

Detection and release of allergenic proteins in *Parietaria judaica* pollen grains

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Summary. Rapid diffusion of allergenic proteins in isotonic media has been demonstrated for different pollen grains. Upon contact with stigmatic secretion or with the mucosa of sensitive individuals, pollen grains absorb water and release soluble low-molecular-weight proteins, these proteins enter in the secretory pathway in order to arrive at the cell surface. In this study we located allergenic proteins in mature and hydrated-activated pollen grains of *Parietaria judaica* L. (Urticaceae) and studied the diffusion of these proteins during the first 20 min of the hydration and activation processes. A combination of transmission electron microscopy and immunocytochemical methods was used to locate these proteins in mature pollen and in pollen grains after different periods of hydration and activation processes. Activated proteins reacting with antibodies in human serum from allergic patients were found in the cytoplasm, wall, and exudates from the pollen grains. The allergenic component of these pollen grains changes according to the pollen state; the presence of these proteins in the exine, the cytoplasm, and especially in the intine and in the material exuded from the pollen grains, is significant in the hydrated-activated studied times, whereas this presence is not significant in mature pollen grains. The rapid activation and release of allergenic proteins of *P. judaica* pollen appears to be the main cause of the allergenic activity of these pollen grains.

Keywords: *Parietaria judaica*; Pollen; Allergenic protein; Localization; Diffusion.

Introduction

Localisation of pollen proteins by immunoelectron microscopic methods has been the aim of several research groups. Knox and Heslop-Harrison (1970) assumed that the pollen proteins were stored in the pollen wall layers (exine and intine). Belin and Rowley (1971) concluded that the allergens are probably stored inside the pollen pro-

toplasm and diffuse through the aperture during germination. Ultrastructural studies performed with pollen grains of different species, i.e., members of the family Poaceae (Staff et al. 1990; Márquez et al. 1997a, b; Grote et al. 2000), *Cryptomeria japonica* (Miki-Hirosige et al. 1994), *Olea europaea* (Rodríguez-García et al. 1995a, b), *Betula verrucosa* (El-Ghazaly et al. 1999, Grote et al. 1999), *Ricinus communis* (Pauri et al. 1999), *Zygophyllum fabago* (Castells et al. 2002), and *Cupressus* species (Suárez-Cervera et al. 2003), have shown that during hydration in isotonic media, pollen grains remain intact, but soluble and low-molecular-weight proteins are eluted in a diffuse manner through the intine and exine. In natural conditions, the proteins are released in the stigma and pollen–stigma recognition takes place (Heslop-Harrison 1987). If this process occurs in the mucosa of sensitive individuals, the released proteins are responsible for allergenic sensitisation because these proteins react with human antibodies (Seoane-Camba and Suárez-Cervera 1986).

Parietaria (Urticaceae) is the genus that includes the most widespread allergenic plants in the Mediterranean region, for example, in southern Italy and around the Mediterranean coast of Spain (D'Amato et al. 1991), and *P. judaica* L. is one of the most common species of this genus. Thus, it appears in many pollen calendars (D'Amato et al. 1991).

Parietaria judaica pollen grains carry a major allergen, Par j 1, that is a glycoprotein of the family known as non-specific lipid transfer proteins (Duro et al. 1997). But Boluda et al. (1998) report that the Par j 1 content of these pollen grains does not correlate with the antigenic potency of the extract; proteins other than Par j 1 contribute to the overall antigenic potency. So, in this study we located allergenic proteins in *P. judaica* pollen grains

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during the hydration and activation processes, using serum of a patient.

Material and methods

Plant material

Mature anthers of *Parietaria judaica* were collected from the area around Barcelona (Spain). A combination of transmission electron microscope and immunocytochemical methods were used to locate allergenic proteins in fresh pollen grains from dehiscent anthers and in vitro hydrated and activated pollen grains.

In vitro hydration and activation

Hydration and activation of mature pollen grains were induced in vitro by sprinkling onto a 0.7% agar germination medium (Walden 1993) for 10, 15, and 20 min at room temperature, to supply similar humidity and temperature conditions to the receptive stigma. After each specific time, the process was interrupted and pieces of agar with pollen grains were removed from the germination medium and fixed.

