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The role of allergenic proteins Pla a 1 and Pla a 2 in the germination of *Platanus acerifolia* pollen grains

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Abstract *Platanus acerifolia* (Aiton) Willdenow is a plane tree, widely grown as an ornamental tree in many cities of the United States and Western Europe, which has become an important source of airborne allergens in our cities. The aim of the present study is to immunolocalize the major allergens in the pollen grain and to examine their potential function in the fertilization process. Observations were made in mature and hydrated, activated pollen of *P. acerifolia* for 5, 15, 30 min and 2 h in the germination medium. Specimens were fixed using freezing protocols for transmission electron microscopy (TEM). For immunogold labelling, cryosections and resin-embedded ultrathin sections were incubated using rabbit antisera against the purified pollen allergens Pla a 1 and Pla a 2. Elution of *P. acerifolia* allergens took place after 5 min of pollen incubation in buffered medium. Intense labelling of Pla a 1 and Pla a 2 was detected after pollen exudates were released. In pollen grains, Pla a 1 was predominantly localized in concentric cisternae of the endoplasmic reticulum (ER), situated between the vegetative nucleus and the generative cell, and was released from pollen grains 5 min after hydration; cytoplasmic localization decreased 15 min after hydration. In pollen grains, glycoprotein Pla a 2 was abundant in

association with Golgi cisternae and vesicles situated in the apertural periphery of the mature pollen grains. Pla a 2 proteins were also detected in ER and in the generative cell wall. Immunolabelling of Pla a 2 decreased 5 min after pollen hydration but was again intense after 15–30 min in germination medium, presumably as a consequence of renewed expression and glycosylation of this protein. Pla a 1 belongs to a new class of allergens related to proteinaceous invertase and pectin methyl esterase inhibitors (PII, PME1) which could be involved in membrane protection and pectin de-esterification control during pollen hydration. Pla a 2 has an exopolygalacturonase (PG) enzymatic activity consistent with pollen-stigma adhesion mechanisms or compatibility systems. Moreover, the expression of Pla a 2 found 15–30 min after hydration might contribute to pollen-tube growth and the modification of transmitting tissue cell walls. The abundant production and elution of Pla a 1 and Pla a 2 proteins may alter the environment in which pollen tube elongation occurs, thus promoting a potential crosstalk between the pollen and the gynoecium.

Keywords Immunocytochemistry · Invertase inhibitor · Pectin methylesterase inhibitor · *Platanus acerifolia* · Pollen grains · Polygalacturonase

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Introduction

Fertilization in flowering plants is initiated with pollination when desiccated pollen grains land on the receptive stigma, adhere, hydrate, germinate and produce a tube that elongates directionally to penetrate the stigma (Lord and Russell 2002). Once the pollen undergoes hydration, its metabolic machinery is activated (Mascarenhas 1993; Heslop-Harrison 1987). These events occur in the lipid-rich environment formed by the pollenkit and stigma exudates, which are essential for successful fertilization (Wolters-Arts et al. 1998).

cPollen grains require carbohydrates to succeed in the maturation process (Clément et al. 1996), so in mature pollen grains of *Platanus acerifolia* (Aiton) Willdenow the cytoplasm contains abundant starch and other polysaccharide granules (Suárez-Cervera et al. 1995). Disturbances in sugar physiology in the anther lead to pollen abortion and subsequent sterility (Dorion et al. 1996). Water stress affects carbohydrate metabolism in the whole plant, and pollen grains are particularly sensitive to the water deficit (Saini 1997; Saini and Lalonde 1998). On the other hand, most of the pollen proteins and glycoproteins that diffuse out of the pollen wall after hydration (Knox and Heslop-Harrison 1969; Casas et al. 1996; Marquez et al. 1997; Castells et al. 2002; Grote et al. 2001; Suárez-Cervera et al. 2003; Vega-Maray et al. 2004) have an enzymatic activity (Knox et al. 1998). The two major allergenic proteins of *P. acerifolia* have enzymatic activities related to sugar metabolism. Pla a 1 belongs to the family of protein invertase inhibitors (PII) and pectin methylesterase inhibitors (PMEI) (Asturias et al. 2002, 2003; SWISS-PROT identifier: Q8GT41 2004; Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2005) and Pla a 2 has exopolygalacturonase (PG) activity (Ibarrola et al. 2004; SWISS-PROT identifier: Q6H9K0 2005). In this study, Pla a 1 and Pla a 2 allergenic proteins are localized using immunogold localization and transmission electron microscopy (TEM) in mature and in vitro hydrated, activated *P. acerifolia* pollen grains. Characterizing the structural role of these proteins may contribute to understanding the role of soluble proteins in pollen rehydration as well as elucidating their function following pollen capture by the stigma. Knowledge of the role of such soluble proteins may be important in elucidating the constraints on pollen viability and fertility in relationship to carbohydrate metabolism.

Materials and methods

Plant materials

Pollen from mature anthers of *P. acerifolia* was collected in Barcelona in the springtime of 2002, 2003 and 2004. The pollen samples used were fresh, without any storage. The observations were made in mature and hydrated-activated pollen in germinating medium.

In vitro hydration and activation

Pollen was sprinkled onto a 0.7% pure agar (PRS Panreac, Barcelona, Spain) or water germination medium supplemented with 0.35 M sucrose, 300 mg/l CaCl₂ and 100 mg/l boric acid, during 5, 15, 30 and 120 min, at 21°C. After these intervals the process was interrupted.

Fixation and embedding

Mature and in vitro hydrated pollen grains were fixed using freezing protocols for TEM.

For cryosections (Figs. 4a–e, 5b–c, 6a–b, 7a,b, 8a–d), pollen grains were chemically fixed at 4°C with a mixture of 4% *p*-formaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. After washing with 0.1 M cacodylate buffer pH 7.3 containing 50 mM glycine, cells were embedded in 10% gelatin and infused in 2.3 M sucrose in 0.1 M cacodylate buffer, pH 7.3 (Raposo et al. 1997). Mounted gelatin blocks were frozen in liquid propane at –187°C (Leica EM CPC, Vienna) and stored in liquid nitrogen at –196°C. Ultrathin sections were prepared with an ultracyromicrotome (Leica EM FCS, Vienna, Austria) and collected in 2% methylcellulose in 2.3 M sucrose (Liou et al. 1996). For high-pressure freezing (HPF), samples were directly frozen using a high-pressure freezer (Leica EMPact, Vienna) and then transferred to liquid nitrogen for storage. Pollen grains were transferred to the planchettes of the EMPact with micropipette tips and filled with 0.7% pure agar (PRS Panreac). Cryosubstitution was performed following two different methods: 1) In 2% osmium tetroxide in anhydrous acetone at –90°C for 72 h and warmed to room temperature, at a rate of 5°C/h. After several acetone rinses, these samples were infiltrated with Spurr resin for 6 days at 21°C and polymerized at 60°C during 48 h (Fig. 5a). 2) In 0.5% uranyl acetate in methanol at –90°C for 72 h and then warmed to –50°C at a rate of 5°C/h. After several methanol rinses, samples were infiltrated in Lowicryl HM20 for 6 days and polymerized at –50°C with UV lamps (Fig. 2c–f). Ultrathin sections were prepared using a Leica ultramicrotome (Leica UCT, Vienna).

