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Jesús Márquez · Juan A. Seoane-Camba
María Suárez-Cervera

Allergenic and antigenic proteins released in the apertural sporoderm during the activation process in grass pollen grains

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Abstract A combination of transmission electron microscopy with immunocytochemical methods was used to localize antigenic and allergenic proteins during the maturation and activation processes of Poaceae pollen grains. The intine undergoes a series of modifications that play a decisive role in these processes. Allergenic and antigenic proteins were detected particularly in the intine of activated in vitro grass pollen grains. Labelling of antigenic proteins was more abundant and less specific than that of allergenic proteins. At the time of hydration, the operculum was lifted up, the intine was swollen and labelling of allergenic proteins appeared highly localized in the *Zwischenkörper*. No significant labelling was observed when the *Zwischenkörper* gelatinized. Immunolocalization of allergenic proteins in the activated *Zwischenkörper* indicated the presence of proteins related to activation of the pollen grains. This confirms that the intine function is involved in the processes of pollen tube formation and fertilization, and also suggests the possible mechanism activated in the pollen grains when allergenic proteins reach the mucosa of sensitive subjects.

Key words Activated pollen grains · Allergenic proteins · Antigenic proteins · Apertural sporoderm · Poaceae

Introduction

Pollinosis due to grasses affects a large proportion of the population. Inhalation of pollen grains leads to allergenic rhinitis and type 1 asthma via allergenic proteins (IgE). The etiology of these phenomena is due to the presence of substances that can induce anaphylactic processes in susceptible individuals. Free amino acids, proteins and enzymes are found in the cytoplasm and in the inner (intine) and outer (exine) walls of pollen grains (Knox 1971, 1973; Knox and Heslop-Harrison 1969, 1970, 1971a, 1976). They are essential for pollen-stigma recognition, formation of the pollen tube and its passage through the style in order for fertilization to take place (Knox 1984). Acid phosphatase, ribonuclease and esterase were observed for the first time in the wall of pollen grains from *Populus*, Poaceae, *Plantago*, *Gladiolus* and Asteraceae using light microscopy (LM) (Knox and Heslop-Harrison 1969, 1970). Further, acid phosphatase was reported in various taxa using transmission electron microscopy (TEM) (Knox and Heslop-Harrison 1969, 1971a; Suárez-Cervera et al. 1992; Knox 1993). Immunocytochemical techniques using TEM have made it possible to locate and recognize the antigens of some pollen grains, principally those of Poaceae (Knox et al. 1970; Knox and Heslop-Harrison 1971b; Howlett and Clarke 1981; Vithanage et al. 1982; Staff et al. 1990; Márquez et al. 1992; Knox 1993; Taylor et al. 1993), Cupressaceae (Southworth et al. 1988; Miki-Hirosige et al. 1994), Betulaceae (Grote 1991) and Urticaceae (Casas et al. 1996).

The aim of this study was to localize by immunocytochemistry the antigenic and allergenic proteins found in the apertural sporoderm of Poaceae pollen grains during the activation processes previous to germination.

J. Márquez · J.A. Seoane-Camba · M. Suárez-Cervera (✉)
Department of Botany, Faculty of Pharmacy,
University of Barcelona, Av. Diagonal s/n.,
E-08028 Barcelona, Spain
Fax +34-3-402 18 86; e-mail: suarez@farmacia.far.ub.es

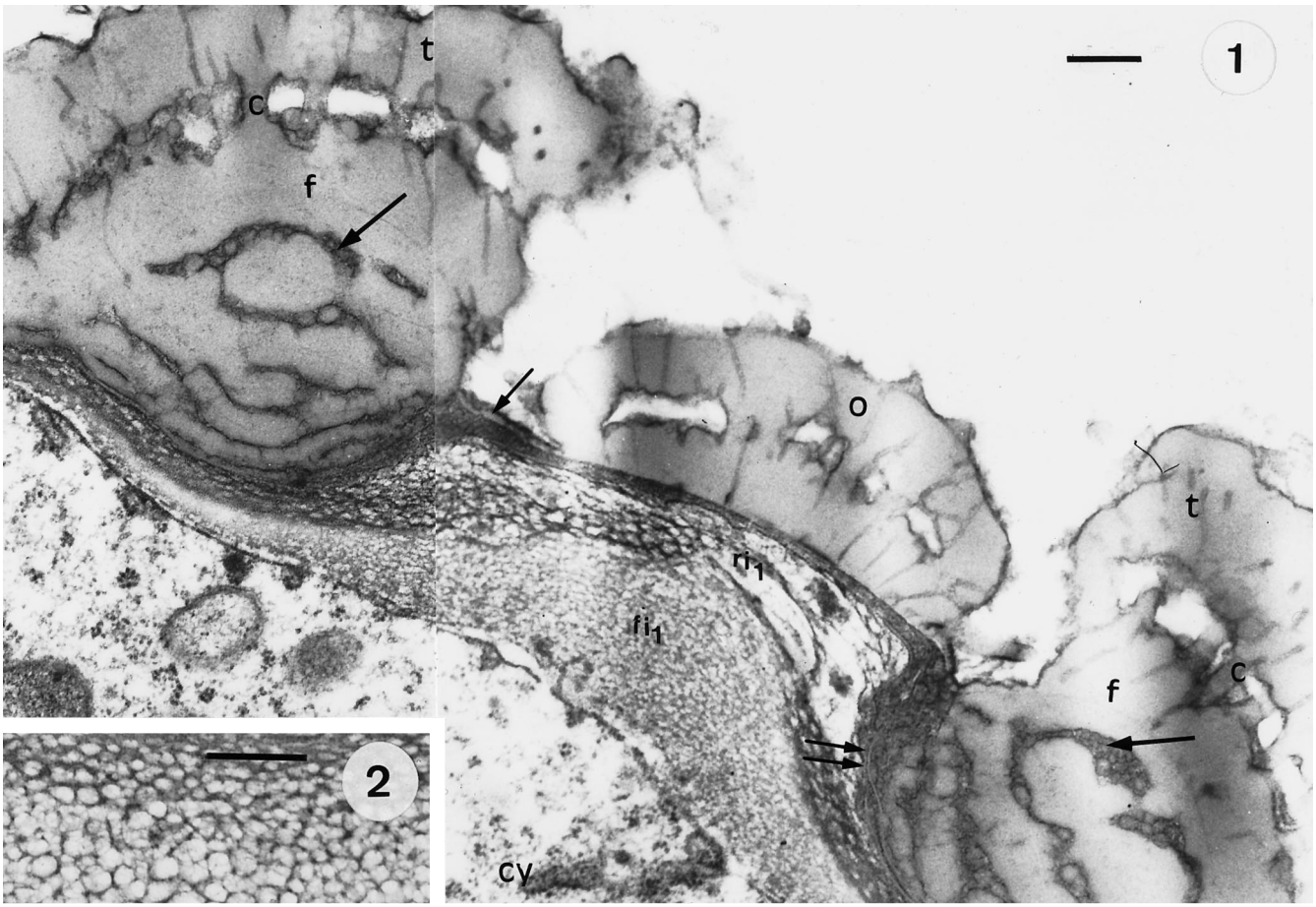
Materials and methods

Plant material

Young and mature anthers of *Paspalum dilatatum* Poir. and *Lolium perenne* L. were collected on the campus of the Faculty of Pharmacy at the University of Barcelona.

