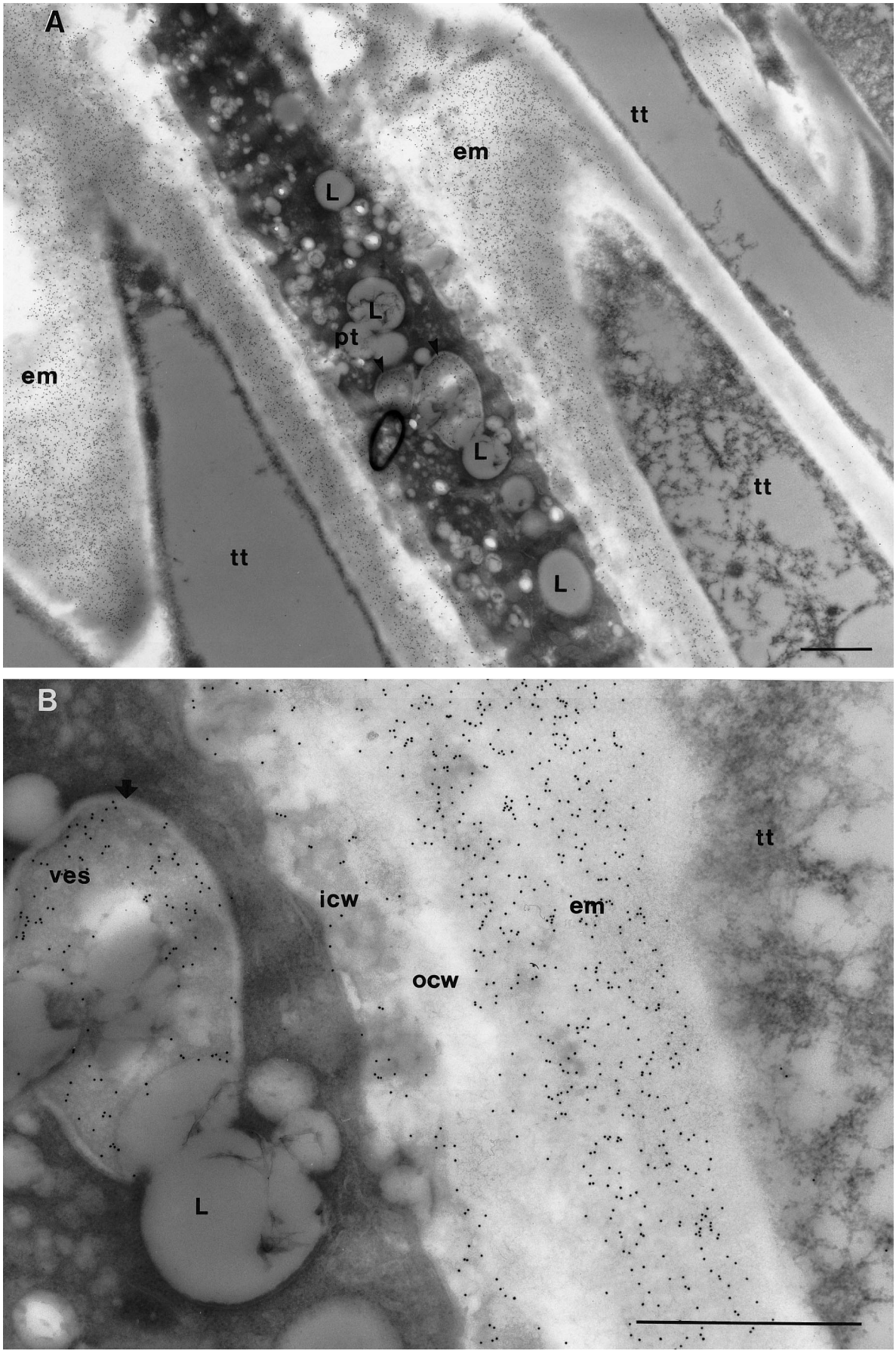
Localization within in vitro-grown pollen tubes