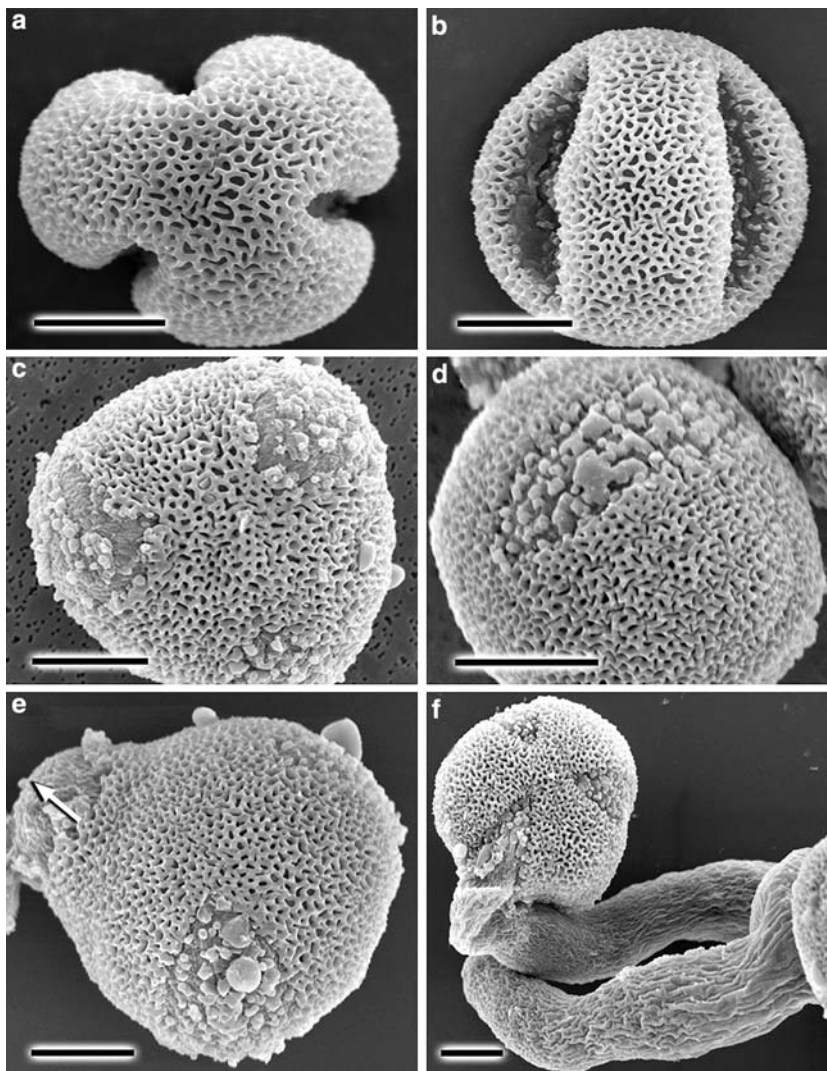
Polyclonal antibodies

Anti-Pla a 1 and anti-Pla a 2 rabbit polyclonal antisera were raised against purified pollen allergens, Pla a 1 and Pla a 2, by immunizing New Zealand white rabbits (Asturias et al. 2002; Ibarrola et al. 2004) and collecting immune serum. Specificity of the primary antibody has been demonstrated using immunoblotting with pre-immune rabbit serum (Asturias et al. 2002; Ibarrola et al. 2004).

Pollen chromatography

Glass columns (8×25 mm) packed with 0.1 g of *P. acerifolia* pollen were washed with phosphate buffered saline (PBS) at a flow rate of 0.5 ml/min. Eluted proteins were analysed using SDS-polyacrylamide gel electrophoresis under reducing conditions and visualized by Coomassie Blue R250 staining. Separated protein bands were electrophoretically transferred to polyvinylene difluoride membranes. After blocking for 1 h with 5% defatted dry milk in Tris-buffered saline, membranes were incubated for 1 h at 37°C with rabbit antisera against Pla a 1 and Pla a 2. Rabbit anti-IgG-peroxidase conjugates were detected by a chemiluminescent method

Fig. 1 Scanning electron microscopy (SEM). **a, b** Dry mature pollen grains. **c, d** 5 min hydrated pollen grains. **e** 15 min hydrated pollen grains. Germinate aperture (arrow). **f** 2 h germinated pollen. Scale bars 5 μ m



as recommended by the manufacturer (ECL-Plus, Amersham Biosciences, Uppsala, Sweden).