Activation

Activation of mature anthers was induced in vitro in a wet chamber for 5, 10 or 30 min at room temperature. After this time activation was interrupted and anthers were removed from the wet chamber and fixed.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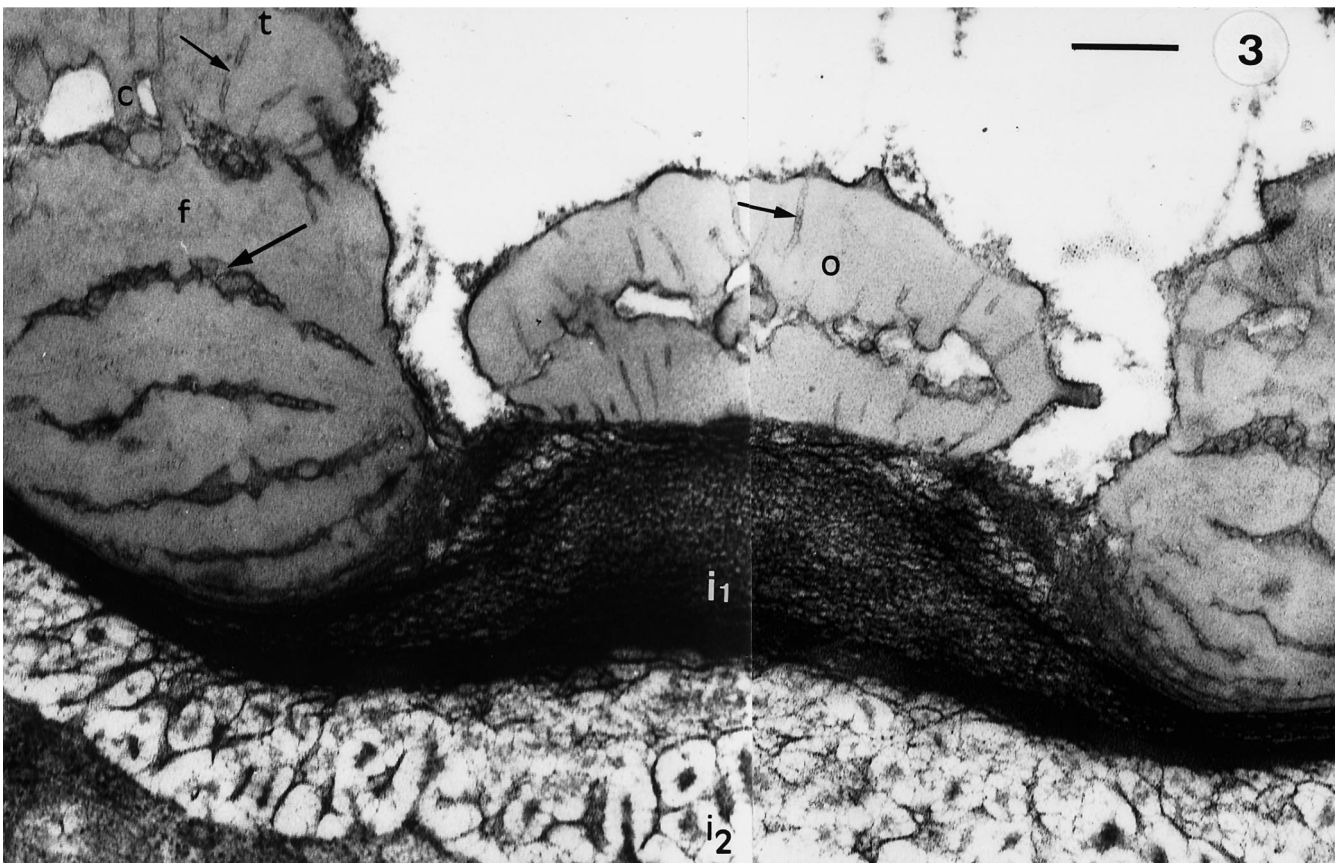
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Fixation and embedding

Young, mature and activated anthers were fixed in a 2% paraformaldehyde and 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer for 12 h at room temperature. Anthers were then washed in 0.1 M cacodylate buffer for 30 min and postfixed with 1% osmium tetroxide in 0.8% phosphate-buffered $K_3Fe(CN)_6$ for 7 h at 4°C. This was followed by dehydration in acetone and embedding in Spurr's resin. Sections of the blocks containing the samples were obtained with a Reichert-Jung Ultracut E, and stained with uranyl acetate and lead citrate. For localization of neutral polysaccharides, sections were treated with periodic acid thiocarbohydrazide silver protein (PA-TCH-SP) according to Thièry (1967).

Human sera

Serum from allergenic patients with specific IgE was supplied by the Allergy Service of the Santiago Apostol Hospital in Vitoria. The specific IgE of the patients against a *Lolium* extract was 100, 97, 31 kU/l. Total IgE was 1327, 1049 and 94 KU/l. (In the Pharmacia CAP system IgE FEIA, 10.39–40.05 kU/l is considered high). Serum from a non-allergenic subject with negative specific IgE and total IgE less than 100 kU/l was used as a control. Serum was stored in Eppendorf tubes at –40°C.

Immunogold labelling and electron microscopy

For detection of allergens (IgE), ultrathin sections of anther were incubated at room temperature on drops of 5% BSA in 0.1 M PBS for 15 min, followed by sensitized patient serum diluted 1:100 in 0.1 M PBS for 2 h. After three 10-min washes, sections were incubated for 1 h with a second antibody bridge, anti-human IgE developed in a goat (Sigma), diluted 1:40 in PBS. After three 10-min washes with drops of 0.1 M PBS, sections were incubated for 1 h with anti-IgG (goat) antibody developed in a rabbit (BIO CELL) conjugated to 10-nm diameter colloidal gold particles using a 1:60 dilution in PBS. This was followed by three washes with drops of 0.1 M PBS for 5 min, two washes with distilled water and air drying. In the control for specific patient antibody, patient serum was replaced by non allergenic subject serum. In the control for non-specific binding of the antibody conjugated to colloidal gold, patient serum and anti-human IgE were omitted. In the control for non-specific binding of the bridge antibody, patient serum was omitted.

For detection of antigens (IgG), ultrathin sections of anther were incubated at room temperature on drops of 5% BSA in 0.1 M PBS for 15 min, followed by sensitized patient serum diluted 1:100 in 0.1 M PBS for 2 h. After three 10-min washes, sections were incubated for 1 h with the antibody, anti-human IgG developed in a goat (Amersham), conjugated to 10-nm diameter colloidal gold particles, using a 1:60 dilution in PBS. This was followed by three 5-min washes with drops of 0.1 M PBS, two washes with distilled water and air drying. In the control for specific patient antibody, patient serum was replaced by non-allergenic subject serum. In the control for non-specific binding of the antibody conjugated to colloidal gold, patient serum was omitted.

Finally, the sections were stained with uranyl acetate for 15 min and lead citrate for 5 min and observed in a Philips EM200 at 60 kV and Philips EM301 at 80 kV.