Extracts from unpollinated styles, compatibly and incompatibly pollinated styles, and in vitro-grown pollen tubes were examined for the presence of the 120-kDa glycoprotein by immunoblotting (Fig. 7). Pollinated and unpollinated styles contained similar amounts of the 120-kDa glycoprotein and pollination had no significant effect on the apparent molecular weight of the glycoprotein. Extracts from in-vitro-grown pollen tubes had no detectable 120-kDa glycoprotein. The 120-kDa glycoprotein was not detected in in-vitro-grown pollen tubes that were sectioned and immunolabelled with antibodies to the 120-kDa glycoprotein (Fig. 8A). In contrast, light but reproducible gold labelling was observed in the cytoplasm and cell walls of pollen tubes that had been grown for 8 h in medium containing added 120-kDa glycoprotein at a concentration of 100 μg/ml (Fig. 8B).

Discussion

The 120-kDa glycoprotein is one of a series of proline- and hydroxyproline-rich glycoproteins that have been identified in the pistils of solanaceous species (Goldman et al. 1992; Chen et al. 1992, 1993; Wang et al. 1993; He et al. 1994; Lind et al. 1994). Their function is largely unknown, although roles in tissue differentiation (Knox 1990), pollen tube nutrition (Labarca and Loewus 1973),

Fig. 5A,B Immunogold localization of the 120-kDa glycoprotein in older regions of compatible pollen tubes. Section from a pistil (genotype S_2S_2) pollinated with compatible pollen (genotype S_6) 7 h prior to fixation and immunolabelled with the antibodies to the 120-kDa glycoprotein, followed by protein A coupled to 10-nm gold. *Bars* 1 μm; **A** ×12 500, **B** ×40 000. **A** Longitudinal section of the style containing three transmitting tract cells (*tt*) and a single pollen tube (*pt*) that is surrounded by extracellular matrix (*em*). The pollen tube has darkly staining cytoplasm and contains large lipid droplets (*L*) and small vesicles as well as two large vesicles (*arrows*) that are heavily labelled with gold particles. **B** One of the large vesicles in **A** at higher magnification. The vesicle (*ves*) appears membrane-bound (*arrow*) and the contents are heterogeneous, with most of the label positioned over the most electron-dense material. The inner cell wall (*icw*) of the pollen tube is also labelled with the antibodies, whilst the outer cell wall (*ocw*) and the cytoplasm around the labelled vesicle are unlabelled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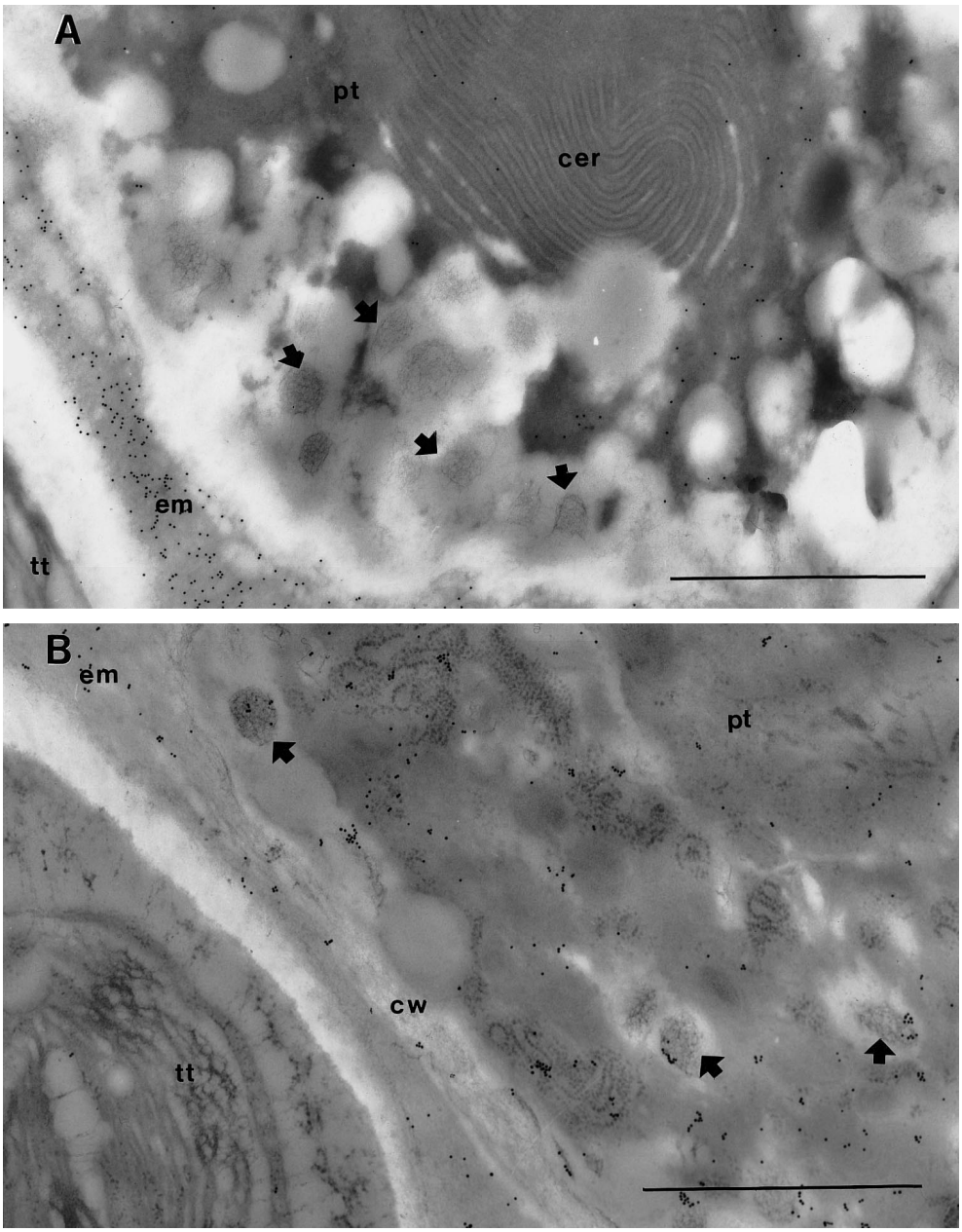