Immunogold labelling

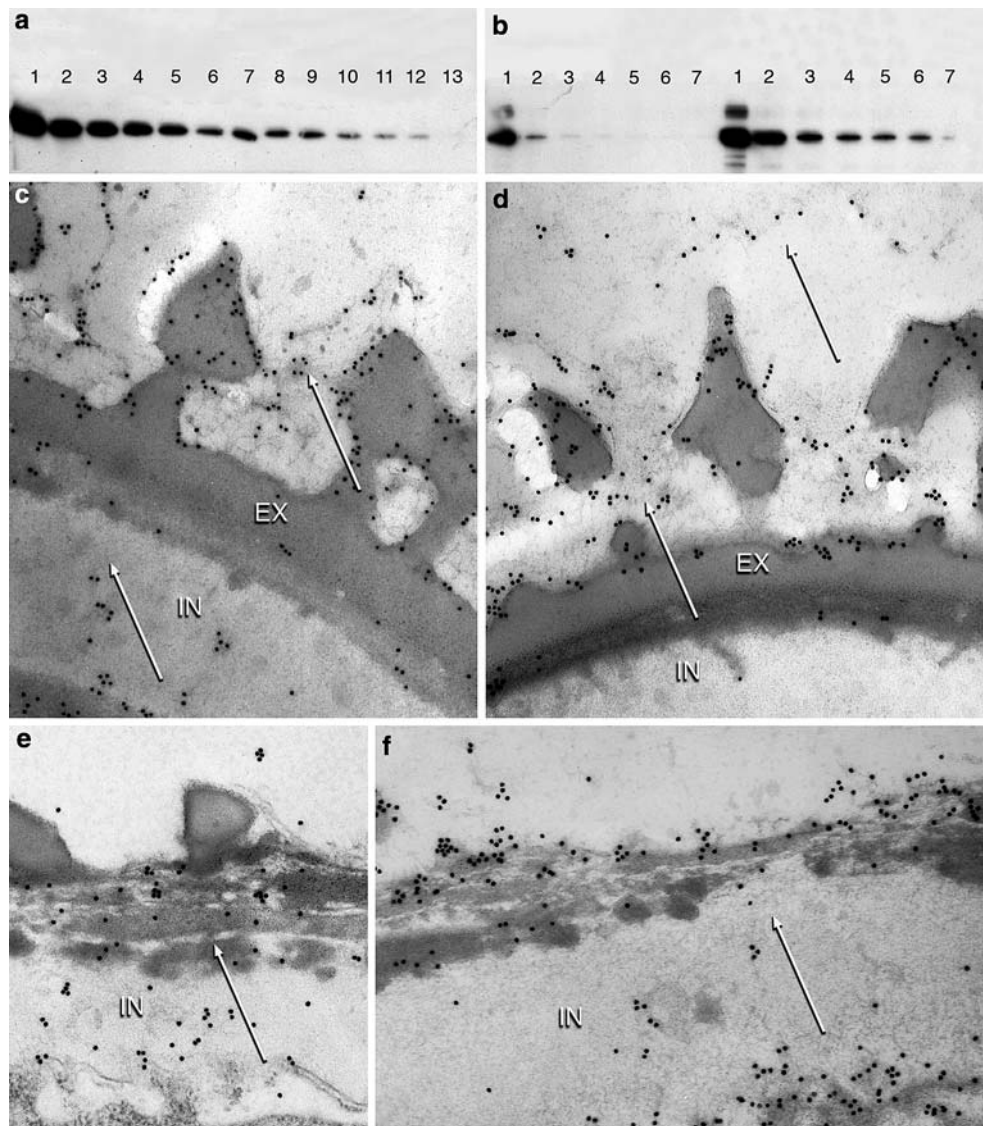
Samples obtained by cryosections were incubated at room temperature on drops of 2% gelatin in phosphate buffer, pH 7.4 for 20 min at 37°C, followed by 50 mM glycine in PBS during 15 min and 10% fetal bovine serum (FBS) in PBS for 10 min. Sections were then incubated with anti-Pla a 1 or anti-Pla a 2 polyclonal antibody in 5% FBS in PBS for 30 min. After three washes with drops of 0.2% FBS in PBS for 20 min, sections were incubated for 20 min using protein A coupled to 10 nm diameter colloidal gold particles (purchased from Dr. J.W. Slot, Department of Cell Biology, Utrecht University Medical School, The Netherlands), using a 1:60 dilution in 5% FBS in PBS. Afterwards, three washes with drops of PBS for 10 min and two washes with distilled water were done. As a control for non-specific binding of the colloidal gold-

conjugated antibody, experiments without primary polyclonal antibody were performed. Sections obtained using HPF were incubated at room temperature on drops of 5% bovine serum albumin (BSA) in PBS for 15 min, followed by anti-Pla a 1 or anti-Pla a 2 antisera for 2 h. After three washes with drops of 0.25% Tween 20 in PBS for 30 min, sections were incubated for 1 h using anti-rabbit IgG (goat) coupled to 10 nm diameter colloidal gold particles (British BioCell International, Cardiff, UK) using a 1:20 dilution in PBS. This was followed by three washes with drops of PBS for 5 min, two washes with distilled water and air-drying. The sections were also incubated with pre-immune serum as a control for specificity and omitting the primary polyclonal antibody to control for non-specific binding of the colloidal gold-conjugated antibody.

Experiments were performed using replicates of two equivalent samples of *P. acerifolia*. We sectioned two blocks and incubated two replicate grids for each sample with both polyclonal antibodies. Labelling was considered significant when all grains presented the same distribution pattern. In order to obtain an estimate of the

Fig. 2 a, b Pollen chromatography.

a Immunoblotting of eluted proteins incubated with rabbit antiserum against Pla a 1. Lanes 1–12 correspond to eluted proteins at 2, 6, 10, 14, 18, 22, 26, 30, 34, 38, 50 and 60 min, respectively. **b** Immunoblotting of eluted proteins incubated with rabbit antiserum against Pla a 2. Lanes 1–7 correspond to eluted proteins at 2, 6, 10, 14, 18, 22 and 26 min, respectively. Right-sided immunoblotting shows lanes 2–7 loaded with four-fold protein amount. **(c–f)** Transmission electron microscopy (TEM). 5 min hydrated pollen grains. Abundant gold particles in the intine, in the exine and in the exudates (arrows). **c, d** Non-apertural region, **e, f** Aperture. Intine (IN) and exine (EX). Gold particles corresponding to Pla a 1 **c, e** and Pla a 2 **d, f** proteins. **c–f** HPF HM20 resin. Scale bars 0.25 μ m



frequency of gold particles associated with various cellular structures, the number of gold particles within the same surface was then counted to determine the frequency of labelling associated with different cellular structures. For each structure, six or more counts were performed.

Finally, the cryosections and resin-embedded ultrathin sections were labelled and embedded in a mixture of methylcellulose and uranyl acetate and observed using a Philips EM 301 (Eindhoven, The Netherlands) and JEOL 1010 (Tokyo, Japan) TEMs.

Scanning Electron Microscopy

Pollen grains were fixed in 1% osmium tetroxide in 0.07 M phosphate buffer, followed by ethanol dehydration and critical point drying. Finally, the pollen grains were sputtered with gold using a Polaron E 5000

sputter coater and observed with a Hitachi S 2300 (Tokyo, Japan) scanning electron microscope.