Results

Apertural intine formation in young pollen grains (bicellular stage)

During the first stage of intine deposit (Fig. 1), the apertural exine has a thick annulus composed of a channeled tectum, a short columella and a large, stratified foot layer. Granules of variable size and form were present in the foot layer. A thin apertural membrane joining the annulus with the operculum was observed. Rudimentary endexine was present. The exintine (*Zwischenkörper*) developed in two layers, fibrillar (inner) and reticular (outer) (Figs. 1, 2). During the second stage of intine deposit (Fig. 3), the exintine (*Zwischenkörper*) was compacted, and the loose and channelled middle intine was deposited. Later, the third intine layer was in place throughout the undulated plasmalemma (Fig. 4). At this stage, the exintine (*Zwischenkörper*) had a fibrillar and compacted appearance and the middle exintine was thick and contained microchannels.

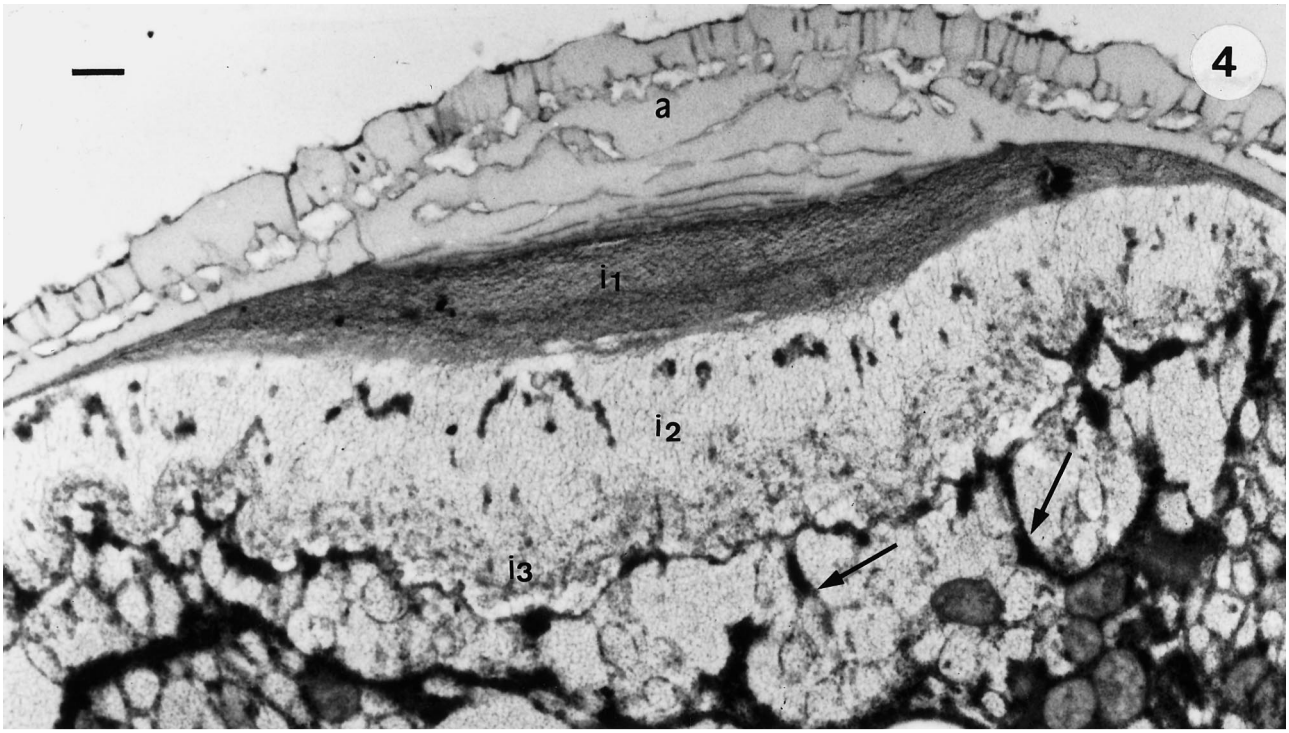
Ripe pollen grains (tricellular stage)

The electron transparent pollenkitt was deposited on the exine and it was delimited by a polysaccharide layer. In the non-apertural sporoderm, the three-layered intine consists of the thin and fibrillar Z-layer (exintine), reticulate and thick middle intine, and thin and fibrillar endintine (Fig. 5). In the apertural sporoderm, the three-layered intine showed a characteristic structure: the *Zwischenkörper* (exintine) was large, the middle intine was reduced in size compared to that of the non-apertural region, and the endintine was thicker than that of the non-apertural region (Fig. 5). Polysaccharide staining was strong in the apertural middle intine and endintine, faint in the *Zwischenkörper* (apertural exintine)

◀ **Fig. 1–3** Apertural sporoderm in young *Paspalum* pollen grain (bicellular stage) during intine deposit. **Fig. 1** The first stage of intine deposit. The annulus is composed of a channeled tectum (*t*), short columella (*c*) and a large foot layer (*f*). Granules (*long arrows*) of variable size and form are present in the stratums of the foot layer. Rudimentary endexine under the foot layer (*double arrow*). A thin apertural membrane (*small arrow*) joins the annulus with the operculum (*o*). The exintine (*Zwischenkörper*) is developed in two layers: fibrillar (fi_1) and reticular (ri_1). Cytoplasm (*cy*). **Fig. 2** Detail of the reticular layer of the exintine (*Zwischenkörper*). **Fig. 3** The second stage of intine deposit. Compacted exintine (*Zwischenkörper*) (I_1), loose and channelled middle intine (I_2), tectum (*t*), short columella (*c*), large foot layer (*f*); granules (*long arrow*). Note the microchannels in the exine (*short arrows*). *Bars* 0.25 μm

Fig. 4 Apertural sporoderm in young *Paspalum* pollen grain (bicellular stage) during the third stage of intine deposit. Section near the pore: annulus (*a*), *Zwischenkörper* or exintine (i_1), middle intine (i_2) and endintine (i_3). The endintine (i_3) is between middle intine (i_2) and undulated plasmalemma (*arrows*) ▶