Fig. 6A,B Immunogold localization of the 120-kDa glycoprotein in incompatible pollen tubes. Sections from pistils (genotype S_2S_2) pollinated with self-pollen for 7 h (**A**) and 24 h (**B**) before fixation were immunolabelled with the antibodies to the 120-kDa glycoprotein followed by protein A coupled to 10-nm gold. *Bars* 1 μm; **A** ×34 000, **B** ×37 000. The pollen tube in **A** has concentric endoplasmic reticulum (*cer*) and numerous P-particles (*arrows*). The extracellular matrix (*em*) and the cytoplasm of the pollen tube are labelled. The older pollen tube (**B**) has a fibrillar cell wall (*cw*) and several P-particles (*arrows*). The cytoplasm contains more gold particles than the 7-h pollen tube, and some of the labelling is positioned over the P-particles

pollen adhesion (Gleeson and Clarke 1979), guidance of pollen tube growth (Wang et al. 1993) and defence against pathogen invasion (Atkinson et al. 1994) have been suggested.

The immunolocalization experiments described in this paper show that the 120-kDa glycoprotein is present in the extracellular matrix of the transmitting tract and that it interacts with and is taken up by pollen tubes growing in vivo. Direct immunocytochemical evidence for in vitro but not in vivo uptake of stylar S-RNases has been presented (Gray et al. 1991). The question of whether other style macromolecules are able to enter pollen tubes relates to pollen tube nutrition and guidance of pollen tube growth in both compatible and incompatible matings.

The 120-kDa glycoprotein is a style-specific component that accumulates to levels of 9% (w/w) of the soluble protein in styles of mature *N. alata* flowers (Lind et al. 1994). Using antibodies directed to the 78-kDa protein backbone of the glycoprotein (Lind et al. 1994), we found that the glycoprotein is evenly distributed throughout the extracellular matrix of the transmitting tract. It was not present in the walls or the cytoplasm of the transmitting tract cells, and therefore has a similar distribution to the S-RNases (Anderson et al. 1989). The lack of antibody binding to the transmitting tract cell walls confirms that the backbone of the 120-kDa glycoprotein is antigenically distinct from both the hydroxyproline-rich glycoproteins present in the cell walls and the salt-extractable extensin precursors (Stuart and Varner 1980; Smith et al. 1984).