Results

Pla a 1 and Pla a 2 protein diffusion

After 5 min of hydration of dry and mature pollen grains (Fig. 1a, b) apertures were exposed due to swelling of the grain (Fig. 1c, d). A pollen aperture protruded 15 min after hydration (Fig. 1e) and long pollen tubes occurred after 2 h (Fig. 1f). Elution of major allergens Pla a 1 and Pla a 2 from *P. acerifolia* pollen grains was observed during the first minutes after inclusion in a buffered medium (Fig. 2a, b). After 5 min in germination medium, intense labelling corresponding to Pla a 1 and Pla a 2 proteins was observed as soluble proteins and were released from apertural and non-apertural regions of pollen

grain wall (Figs. 2c–f, 3). After 15 and 30 min, gold particles recognizing Pla a 2 proteins were abundant in the germinal aperture (Figs. 4d, e, 3). In mature non-hydrated pollen grains, scarce and no significant labelling was observed in the walls (Figs. 4a–c, 3, 6a), respectively.

Pla a 1 and Pla a 2 protein localization

Mature pollen grains of *P. acerifolia* are bicellular: they contain a large vegetative cell with abundant storage material and a small generative cell (Fig. 5a). Vegetative nucleus and generative cell are situated in the centre of pollen grains, associated with concentric stacks of ER (Fig. 5a). Some single cisternae of ER are dispersed through the cytoplasm and near the plasma membrane of the vegetative cell. Numerous Golgi stacks were located in the apertural region near the plasma membrane and oriented parallel or perpendicular to the long axis of the vegetative cell (Fig. 6a).

Pla a 1 protein was predominantly found associated with the ER concentric cisternae, which are situated between the vegetative nucleus and the generative cell in mature, non-hydrated pollen grains (Figs. 5b, 3). After 5, 15 and 30 min and 2 h of hydration, labelling was noticeably decreased (Figs. 5c, 3).

Fig. 3 The distribution of 10 nm gold particles in the cytoplasm, wall and exudates at mature non-hydrated pollen grains and after 5, 15, 30 min and 2 h of pollen grains incubated in germinated medium

Pla a 2 labelled abundantly in cisternae and vesicles of Golgi in the apertural region of mature, non-hydrated pollen grains (Fig. 6a). This protein was sparse in the centre of the cytoplasm, associated with cisternae and vesicles of the ER and abundant in the generative cell wall (Fig. 6b) as well. After 5 min of hydration, immunogold labelling decreased (Fig. 3). Unlike Pla a 1, Pla a 2 was also recognized in the centre of the vegetative cell, both in the ER and Golgi after 15 and 30 min of hydration (Figs. 7a, b, 3). Pla a 2 displayed abundant labelling 15–30 min after hydration, concomitant with changes in the ER cisternae, which showed a disorganized and interrupted concentric structure with numerous ER vesicles linked to Golgi vesicles (Fig. 7a, b). After 2 h in germinating medium, the labelling decreased in abundance (Fig. 3) and ER–Golgi activity was not observed (data not shown). No significant labelling was observed in the pre-immune controls (Fig. 8a–d).

Discussion

The natural function of most pollen allergens remains largely unknown. A relatively small number of pollen

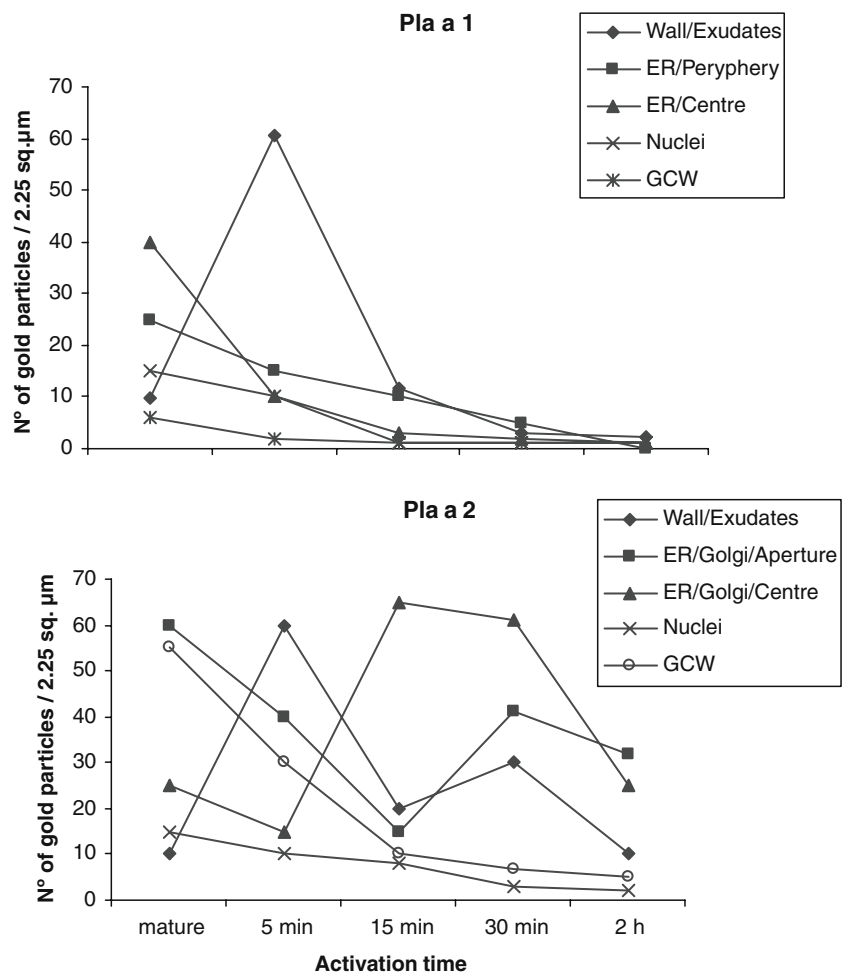
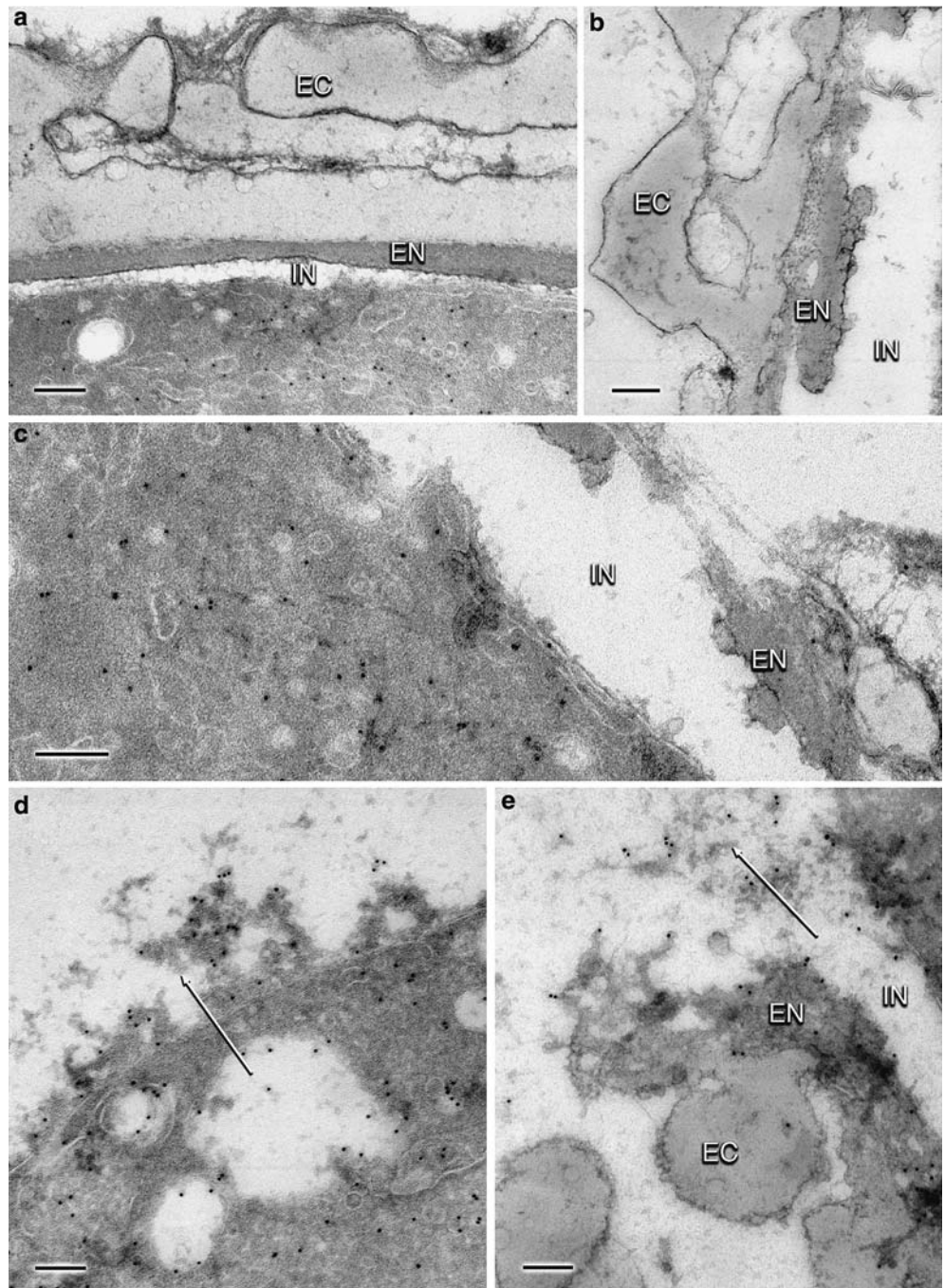