Fig. 5 Ripe *Lolium* pollen grains (tricellular stage). Thièry test. Non-apertural sporoderm: Z-layer or exintine (*arrow*), middle intine (i_2) and endintine (*arrowhead*). Apertural sporoderm: *Zwischenkörper* or exintine (i_1), middle intine (i_2) and endintine (i_3). The polysaccharide staining is strong in the apertural middle intine and endintine (i_2,i_3) and faint in the *Zwischenkörper* (i_1). Operculum (*o*); tapetum (*T*), Ubisch bodies (*ub*). Note the electron-transparent pollenkitt (*pk*). Observe the abundant granular P-particles (*gp*) and starch granules (*s*) in the cytoplasm. *Bars* 0.25 μm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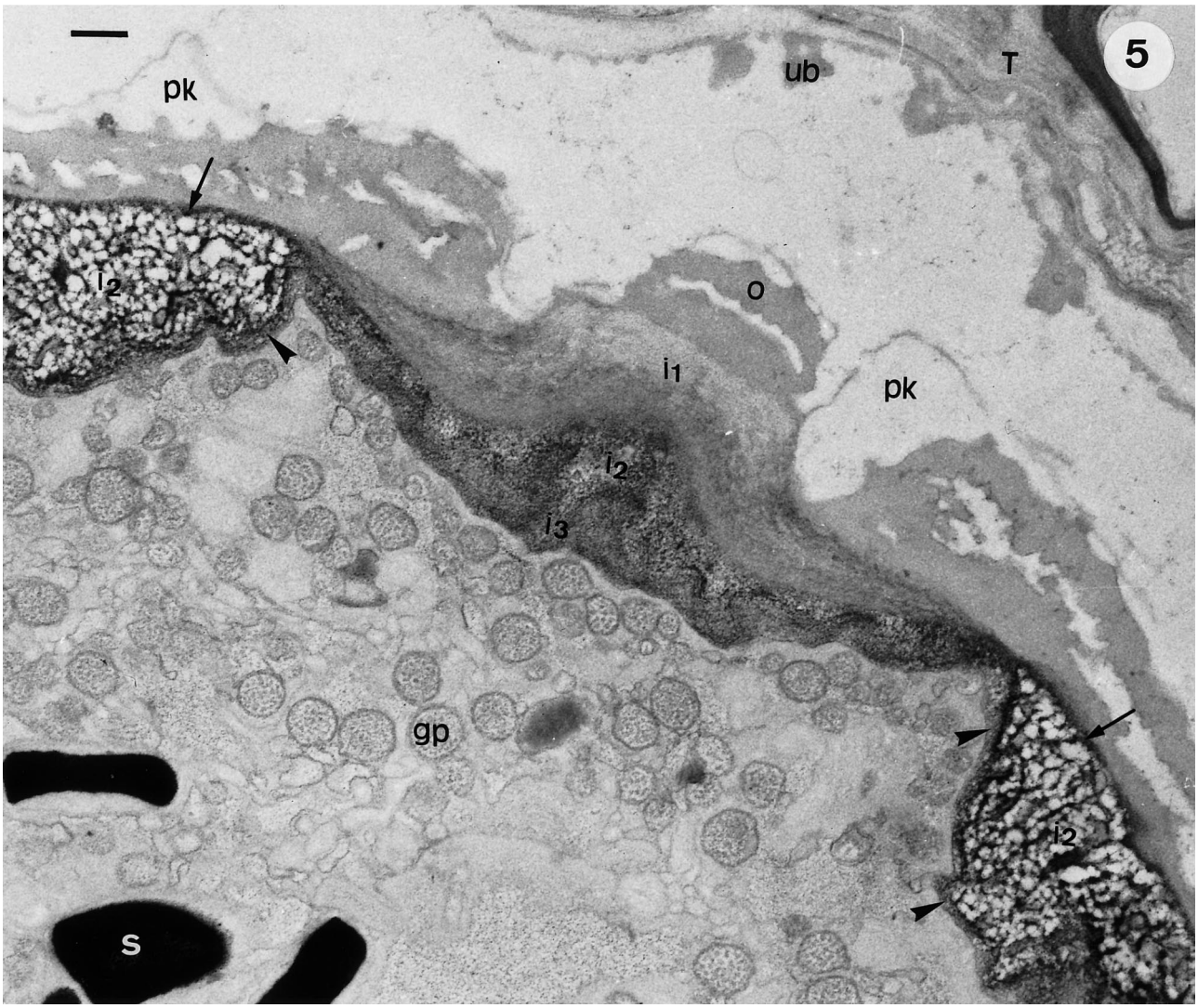
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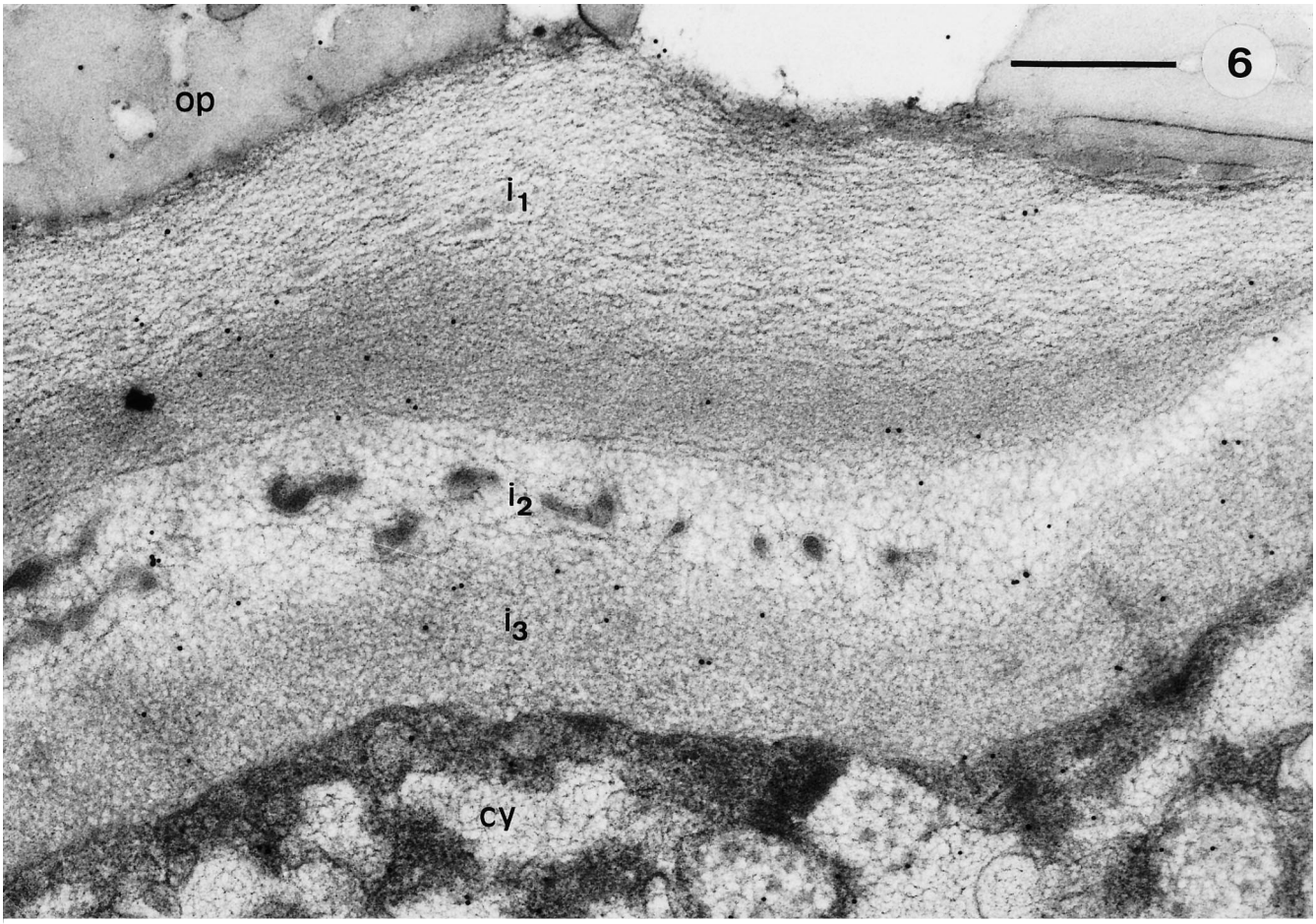
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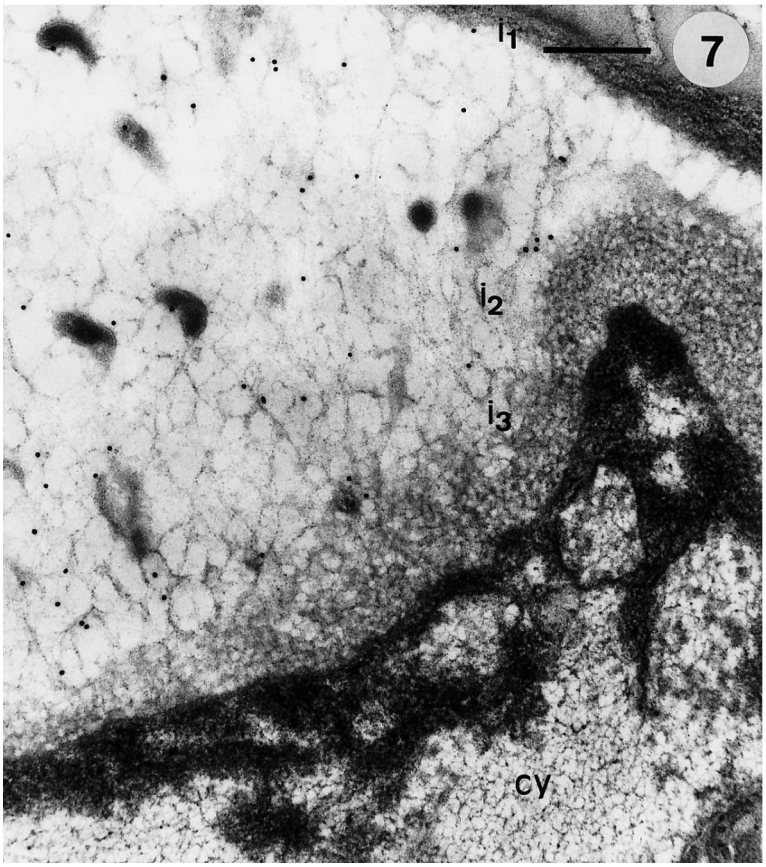
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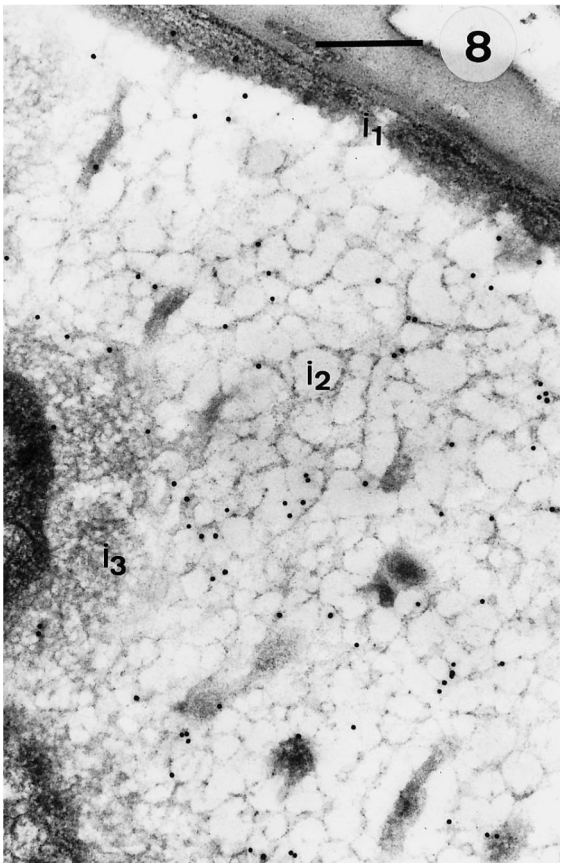
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and moderate in the Z-layer (non-apertural exintine) (Fig. 5).