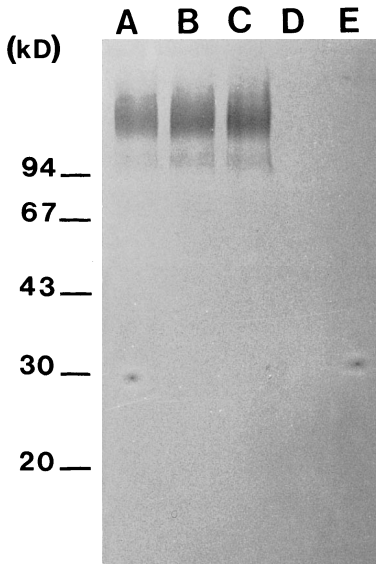
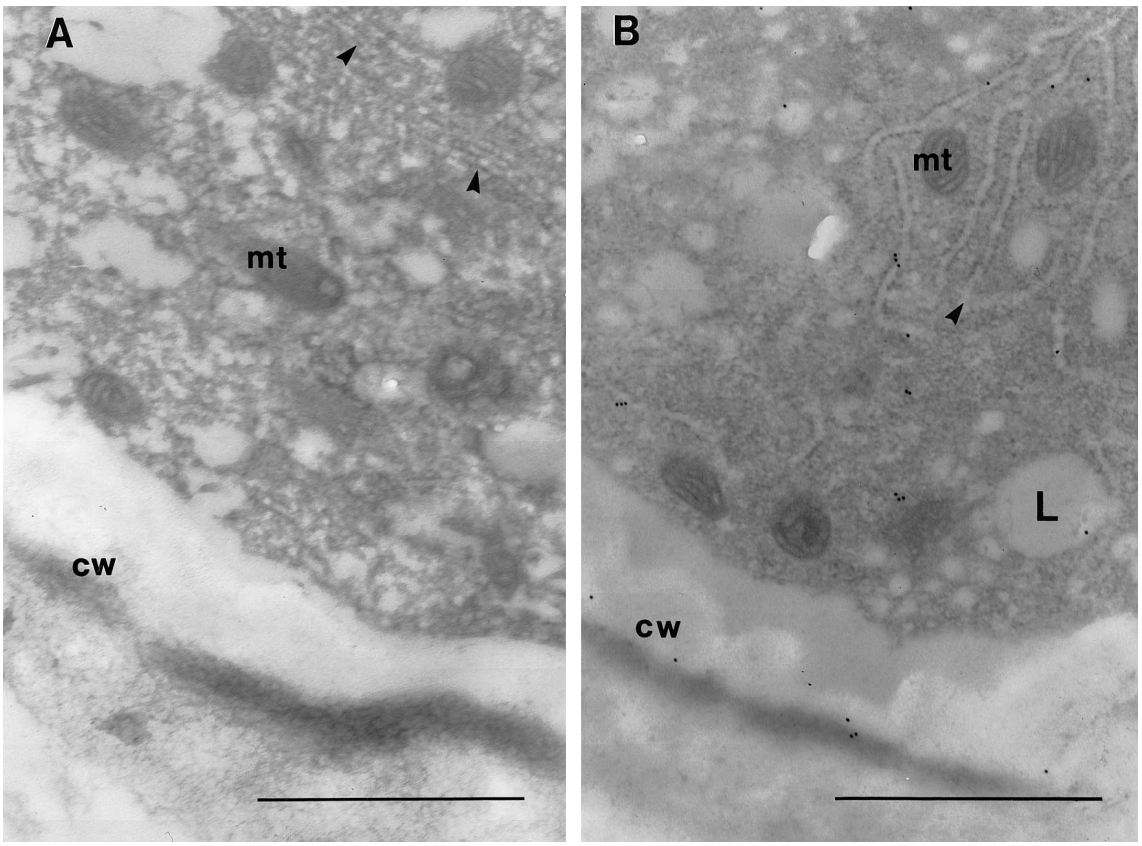