Fig. 4 TEM. Immunogold labelling corresponding to Pla a 2 proteins. **a–c** Mature non-hydrated pollen, abundant gold particles in the cytoplasm, and sparse occurrence in the wall.

a Non-apertural region. **b** Apertural margin. **c** Apertural region. **d** Pollen wall after 15 min hydration. Germinal aperture. Note abundant association of gold particles with the fibrillar material around emergent pollen tube (arrow). **e** Pollen wall after 30 min hydration. Gold particles in juncture between pollen wall and pollen tube (arrow). IN, EN and ectexine (EC). Cryosections. Scale bars 0.25 μ m

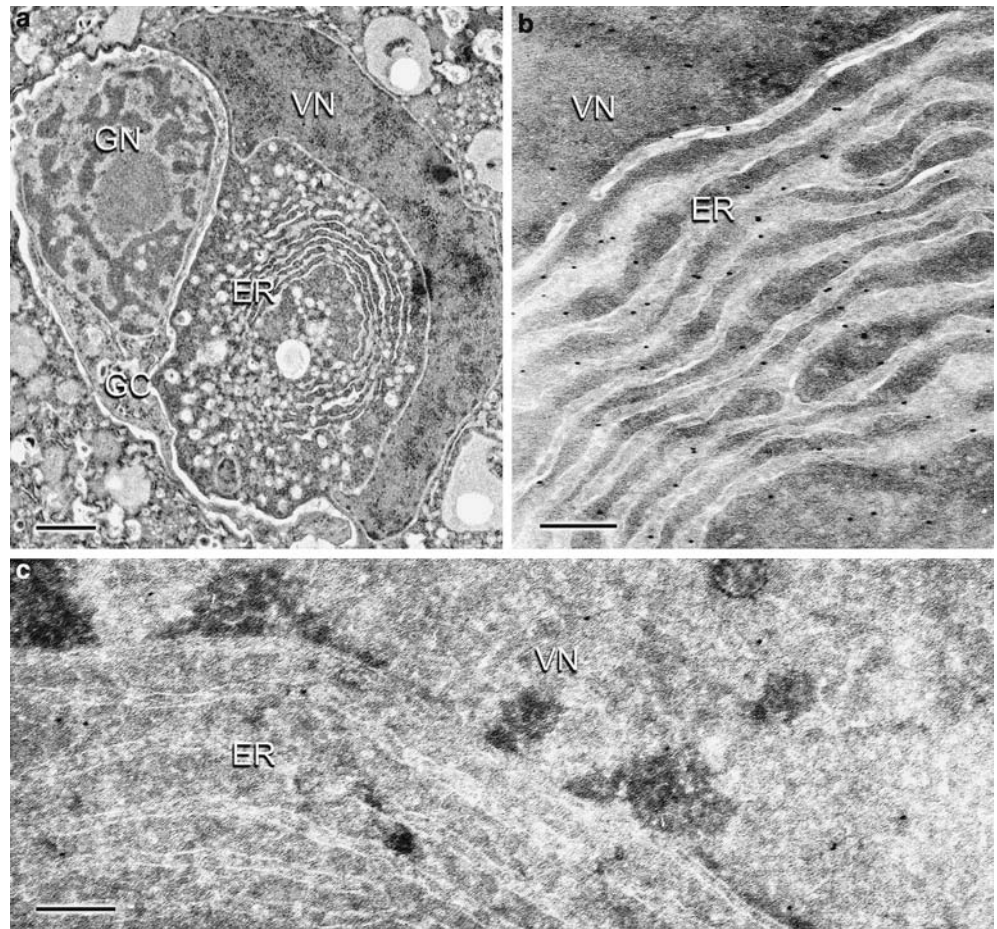


allergens have been biochemically characterized as active enzymes, including 1,3- β -glucanase of Ole e 9 (Huecas et al. 2001), PG of Cry j 2, Pla a 2, Cyn d 13 and Phl p 13 (Ohtsuki et al. 1995; Ibarrola et al. 2004; SWISS-PROT identifier: Q6H9K0 2005; Swoboda et al. 2004), pectate lyases of Cry j 1 and Cup a 1 (Taniguchi et al. 1995; Aceituno et al. 2000), cyclophilin of Bet v 7 (Cadot et al. 2000) and PII of Pla a 1 (Asturias et al. 2003; SWISS-PROT identifier: Q8GT41 2004; Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2005). Most of these allergens have enzymatic activity related to carbohydrate metabolism.