Fixation and embedding

Dehiscent anthers and in vitro hydrated and activated pollen grains were fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde buffered by 0.1 M cacodylate, pH 7.3, for 2 h at room temperature. Later, the samples were washed in 0.1 M cacodylate buffer, pH 7.3, for 40 min and postfixed with 1% osmium tetroxide in 0.8% phosphate-buffered $K_3Fe(CN)_6$ at pH 7.3, for 3 h at 4 °C. This was followed by dehydration in an acetone series and embedding in Spurr resin. Sections of the blocks containing the samples were obtained with a Reichert-Jung Ultracut E microtome. These sections were mounted on gold grids.

Human sera

Serum from an allergic patient with specific immunoglobulin E (IgE), and serum from a nonallergic subject with negative specific IgE were supplied by the El Bierzo Hospital in Ponferrada (León, Spain). The radioallergosorbent test revealed that the specific IgE against *P. judaica* extract belonged to class two (0.7–3.5 PRU/ml). Serum from a nonallergic subject was used as control. These sera were stored in Eppendorf tubes at –40 °C.

Immunogold labelling

For detection of allergenic proteins, ultrathin sections were incubated at room temperature on drops of 5% bovine serum albumin in 0.1 M Tris-buffered saline (TBS) for 15 min, followed by sensitised-patient serum for 2 h. After three 10 min washes with drops of 0.1 M TBS, sections were incubated for 1 h with a second antibody bridge, in this case, anti-human IgE developed in a goat (Sigma), diluted 1:40 in TBS. After three 10 min washes with drops of 0.1 M TBS, sections were incubated for 1 h with the antibody conjugated to 10 nm diameter colloidal gold particles, using anti-IgG (goat) passaged in a rabbit (BioCell) using a 1:60 dilution in TBS. This was followed by two washes with drops of 0.1 M TBS for 5 min, two washes with distilled water and air-drying. In the control for specific patient antibody, the patient serum was replaced by nonallergic-subject serum. In the control for nonspecific binding of the antibody conjugated to colloidal gold, the patient serum and the anti-human IgE were omitted. In the control for nonspecific binding of the bridge antibody, the patient serum was omitted.

We sectioned two blocks and incubated two replicate grids for each sample. Each grid had 8–9 sections and each section had 15–20 sectioned grains. Labelling was considered significant when all grains

presented the same distribution pattern. Finally, sections were stained with uranyl acetate for 15 min and lead citrate for 5 min and observed in a Philips EM 301 transmission electron microscope.

Results

Mature pollen grain

Parietaria judaica pollen grains are three-pored and bicellular, they contain a little generative cell and a big vegetative cell (Fig. 1A). The wall is formed by a thin outer layer (exine) and an inner layer (intine) which is thin in the non-apertural region but thickens below the apertures to form a convex structure (oncus) (Fig. 1A–C). In the cytoplasm, vacuoles, lipid bodies, mitochondria, rough endoplasmic reticulum, Golgi apparatus, and ribosomes were observed (Fig. 1A, B).

In the immunogold-labelled sections, gold particles were not detected in the wall and cytoplasm. We only observed slight labelling in the intine of the apertural region (Fig. 1C).

Hydration and activation

After 10 min in culture medium, labelling was extensive in nonapertural and apertural exine and intine and was abundant in the material exuded by the pollen grain through the microchannels of the tectum showing the traffic of proteins (Fig. 2A, B). Moreover, grouped gold particles were observed in the intine and exine (Fig. 2B). In the vegetative cytoplasm, abundant labelling was observed in the ribosomes, vacuoles, lipid bodies, rough endoplasmic reticulum, and in the Golgi stacks and associated vesicles predominantly located near the plasma membrane (Fig. 2A). Clusters of colloidal gold particles were observed in this cytoplasm (Fig. 2A). After 15 min in the agar germination medium, an increase in labelling was observed in the material exuded from the pollen grain (Fig. 3A). In the rest of the pollen grain, labelling was similar to that found at 10 min. After 20 min, the only difference was the noticeable decrease in the labelling detected in exudates (Fig. 3B). No significant labelling was observed in any of the controls (Fig. 1B).