Fig. 7 Comparison of the levels of the 120-kDa glycoprotein in pollinated and unpollinated pistils and in vitro-grown pollen tubes. Buffer soluble proteins (4 μg) extracted from S_2S_2-genotype pistils that were: (*A*) unpollinated, (*B*) pollinated with incompatible pollen for 24 h, (*C*) pollinated with compatible pollen (S_6 genotype) for 24 h; and from (*D*) S_2 pollen grown in vitro for 8 h; and (*E*) S_6 pollen grown in vitro for 8 h. The proteins were separated on an SDS 12.5% polyacrylamide gel, electroblotted onto nitrocellulose and immunostained with the antibodies to the 120-kDa glycoprotein

The 120-kDa glycoprotein was only detected in in vitro-grown tubes after the glycoprotein was added to the growth medium. This supports the hypothesis that the 120-kDa glycoprotein detected in stylar-grown pollen tubes is derived from the extracellular matrix of the pistil. An alternative interpretation is that pollen tubes synthesise the glycoprotein when in contact with the glycoprotein, either in the transmitting tract or when added exogenously in vitro.

In contrast to the transmitting tract cells, pollen tubes growing extracellularly through the transmitting tract contain the 120-kDa glycoprotein in their cytoplasm and cell walls. The glycoprotein was most abundant in cytoplasm more than 5 μm distal to the pollen tube tip, which is rich in secretory P-particles. Regions of the cytoplasm that contained few P-particles, or were rich in lipid drop-

Fig. 8A,B Immunogold localization of the 120-kDa glycoprotein in pollen tubes grown in vitro. Pollen (S_2-genotype) was germinated and grown in vitro for 8 h in the presence of **A** BSA (100 μg protein/ml) or **B** the 120-kDa glycoprotein (100 μg protein/ml). The pollen tubes were immunolabelled with antibodies to the 120-kDa glycoprotein followed by protein A coupled to 10-nm gold. *Bar* 1 μm; ×35 000. The pollen tubes were sectioned through areas of cytoplasm containing mitochondria (*mt*), lipid droplets (*L*), and endoplasmic reticulum (*arrowheads*). The section of the pollen tube that had been grown in the presence of BSA (**A**) contained no gold particles, whereas the pollen tube grown in the presence of the 120-kDa glycoprotein (**B**) contained gold particles over the cytoplasm and cell wall



lets, contained very low levels of the 120-kDa glycoprotein. Further back from the pollen tube tip, in areas of darkly staining cytoplasm and numerous lipid droplets, the glycoprotein was detected in electron-opaque inclusions within the callosic cell wall layer. These inclusions are similar in appearance to those produced when P-particles fuse with the plasma membrane and dispense their contents into the cell wall (de Nettancourt et al. 1973; Cresti and van Went 1976). One explanation for the observations is that the 120-kDa glycoprotein is taken up from the style extracellular matrix into the pollen tube cytoplasm and then secreted into the callosic layer of the pollen tube cell wall. The co-localization of the 120-kDa glycoprotein with material secreted from P-particles into the cell wall suggests that the glycoprotein is either present in intact P-particles or in another type of vesicle that also deposits its contents into the callosic wall layer. Direct evidence of co-localization of the 120-kDa glycoprotein with intact P-particles was not obtained, as osmium tetroxide treatment is required to visualise the membrane of the P-particles and this treatment is not compatible with immunolocalization using the antibodies to the 120-kDa glycoprotein.

In general, incompatible tubes contained less 120-kDa glycoprotein than compatible tubes of the same age although this observation was not quantified. This apparent difference could be specific to incompatible pollen tubes, or could occur in all pollen tubes that are growing slowly. Incompatible pollen tubes grown for 24 h generally contained more of the 120-kDa glycoprotein than those grown for 7 h. The increase could be due to a ″leakiness″ of the cell membrane induced by the self-incompatibility response, or to continued accumulation of the 120-kDa glycoprotein during growth.