Activated pollen grains

Activated pollen grains were always in the tricellular stage (Fig. 10). Three activation stages were observed in the apertural sporoderm. During the first stage of activation, the three intine layers developed, the apertural membrane ruptured, and the operculum separated from the apertural margins (Fig. 6). The antigenic and allergenic proteins (IgG and IgE) were detected by incubation with human serum against *Lolium*. In the pore, significant labelling was observed in the exintine (*Zwischenkörper*) and endintine (Figs. 6, 9). Around the pore, the middle layer of intine showed more abundant labelling (Figs. 7, 8, 11, 12). At this time, IgE labelling was less extensive than IgG, and had a tendency to form groups (Figs. 9, 11, 12). During the middle stage of activation in *Lolium* pollen grains, the operculum was lifted up, the intine was swollen and immunolabelling of allergenic proteins (IgE) appeared highly localized in the *Zwischenkörper* (Fig. 13). Concurrently, the intensity of IgG labelling decreased notably in the apertural intine. During the final stage of activation the *Zwischenkörper* gelation occurred. The *Zwischenkörper* disappeared and no significant labelling of either glycoprotein was observed (Fig. 14).

Discussion

During the processes of maturation and activation of Poaceae pollen grains that occur prior to germination, the intine is extremely active and undergoes a series of modifications that play a decisive role in both processes.

Antigenic and allergenic proteins in activated Poaceae pollen grains have been detected by immunocytochemical methods. These proteins were significantly present in the intine, even though we used conventional resins. The use of conventional resins (Spurr, Araldite, Epon) has been considered unsuitable for immunocytochemistry. Lowicryl K4M and LR white are the resins currently most widely used. However, these resins do not preserve cytoplasmic organelles as well as Spurr's resin; therefore, sectioning is difficult. We used Spurr's resin to localize wall proteins (Rubistein et al. 1995; Casas et al. 1996), and we found labelling similar to that obtained by other authors using Lowicryl or LR white resin.

Intine formation

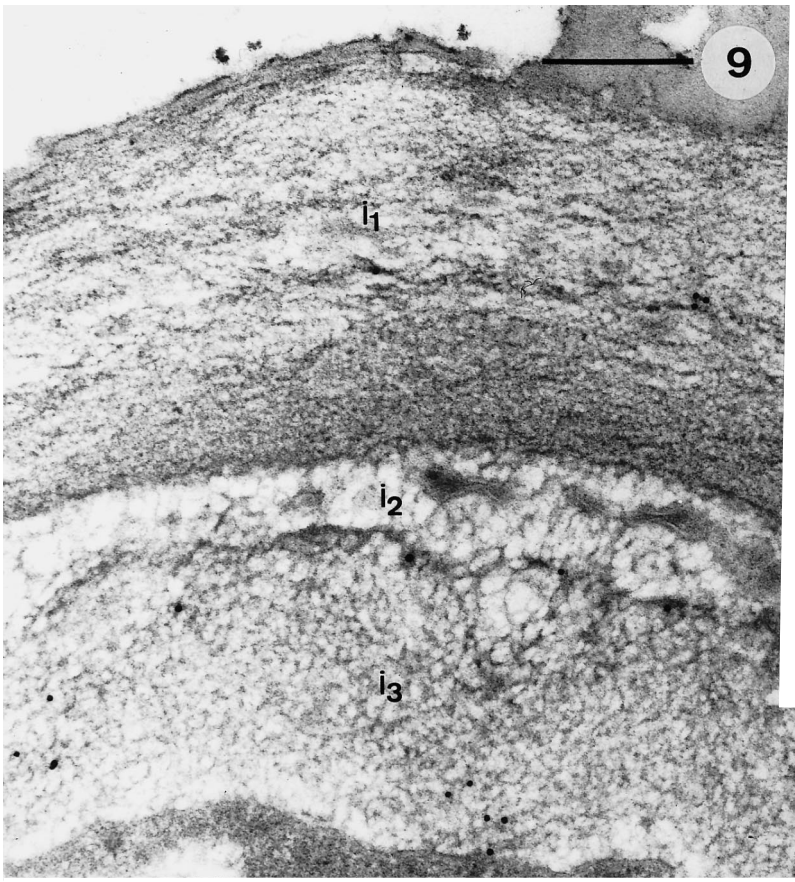
Deposition of the successive layers, the middle intine and endintine, corresponds to a strong digitalization of the plasmalemma in *Paspalum*. This mechanism is very similar to that observed in *Triticum* (El-Ghazaly and Jensen 1986, 1987) and in *Lolium* (Pacini et al. 1992). In *Lolium*, deposition of the three intine layers corresponds to intine stratification in the Poaceae (Heslop-Harrison and Heslop-Harrison 1991).