Discussion

We located allergenic proteins in the material exuded from the pollen grains because the two exine layers (ectexine and endexine) show considerable elasticity and permeability to allow the diffusion of allergenic proteins through the microchannels of the exine (Rowley 1975, Seoane-Camba and Suárez-Cervera 1986, Knox et al.

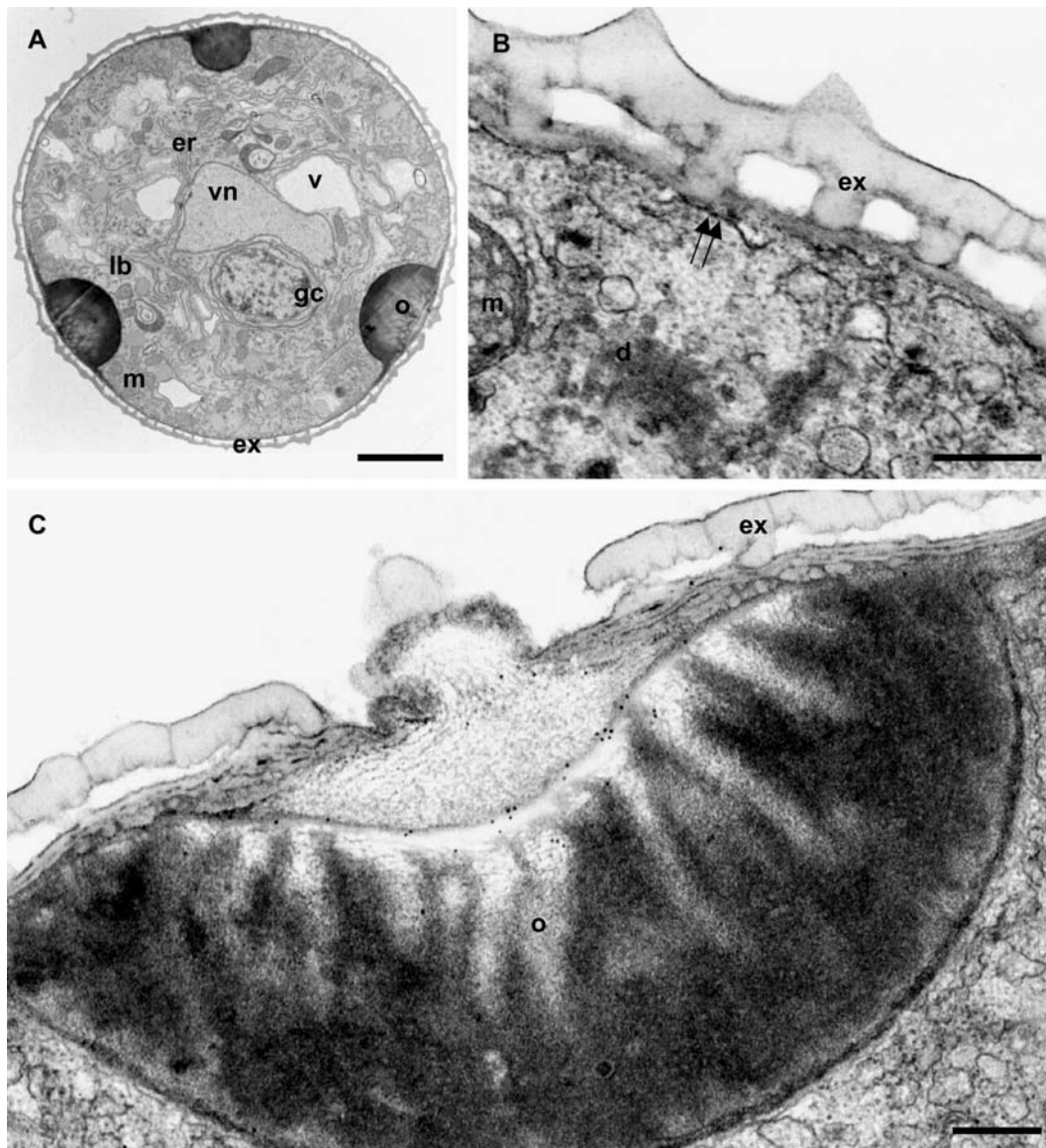


Fig. 1 A–C. Immunocytochemical localisation of allergenic proteins in *P. judaica* mature pollen grain. Conventional protocol. **A** General view of a transverse section showing three apertures. The wall is formed by the exine and the intine that is thin in the nonapertural region but thickens below the apertures forming the onci. Cytoplasm with a little generative cell and a big vegetative cell. **B** Control for specific patient antibody. No significant labelling was observed in the controls. **C** Slight labelling was observed in the oncus of the mature pollen grains. *d* Dictyosome, *er* rough endoplasmic reticulum, *ex* exine, *gc* generative cell, *lb* lipid bodies, *m* mitochondrion, *o* oncus, *v* vacuole, *vn* vegetative nucleus. Double arrow, intine. Bars: A, 1 μm ; B and C, 0.25 μm

1998). The abundant trafficking through the intine confirms the functions attributed to this layer in the process of pollination and fertilisation (Heslop-Harrison and Heslop-Harrison 1991, Suárez-Cervera et al. 2000).

The allergenic proteins localised in the cytoplasm of *P. judaica* pollen grains were found in those areas in the pollen grain that are known to represent sites of protein metabolism and energy supply. Most of the gold markers were located in the cytosol, which was closely packed with ribosomes. Moreover, these soluble proteins enter in the secre-

tory pathway via the endoplasmic reticulum and the Golgi complex and are rapidly exuded from the pollen grain during the hydration and activation processes (Pfeffer and Rothman 1987, Denecke et al. 1990), although the storage of many proteins in angiosperms seems to be in the rough endoplasmic reticulum (Galili et al. 1998; Alché et al. 1999, 2002; Castells et al. 2002). In *Olea* species (Rodríguez-García et al. 1995a, b; Alché et al. 1999, 2002) and in *Betula verrucosa* (Grote et al. 1993, 1999; El-Ghazaly et al. 1999) significant labelling has also been detected in