Loewus and Labarca were the first to study uptake of pistil glycoconjugates into pollen tubes (Labarca and Loewus 1972, 1973; Loewus and Labarca 1973). They incubated pistils with ^{14}C-glucose, extracted the labelled pistil extract and isolated a fraction that was composed in part of proteoglycans with arabinogalactan-like side chains (Aspinall and Rosell 1978). When pollen tubes growing in vitro or in vivo were exposed to this macromolecular fraction, labelled arabinose and galactose residues from the proteoglycans accumulated in the cell wall polysaccharides of the pollen tubes. It was not clear, however, whether intact proteoglycan molecules had entered the pollen tubes and been deposited more or less intact into the cell wall, or whether the labelled monosaccharides had been released from degraded proteoglycans before uptake and incorporated into newly synthesised cell wall polymers. The 120-kDa glycoprotein detected in the pollen tubes in this study was localized with antibodies directed to the protein backbone of the molecule. The fate of the carbohydrate side chains has not been investigated, but continued antibody binding suggests that the protein backbone, at least, has not undergone substantial degradation after uptake. Other studies on uptake of macromolecules into pollen tubes have been performed on in vitro-grown tubes. Gray and co-workers (1991), for example, have shown that the style S-RNases involved in self-incompatibility in *N. alata* are taken up by pollen tubes cultured in vitro. Immunocytochemical evidence for the uptake of S-RNases into in vivo-grown tubes has not been reported, but the absolute requirement of the RNase activity for self-incompatibility (Huang et al. 1994; Kowyama et al. 1994; Royo et al. 1994) and the observation that RNA is degraded in incompatible pollen tubes (McClure et al. 1990) indicates that S-RNases must enter the pollen tube cytoplasm to mediate their cytotoxic effect. An understanding of the mechanism of uptake of S-RNases into pollen tubes will play an important role in deciphering the role of the pollen *S*-gene product in the self-incompatibility reaction. It has been suggested that the pollen product of the *S*-gene is a specific receptor or transporter located on the surface of the pollen tube that recognises and leads to uptake of the style S-RNases in an allele-specific manner (see Newbigin et al. 1994 for review). An alternative hypothesis is that S-RNases enter pollen tubes in a non-specific manner and are either activated or inhibited after interaction with the pollen-S component in the cytoplasm. If non-specific uptake occurs it is likely that the 120-kDa glycoprotein, the style S-RNases and other macromolecules enter pollen tubes by a similar mechanism. Non-specific endocytosis has been demonstrated in vitro with pollen tubes of *Nicotiana tabacum* using labelled dextrans equivalent in size to a 30-kDa globular protein (O'Driscoll et al. 1993a,b).

Interestingly, the 120-kDa glycoprotein in the extracellular matrix often appeared to be concentrated on the outer face of the pollen tube cell wall. This apparently close association may be due to non-specific ionic interactions, since the 120-kDa glycoprotein is highly positively charged (Lind et al. 1994) and the surface of pollen tubes is negatively charged (Li et al. 1994). Alternatively, the association may reflect a specific interaction between the 120-kDa glycoprotein and a component of the pollen tube wall. The glycoprotein must be able to pass through the pollen tube wall before endocytosis can occur. Whereas globular proteins of 15–25 kDa can probably diffuse through pollen tube walls (Hoggart and Clarke 1984), passage of a significantly larger molecule would be very slow (Read and Bacic 1995) unless it has a linear conformation. The very low level of cysteine in the 120-kDa glycoprotein (Lind et al. 1994) is consistent with its having a linear rather than a globular conformation. It is possible that uptake of macromolecules from the extracellular matrix of the style into pollen tubes is a common process, providing the molecules are of an appropriate size, shape and charge to pass through the cell wall to the plasma membrane.

The role of the 120-kDa glycoprotein in pollen tube physiology is not known. Uptake and incorporation into the wall does not necessarily indicate a primary role in pollen tube nutrition. A morphogenetic role, or roles in pollen tube elongation and guidance, are also possible.