Activation process

Grass pollen is released in a tricellular state. The gametophyte is completely developed at the moment of anthesis. Precursor materials for the formation of the pollen tube have already been synthesized. Before anthesis, rapid and dramatic dehydration occurs, facilitating dispersion of the pollen grains by the wind (Stanley and Linskens 1974). In grasses, this dehydration reduces the moisture content in the pollen grains by 35% (Heslop-Harrison 1979; Dumas et al. 1984). After dispersion and pollination, pollen grains undergo rehydration on the surface of the stigma. Hydration is achieved by contact between the stigma and the pollen grain, through stigmatic secretions and the pollenkitt. These substances are proteins, lipids and polysaccharides which form a fluid film that facilitates the flow of water through the pollen grain aperture. As the pollen grain grows, the pectidic *Zwischenkörper* undergoes rapid gelation; proteins are released from the pore and the operculum is expelled. At the same time, large amounts of secretions are produced which pass through the pollen grain wall, leaving through the microchannels of the exine. If a compatible stigma is found, the pollen grain forms a tube, the first morphological indication of germination. In *Lolium*, after moisture activation, we were able to follow very clearly by TEM the three step process prior to germination. During the first stage of the intine hydration, the operculum was separate from the annulus and the *Zwischenkörper* protruded slightly; this was followed by expulsion of the operculum. When the intine increased in volume, the immunolabelling of proteins appeared highly localized in the *Zwischenkörper*. Finally, *Zwischenkörper* gelation occurred, the *Zwischenkörper* disappeared and no significant labelling was detected in the slight halo observed between the operculum and intine remnants. Immunolocalization of antigenic and allergenic proteins in the activated *Zwischenkörper* indicated the presence of proteins related to the activation of the pollen grains. Re-

◀ **Fig. 6–8** First stage of activation in *Lolium* pollen grains. Detection of antigenic proteins (IgG) by incubation with human serum against *Lolium*. **Fig. 6** Apertural region. Significant labelling in the apertural intine (i_1, i_2, i_3). Sparse labelling in the cytoplasm (*cy*) and operculum (*op*). **Figs. 7, 8** Intine close the pore (i_1, i_2, i_3). Significant labelling in the middle intine (i_2). *Bars* 0.25 μm