Role of invertase and pectin methyl esterase inhibitors (Pla a 1) during the germination process

Pla a 1 allergen is a secreted protein that is presumed to be synthesized in the ER. Its localization in the ER agrees with its non-glycosylated and basic protein structure (Asturias et al. 2002). After the first 5 min of hydration–activation, Pla a 1 was released from pollen grains by a diffusion-like process. Pla a 1 belongs to a new class of allergens related to PII (Asturias et al. 2003; SWISS-PROT identifier: Q8GT41 2004) and to PME1 (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al.

Fig. 5 TEM. **a** Bicellular mature pollen grain. Vegetative nucleus (VN), generative cell (GC) and generative nucleus (GN) situated in the centre of pollen grains and connected by concentric stacks of endoplasmic reticulum (ER). **b, c** Immunogold labelling corresponding to Pla a 1 proteins. **b** Mature non-hydrated pollen grains. Gold particles associated with ER cisternae and the VN. **c** After 5 min hydration. No significant labelling is observed in the ER and VN. **a** HPF, Spurr's resin; **b–c** cryosections. **a** Scale bar 1 μm ; **b, c** scale bars 0.25 μm

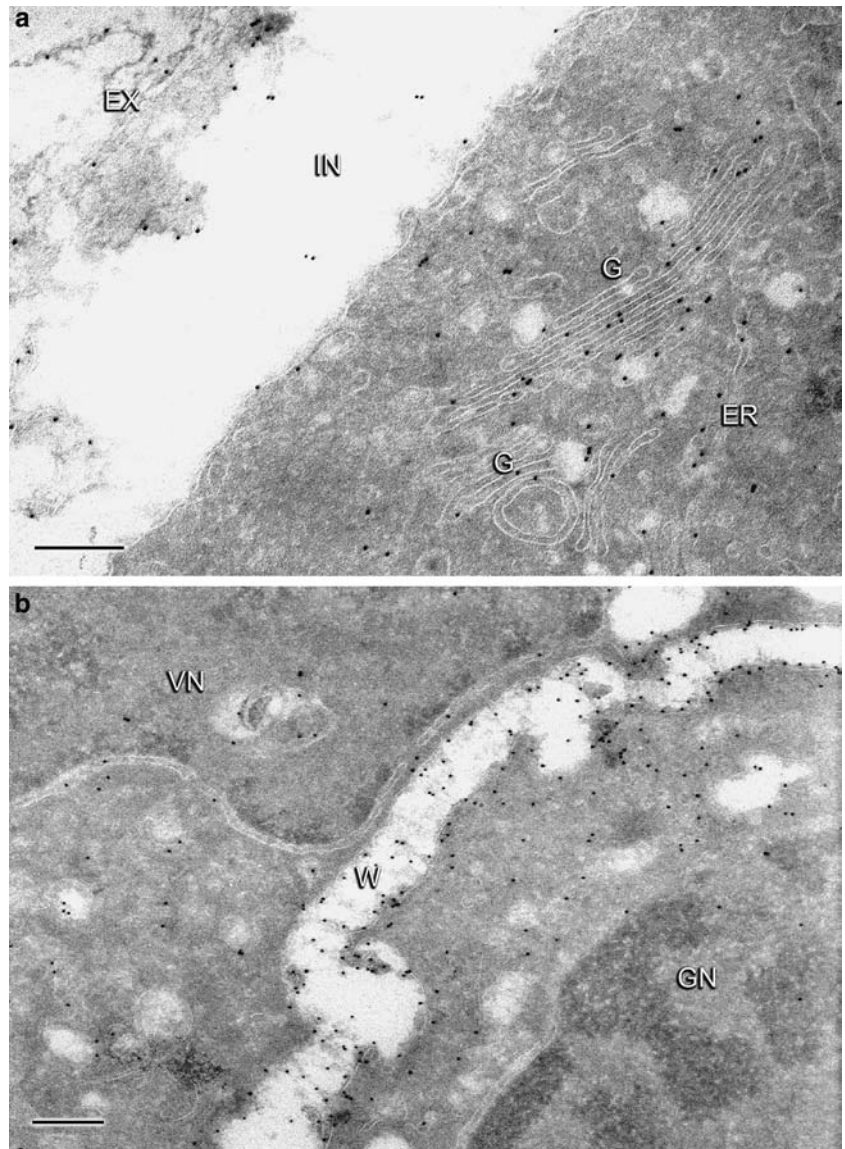


2005). Various enzymatic activities related to sugar metabolism were detected in mature pollen grains, most of them linked to germinating processes. These enzymatic activities include invertases (Singh and Knox 1984), which play a key role in the use of stigmatic exudates for pollen tube penetration into the style tissues. During pollen development, having reached the anther, sucrose is cleaved into glucose and fructose by anther wall invertases (Miller and Ranwala 1994; Dorion et al. 1996), leading to identical amounts of both monosaccharides (Roberts-Oehlschlanger et al. 1990; Dorion et al. 1996; Clément and Audran 1999). Germination assays in *Petunia hybrida* pollen showed the presence of wall-bound invertase activity and the uptake of sugars in the form of monosaccharides into the growing pollen tube (Ylstra et al. 1998). Nevertheless, in isolated mature pollen grains, sucrose is the major form of sugar identified in angiosperm pollen (Hoekstra et al. 1992; Speranza et al. 1997; Stadler et al. 1999). High concentrations of sucrose in dehydrated pollen grains protect the integrity of cell membrane phospholipids against desiccation (Hoekstra and van Roekel 1988; Hoekstra et al. 1992). The release of Pla a 1 (PII) from *P. acerifolia* hydrated pollen grains may contribute to the process of accumulation of sucrose surrounding the pollen-stigma region. Most likely, sucrose protects the

membrane systems maintaining pollen turgidity during the first stages of pollen hydration. The inactivation of invertases, via PII, during critical stages of plant development has been already proposed (Grenier et al. 2000). These inhibitors could allow a rapid transition from a phase of a high to a low sucrose/hexose ratio when it is required (Wobus and Weber 1999).