The next steps in furthering an understanding of the uptake of the 120-kDa glycoprotein and its physiological role will be labelling experiments to follow the fate of both the carbohydrate chains and the protein backbone. These would be refinements of the pioneering experiments of Loewus and Labarca whose work in the early 1970s indicated that uptake and incorporation of proteoglycans from styles into pollen tubes was possible (Labarca et al. 1970; Labarca and Loewus 1972, 1973).

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References

Anderson MA, Harris PJ, Bönig I, Clarke AE (1987) Immunogold localization of α-L-arabinofuranosyl residues in pollen tubes of *Nicotiana alata* Link et Otto. Planta 171:438–442

Anderson MA, McFadden GI, Bernatzky R, Atkinson A, Orpin T, Dedman H, Tregear G, Fernley R, Clarke AE (1989) Sequence variability of three alleles of the self-incompatibility gene of *Nicotiana alata.* Plant Cell 1:483–491

Aspinall GO, Rosell KG (1978) Polysaccharide component in the stigmatic exudate from *Lilium longiflorum*. Phytochemistry 17:919–921

Atkinson AH, Lind JL, Clarke AE, Anderson MA (1994) Molecular and structural features of the pistil of *Nicotiana alata*. Biochem Soc Symp 60:15–26

Capkova V, Hrabetova E, Tupy J, Rihova L (1983) Amino acid uptake and protein synthesis in cultured tobacco pollen. Biochem Physiol Pflan 178:511–520

Chen C-G, Cornish EC, Clarke AE (1992) Specific expression of an extensin-like gene in the style of *Nicotiana alata*. Plant Cell 4:1053–1062

Chen C-G, Mau S-L, Clarke AE (1993) Nucleotide sequence and style-specific expression of a novel proline-rich gene from *Nicotiana alata*. Plant Mol Biol 21:391–395

Chen M-S, Loewus FA (1977) *myo*-Inositol metabolism in *Lilium longiflorum* pollen. Uptake and incorporation of *myo*-inositol-2-^{3}H. Plant Physiol 59:653–657

Cresti M, Keijzer CJ, Tiezzi A, Ciampolini F, Focardi S (1986) Stigma of *Nicotiana*: ultrastructural and biochemical studies. Am J Bot 73:1713–1722

Cresti M, Went JL van (1976) Callose deposition and plug formation in *Petunia* pollen tubes in situ. Planta 133:35–40

Deshusses J, Gumber SC, Loewus FA (1981) Sugar uptake in lily pollen. Plant Physiol 67:793–796

Gleeson PA, Clarke AE (1979) Structural studies on the major component of *Gladiolus* style mucilage, an arabinogalactan-protein. Biochem J 181:607–621

Goldman MH de S, Pezzotti M, Seurinck J, Mariani C (1992) Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. Plant Cell 4:1041–1051

Gray JE, McClure BA, Bönig I, Anderson MA, Clarke AE (1991) Action of the style product of the self-incompatibility gene of *Nicotiana alata* (S-RNase) on in vitro-grown pollen tubes. Plant Cell 3:271–283

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

He D, Simpson RJ, Moritz RL, Clarke AE, Bacic A (1994) Isolation of the protein backbone of an arabinogalactan-protein from the styles of *Nicotiana alata* and characterisation of a corresponding cDNA. Plant Cell 6:1643–1653

Hoggart RM, Clarke AE (1984) Porosity of *Gladiolus* stigmatic papillae and pollen tube walls. Ann Bot 53:271–277

Huang S, Lee H-S, Karunanandaa B, Kao T-H (1994) Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. Plant Cell 6:1021–1028

Knox JP (1990) Emerging patterns of organisation at the plant cell surface. J Cell Sci 96:557–561

Konar RN, Liskens HF (1966) Physiology and biochemistry of the stigmatic fluid of *Petunia hybrida.* Planta 71:372–387

Kowyama Y, Kunz C, Lewis I, Newbigin E, Clarke AE, Anderson MA (1994) Self-compatibility in a *Lycopersicon peruvianum* variant (LA2157) is associated with lack of style S-RNase activity. Theor Appl Genet 88:859–864