Fig. 9–12 First stage of activation in *Lolium* pollen grains. Detection of allergenic proteins (IgE) by incubation with human serum against *Lolium*. **Fig. 9** Apertural intine (i_1, i_2, i_3). Scarce labelling in the *Zwischenkörper* (i_1) and endintine (i_3). **Fig. 10** Tri-nucleate pollen grain. Note the vegetative nucleus (*vn*), two spermatic nuclei (*arrows*) and thick intine (*double arrow*). **Figs. 11, 12** Intine close to the pore (i_1, i_2, i_3). Note grouped labelling in the middle intine (*arrows*). No significant labelling in the cytoplasm (*cy*) and exine (*ex*). *Bars* 0.25 μm ▶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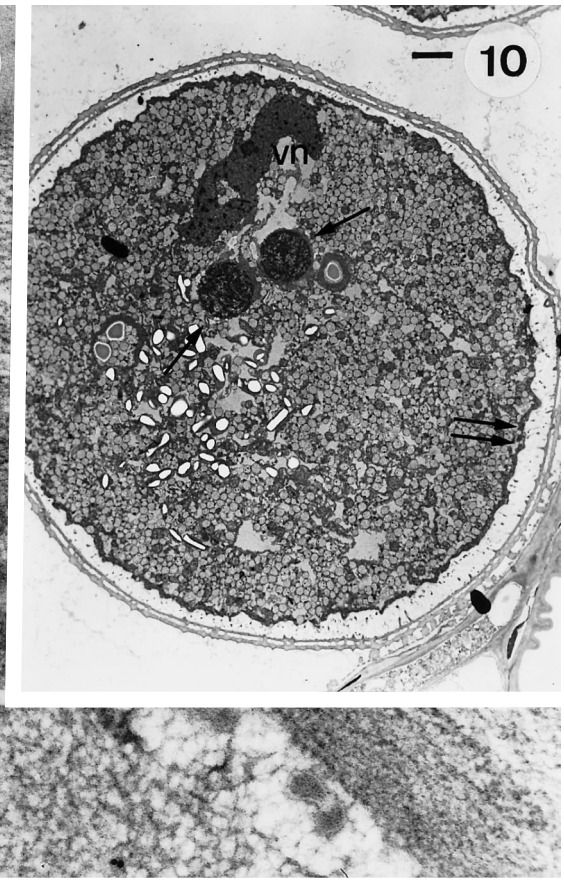
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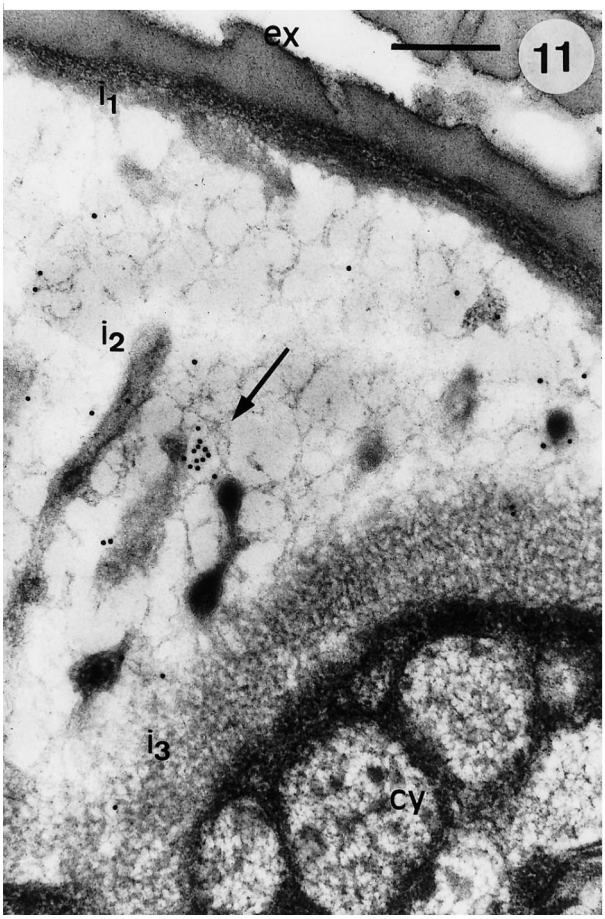
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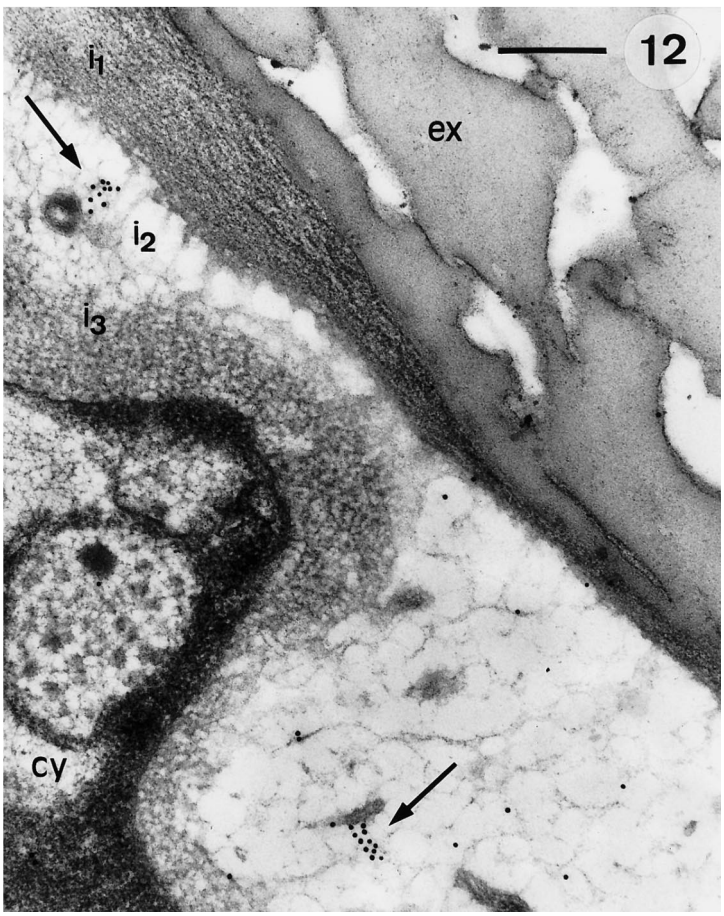
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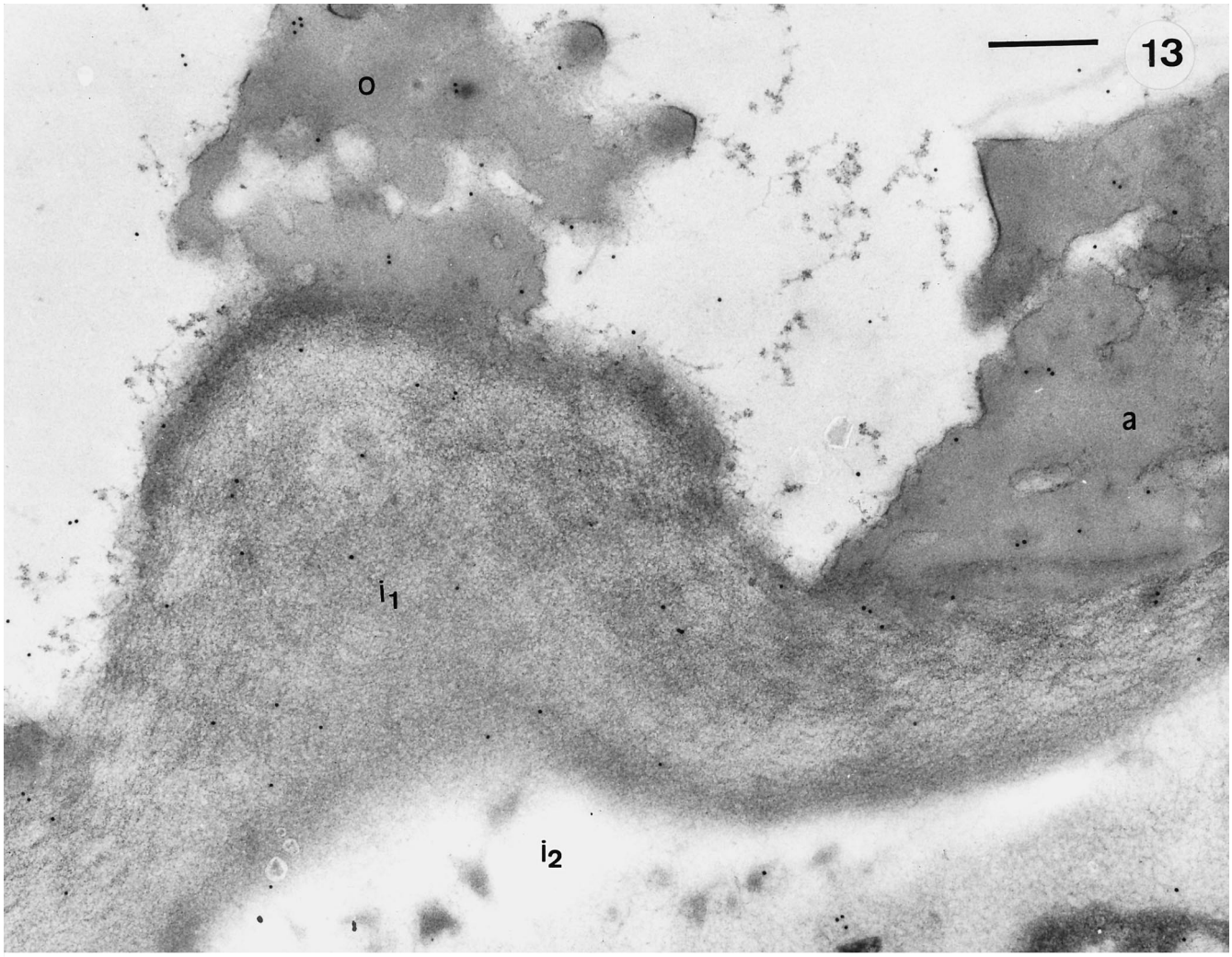
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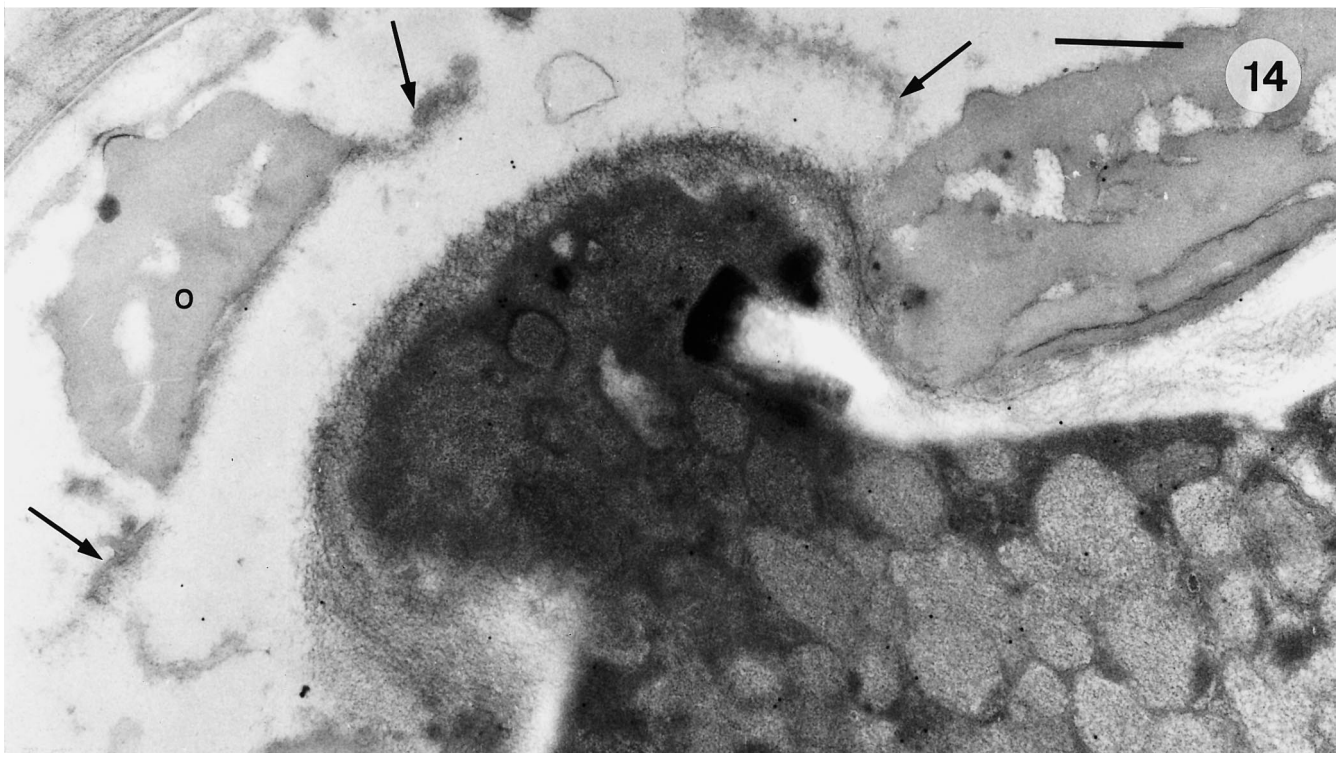
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cently, Casas et al. (1996) subjected the pollen grains of *Parietaria judaica* to activation, in order to detect proteins in the intine and in material extruded from the pollen grains that are related to pollen-stigma recognition. The allergenic proteins of *P. judaica* pollen grains were activated after 10 min of hydration, prior to pollen tube formation, and these activated proteins reacted with the antibodies. Immunogold labelling data from *Lolium* localized these proteins in the activated *Zwischenkörper*. However, no significant labelling was observed when gelation occured and the *Zwischenkörper* disappeared; at this time, the specific proteins of the activation process were diffused. Our results with activated *Lolium* pollen grains confirm the function attributed to the *Zwischenkörper* in the processes of pollination and fertilization (Heslop-Harrison and Heslop-Harrison 1980).