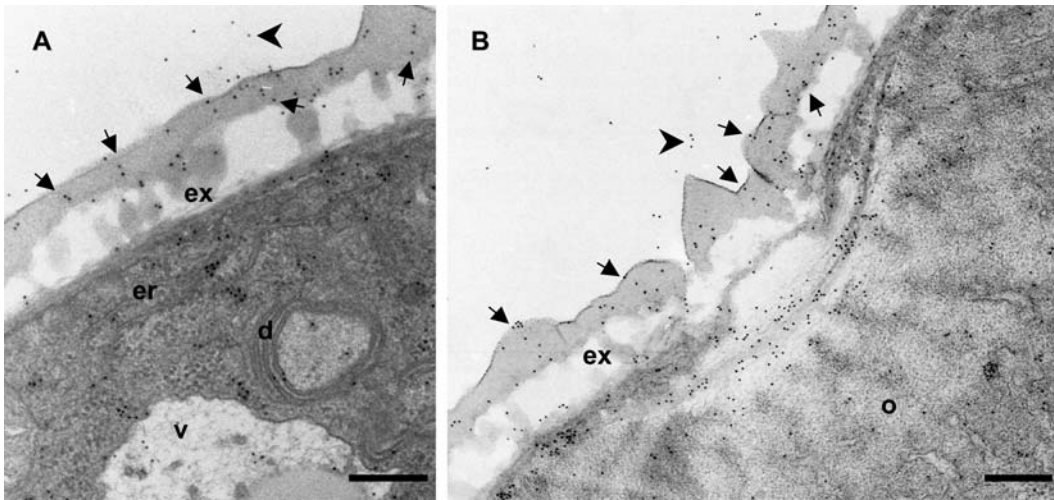


Fig. 2 A, B. Sections of *P. judaica* pollen grains after 10 min in germination medium. Abundant labelling was observed in nonapertural (A) and apertural (B) exine and intine and in the material exuded by the pollen grain. In the cytoplasm gold particles were observed in rough endoplasmic reticulum, dictyosomes, and vacuoles. Clusters of colloidal gold particles were observed in the exine, intine, and cytoplasm (arrowheads). Note the labelling in the microchannels of the exine (arrows). *d* Dictyosome, *er* rough endoplasmic reticulum, *ex* exine, *o* oncus, *v* vacuole. Bars: 0.25 µm

the cisterns of the rough endoplasmic reticulum. In *Cryptomeria japonica* (Miki-Hirosige et al. 1994) and *Cupressus arizonica* and *C. sempervirens* (Suárez-Cervera et al.

2003), colloidal gold particles were found in the Golgi apparatus and in the Golgi vesicles. In *Zygophyllum fabago*, Castells et al. (2002) observed colloidal gold particles in the rough endoplasmic reticulum, in the dictyosomes, and in the Golgi vesicles of the pollen grains.

Labelling in the vacuoles and in the lipid bodies revealed the presence of the major allergen of *P. judaica* pollen grains (Par j 1) in both organelles, suggesting traffic of lipid transfer proteins between them (Vega-Maray et al. 2004).

The extracellular sites of pollen allergens recognised by the patient serum, especially in the exine, intine, and in the exudates, may implicate their role as proteins that are present in the first steps of male–female gametophyte recognition. Similar results have been obtained for allergenic proteins of pollen grains from other species like *Cryptomeria japonica* (Miki-Hirosige et al. 1994), members of the family Poaceae (Taylor et al. 1994; Márquez et al. 1997a, b), *Betula verrucosa* (Grote et al. 1999), *Zygophyllum fabago* (Castells et al. 2002), *Cupressus arizonica* and *C. sempervirens* (Suárez-Cervera et al. 2003).

The allergenic component of pollen grains changes according to the pollen state; pollen from immature flowers contain fewer allergenic proteins than pollen from mature flowers and hydrated and activated pollen grains have more allergenic proteins than mature pollen (Grote et al. 1993). So, in *P. judaica*, the presence of these proteins in the exine, the cytoplasm, and especially in the intine and in the material exuded from the pollen grains is significant in the hydrated and activated studied times, whereas this

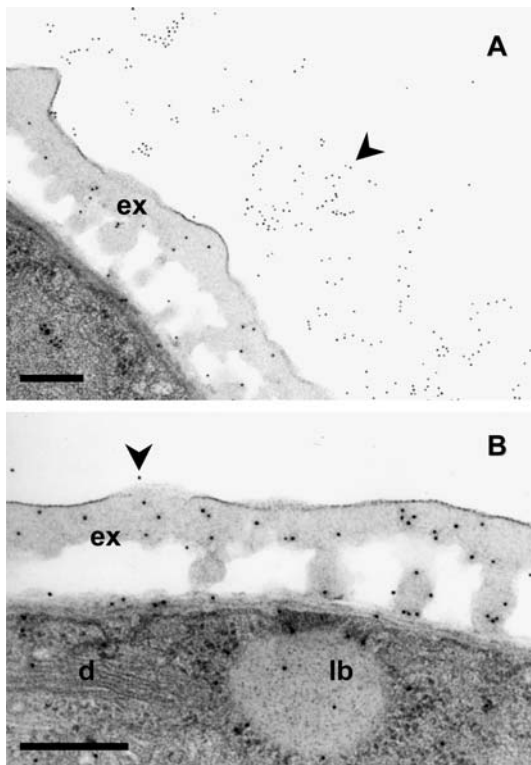


Fig. 3. A After 15 min in germination medium an increase of labelling was observed in the material exuded by the pollen grain (arrowheads). B After 20 min in germination medium labelling decreases on the exudates (arrowheads). *d* Dictyosome, *ex* exine, *lb* lipid body. Bars: 0.25 µm

presence is not significant in mature pollen grains. These allergenic proteins are suddenly activated, released, and detected during the first minutes of the hydration and activation processes. This rapid diffusion of the allergenic proteins in *P. judaica* should be related to the significant cytoplasm changes observed at the beginning of the hydration and activation processes (Vega-Maray et al. 2003).