Labarca C, Kroh M, Loewus F (1970) The composition of stigmatic exudate from *Lilium longiflorum*. Labelling studies with *myo*-inositol, D-glucose and L-proline. Plant Physiol 46: 150–156

Labarca C, Loewus F (1972) The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longiflorum*. I. Utilization of injected stigmatic exudate. Plant Physiol 50:7–14

Labarca C, Loewus F (1973) The nutritional role of pistil exudate in pollen tube wall formation in *Lilium longiflorum*. II. Production and utilization of exudate from the stigma and stylar canal. Plant Physiol 52:87–92

Li YA, Chen F, Linskens HF, Cresti M (1994) Distribution of unesterified and esterified pectins in cell walls of pollen tubes of flowering plants. Sex Plant Reprod 7:145–152

Lind JL, Bacic A, Clarke AE, Anderson MA (1994) A style-specific hydroxyproline-rich glycoprotein with properties of both extensins and arabinogalactan proteins. Plant J 6:491–502

Loewus F, Labarca C (1973) Pistil secretion product and pollen tube wall formation. In: Loewus F (ed) Biogenesis of plant cell wall polysaccharides. Academic Press, New York, pp 175–193

Maheswaran G, Perryman T, Williams EG (1986) Use of an interspecific hybrid in identifying a new allelic specificity generated at the self-incompatibility locus after inbreeding in *Lycopersicon peruvianum.* Theor Appl Genet 73:236–245

McClure BA, Gray JE, Anderson MA, Clarke AE (1990) Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. Nature 347:757–760

Nettancourt D de, Devreux M, Bozzini A, Cresti M, Pacini E, Sarfatti G (1973) Ultrastructural aspects of the self-incompatibility mechanism in *Lycopersicon peruvianum* Millennium. J Cell Sci 12:430–419

Newbigin E, Anderson MA, Clarke AE (1993) Gametophytic self-incompatibility systems. Plant Cell 5:1315–1324

O'Driscoll D, Hann C, Read SM, Steer MW (1993a) Endocytotic uptake of fluorescent dextrans by pollen tubes grown in vitro. Protoplasma 175:126–130

O'Driscoll D, Read SM, Steer MW (1993b) Determination of cell-wall porosity by microscopy: walls of cultured cells and pollen tubes. Acta Bot Neerl 42:237–244

Read SM, Bacic A (1995) Cell wall porosity and its determination. In: Jackson JF, Linskens H-F (eds) Plant cell wall analysis. (Modern methods of plant analysis, vol 17). Springer, Berlin Heidelberg New York (in press)

Read SM, Bacic A, Clarke AE (1992) Pollen tube growth in culture. I. Control of morphology and generative nucleus division in cultured pollen tubes of *Nicotiana*. In: Ottaviano E, Mulcahy DL, Sari Gorla M, Mulcahy GB (eds) Angiosperm pollen and ovules. Springer, Berlin Heidelberg New York, pp 162–167

Royo J, Kunz C, Kowyama Y, Anderson M, Clarke AE, Newbigin E (1994) Loss of a histidine residue at the active site of S-locus ribonuclease is associated with self-compatibility in *Lycopersicon peruvianum*. Proc Natl Acad Sci USA 91:6511–6514

Schlüpmann H, Bacic A, Read SM (1994) Uridine diphosphate glucose metabolism and callose synthesis in cultured pollen tubes of *Nicotiana alata* Link et Otto. Plant Physiol 105: 659–670

Smith JJ, Muldoon EP, Lamport DTA (1984) Isolation of extensin precursors by direct elution of intact tomato cell suspension cultures. Phytochemistry 23:1233–1239

Stuart DA, Varner JE (1980) Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. Plant Physiol 66:787–792

Tupy J (1961) Investigation of free amino-acids in cross-, self-, and non-pollinated pistils of *Nicotiana alata*. Biol Plantarum 3:47–64

Wang H, Wu H-M, Cheung AY (1993) Developmental and pollination regulated accumulation and glycosylation of a stylar transmitting tissue-specific proline-rich protein. Plant Cell 5: 1639–1650