Labelling shown by the antigenic proteins (IgG) was very abundant, but less specific than that of the allergenic proteins (IgE). The IgG proteins were activated prior to the IgE proteins and their diffusion was almost complete at the middle stage of pollen grain activation. These results with Poaceae agree with similar observations with Urticaceae (Casas et al. 1996).

Mobility of the antigenic and allergenic proteins

The two layers of the pollen grain wall, the exine and intine, are morphologically and chemically distinct. The dominant material of the exine is sporopollenin. Specific proteins have been detected in this layer using monoclonal antibodies in Cupressaceae, *Calocedrus decurrens* (Southworth et al. 1988) and *Cryptomeria japonica* (Miki-Hirosige et al. 1994). Labelling has also been obtained with polyclonal antibodies (Howlett et al. 1979; Vithanage et al. 1980, 1982; Grote et al. 1983; Grote and Fromme 1984; Grote 1991, 1992; Márquez et al. 1992; Knox 1993). The intine functions as a mediator in gametophyte-sporophyte relations (Knox 1984), specifically in (1) protection of the gametophyte from desiccation (polysaccharide component of the intine), (2) biochemical pollen-stigma recognition, and (3) storage of proteolytic and hydrolytic enzymes for pollen tube formation and subsequent penetration into the stigma. However, in Poaceae (Howlett and Clarke 1981; Abadie et al. 1988; Vithanage et al. 1982; Knox 1993; Staff et al. 1990; Márquez et. al 1992), Cupressaceae (Southworth et al. 1988; Miki-Hirosige et al. 1994) and Betulaceae (Grote 1991) significant labelling with polyclonal and monoclonal antibodies has been obtained only in the exine, Ubisch bodies and cytoplasm of the pollen grain, and not in the intine. The authors cited attribute the lack of labelling in the intine to mobility of the proteins. This mobility may be due to the degree of hydration of the aqueous fixatives or exposure to aqueous media. An anhydrous fixation technique for localization of water-soluble antigens of starch granules of pollen grains was developed (Taylor et al. 1993); detection of allergens in the starch granules and the mobility of water-soluble antigens with standard aqueous methods seemed to be evident. Márquez et al. (1992) fixed anthers of Poaceae pollen grains with paraformaldehyde vapors and placed the sections in an aqueous medium for 3 days. They were able to demonstrate that the proteins had a degree of mobility and they could observe significant labelling in the cytoplasm, exine and Ubisch bodies, but not in the intine. However, the lack of labelling, or the inability to detect antigenic and allergenic proteins in the intine, conflicts with studies in which enzymes and proteins were clearly detected in the intine. Until now, mature pollen grains, such as those found in the anther at the moment of dehiscence, have been studied. Now we subject the pollen grains to an activation process in order to detect proteins in the intine and in the material extruded from the pollen grains that is related to pollen-stigma recognition. The activated proteins may react with patient serum antibodies. Our results suggest that proteins of Poaceae pollen grains are activated at the moment of hydration, prior to pollen tube formation, and are rapidly diffused. Sensitive patients are normally exposed to pollen grains through the respiratory tract, and this contact provides the pollen grains with a warm, wet medium, one that is very similar to the medium they require to germinate upon arrival at the stigma. Pollen grains, upon contact with an aqueous medium or stigmatic secretion, absorb water and release low molecular weight proteins, which easily diffuse in an aqueous medium, and pollen-stigma recognition takes place (Heslop-Harrison 1987). Subsequently, if pollen and stigma are compatible, germination occurs, with the formation of the pollen tube. Presumably, in sensitive individuals the first part of the process takes place in the mucosa. The humidity and temperature of the mucosa may facilitate release of the same proteins that would be activated or liberated in pollen-stigma recognition. These proteins may be responsible for allergic sensitization (Seoane-Camba and Suárez-Cervera 1986; Casas et al. 1996). This confirms the function attributed to the intine in the processes of pollination and fertilization, and also suggests the possible mechanism activated when pollen grains reach the mucosa of sensitive individuals.

◀ **Fig. 13** Middle stage of activation in *Lolium* pollen grains. Detection of allergenic proteins (IgE) by incubation with human serum against *Lolium*. Apertural region. Significant labelling in the protruding *Zwischenkörper* (i_1), operculum (*o*), annulus (*a*) and exudates. No significant labelling in the middle intine (I_2)

Fig. 14 Complete stage of activation in *Lolium* pollen grains. Detection of allergenic proteins (IgE) by incubation with human serum against *Lolium*. Note the remnants of *Zwischenkörper* gelation (*arrows*). No significant labelling was observed in the wall. Operculum (*o*). *Bars* 0.25 μm

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