Pectin methylesterases (PME) catalyze the de-esterification of O6 methyl-esterified D-galactosiduronic acid units in pectic compounds (Markovič and Janeček 2004). Pectins are synthesized with a high degree of methyl-esterification, and subsequently secreted into the pollen wall where some of the methylester groups are cleaved by PMEs (Zhang and Staehelin 1992; Goldberg et al. 1996; Geitmann 1999). During pollen grain hydration, an abundant accumulation of unesterified pectins was observed in the apertural intine, forming an elastic gel material, which could facilitate the necessary expansion to the pollen tube emission (Suárez-Cervera et al. 2002; Castells et al. 2003). Moreover, the pectins located at the tip of the pollen tube are highly esterified, whereas they are unesterified in areas away from the tip (Li et al. 1994, 2002; Jauh and Lord 1996; Lennon and Lord 2000). Studies using pectinase at higher concentrations revealed an inhibitory effect of this enzyme on pollen germination (Brewbaker and Kwack 1964); nev-

Fig. 6 TEM. Mature non-hydrated pollen grains. Immunogold labelling corresponding to Pla a 2 proteins. **a** Abundant gold particles associated with peripheral Golgi apparatus (*G*) and *ER*. Sparse labelling in the pollen wall, *IN* and *EX*. **b** Abundant gold particles in the GC wall (*W*). No significant labelling in the *VN* and *GN*. Cryosections. Scale bars 0.25 μ m



ertheless, a stimulation of pollen tube germination and pollen tube growth was obtained applying a moderate concentration of PME, demonstrating that pectin is a key component in the pollen tube development (Parre and Geitmann 2005). Recently, a *VANGUARD1* protein has been identified and characterized in *Arabidopsis thaliana* which encodes a PME required for enhancing the growth of pollen tubes in the transmitting tract tissues (Jian et al. 2005). Assuming the role of pectins in controlling cell-wall polymers, the PME activity of Pla a 1 could contribute to maintain the equilibrium between esterified and unesterified pectins at the critical start of pollen germination.

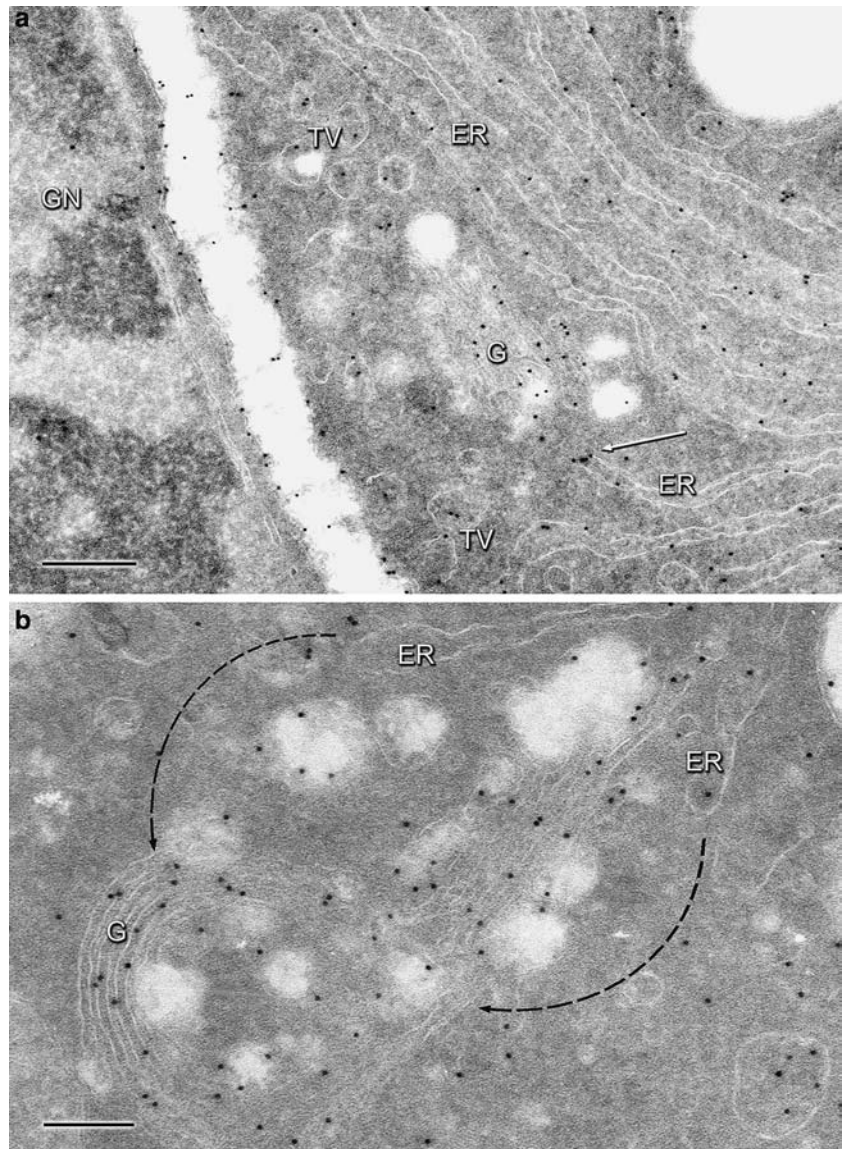
Role of PG (Pla a 2) during the germination process

Pla a 2 is a glycoprotein intensely secreted through the pollen wall after the first 5 min of the hydration-acti-

vation process. Its localization in the Golgi apparatus is consistent with its identification as a glycosylated protein (Ibarrola et al. 2004). The abundant Golgi stacks and associated vesicles situated at the apertural region might be related to the secretion of Pla a 2 protein, which takes place during the first steps of pollen hydration and probably during pollen-stigma activity. After 15–30 min in the germinating medium, the peripheral Pla a 2 activity was notably decreased (Fig. 3), although the ER and Golgi apparatus situated in the centre of the cytoplasm displayed a change of conformation, which could indicate significant activity in those locations. The localization of Pla a 2 in both organelles suggests an active process of transport from the ER to the Golgi apparatus, as described for other plant cells during the process of glycosylation (Staehelin and Moore 1995; Nebenführ and Staehelin 2001).

Exopolysaccharidases have the capacity to reduce the apparent molecular size of pectic polymers by

Fig. 7 TEM. Hydrated pollen grains. Immunogold labelling corresponding to Pla a 2 proteins. Central vegetative cytoplasm. **a** 15 min, **b** 30 min hydrated. **a** Abundant gold particles located in dissociated ER cisternae which form numerous ER transport vesicles (TV) and in active G. Note the link between ER and G (arrow) and no significant labelling in the GN. **b** Gold particles in ER and G. Note the link between the dissociated ER cisternae and G (arrows). Cryosections. Scale bars 0.25 μ m



cleaving neutral chain residues (De Veau et al. 1993). High levels of PG activity have been detected in pollen of many species of grasses (Pressey and Reger 1989; Swoboda et al. 2004). A pollen-specific *Brassica* PG also has been detected in ungerminated pollen grains, stigmatic papillae and in the tip of pollen tubes (Dearnaley and Daggard 2001). Immunolocalization of PG in maize pollen grains, using antiserum raised to maize PG, revealed the presence of PG in walls of cells undergoing modifications such as lysis in the tapetum or expansion in the growing pollen tubes (Dubald et al. 1993). Alignment of Pla a 2 with PG sequences reveals high conservation of regions involved in the catalytic sites of these enzymes, and furthermore, Pla a 2 is a functionally active PG (Ibarrola et al. 2004; SWISS-PROT identifier: Q6H9K0 2005). The intense secreted Pla a 2 PG during the first 5 min of hydration could be involved in pollen-stigma adhesion mechanisms (Lennon et al. 1998; Mollet et al. 2000) or compatibility

systems (Athanasίου et al. 2003). Moreover, the new expression of Pla a 2, after 15–30 min of germination, allows the PG activity during the pollen tube growth, which contributes to the degradation of the style cell wall (Khosravi et al. 2003).

Conclusions

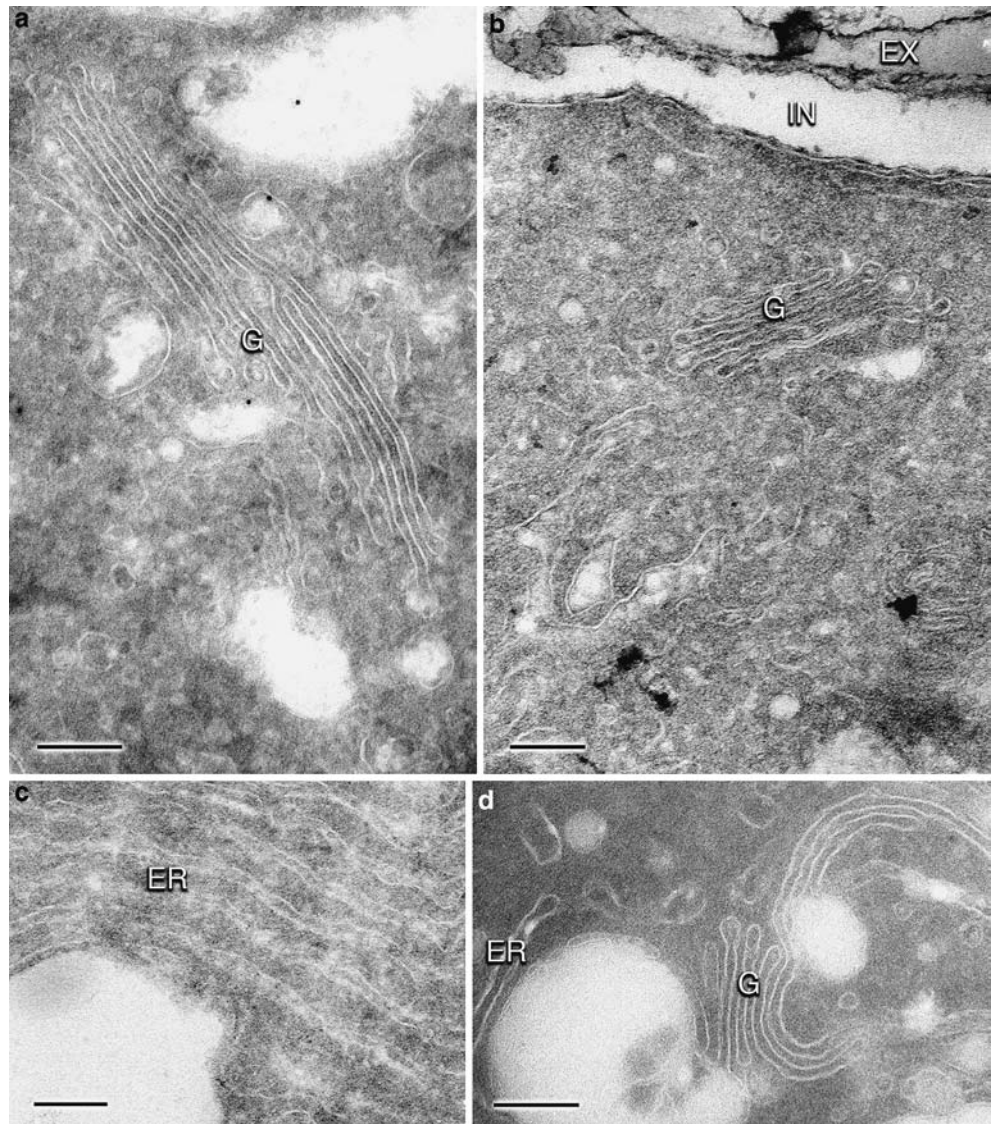
Pla a 1 is a secreted protein that may have a role in modifying cell walls in the extra-cellular space.

The early diffusion of Pla a 2 protein in hydrated pollen may be related to pollen-stigma adhesion.

The new expression and diffusion-like processes of Pla a 2 shown at late time points may be involved in the pollen-tube growth and the modification of the transmitting tissue cell walls.

The abundant production and elution of Pla a 1 and Pla a 2 proteins may alter the environment in which

Fig. 8 TEM. **a, b** Incubation with pre-immune antibodies. **a** Mature pollen grains, **b** 15 min hydrated pollen. **c, d** Controls omitting the primary antibodies. **c** Mature pollen grains, control corresponding to Pla a 1 proteins. **d** 15 min hydrated pollen, control corresponding to Pla a 2 proteins. **EX, IN, ER** and Golgi (**G**). Cryosections. Scale bars 0.25 μ m



pollen tube elongation occurs, thus promoting a potential crosstalk between the pollen and the gynoecium.

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