Besides allergenic proteins, for *P. judaica* pollen grain antigenic proteins (Casas et al. 1996) and the major allergen, Par j 1 (Vega-Maray et al. 2004), have been studied by immunocytochemical methods at different activation times. Activation of antigenic proteins recognised by rabbit sera IgG (Casas et al. 1996) was faster than activation of those recognised by IgE. This may be due to a longer activation period of the proteins recognised by IgE. Moreover, labelling shown by the antigenic proteins (IgG) was very abundant but less specific than that of the allergenic proteins, and labelling observed with allergenic proteins was more abundant and less specific than that observed with Par j 1 (Vega-Maray et al. 2004). Possible causes of the nonspecificity of IgG compared with IgE may be that rabbits were immunized with a heterogeneous mixture of different macromolecules obtained from an extract of *P. judaica* pollen grains (Casas et al. 1996). Pollen grains contain free amino acids, proteins, and enzymes in their cytoplasm and in their wall (Knox and Heslop-Harrison 1970). This provided sera with heterogeneous specificity, and therefore labelling was much wider and less specific than labelling obtained with sera from allergic patients. The sera of allergic patients appear to be sensitised to fewer proteins and hence their response could be more specific. But the most specific labelling and the lowest labelling were observed when the major allergen was detected (Vega-Maray et al. 2004), because in this case, only one protein was involved.

The behaviour of these proteins in the hydration and activation processes was also different. The antigenic proteins detected by Casas et al. (1996) had abundant labelling in the 10 min hydrated-activated pollen grains, and this intensity decreased at 30 min. With the allergenic proteins, our results showed similar labelling intensity during the 20 min studies. And the studies with the major allergen Par j 1 revealed a noticeable decrease of the labelling from the 10 min to the 20 min hydrated-activated pollen grains (Vega-Maray et al. 2004).

Another difference are the aggregates of colloidal gold particles that appeared when we detected allergenic proteins. According to Grote et al. (1993) the aggregates are caused by real aggregation of protein molecules, so allergenic proteins of *P. judaica* appear in aggregates, whereas Par j 1 does not present this characteristic. However, in

members of the family Poaceae (Márquez et al. 1997a, b) and *Zygophyllum fabago* (Castells et al. 2002), aggregates of allergenic proteins were observed, whereas in *Betula verrucosa*, Grote et al. (1993) detected aggregates of the major allergen Bet v 1.

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References

- Alché JD, Castro AJ, Olmedilla A, Fernández MC, Rodríguez R, Villalba M, Rodríguez-García MI (1999) The major olive pollen allergen (Ole e 1) shows both gametophytic and sporophytic expression during anther development and its synthesis and storage takes place in the RER. *J Cell Sci* 112: 2501–2509
- Alché JD, Castro AJ, Rodríguez García MI (2002) Localization of transcripts corresponding to the major allergen from olive pollen (Ole e 1) by electron microscopic non-radioactive in situ RT-PCR. *Micron* 33: 33–37
- Belin L, Rowley JL (1971) Demonstration of birch pollen allergen from isolated pollen grains using immunofluorescence and single radial immunodiffusion technique. *Int Arch Allergy Appl Immunol* 40: 754–769
- Boluda L, Casanovas M, Prieto JL, Fernández-Caldas E (1998) Determinations of Par j 1 by a competitive enzyme immunoassay using human specific IgE and IgG. Validation by skin pricks testing. *J Invest Allergol Clin Immunol* 8: 207–213
- Casas C, Márquez J, Suárez-Cervera M, Seoane-Camba JA (1996) Immunocytochemical localisation of allergenic proteins in *Parietaria judaica* L. (Urticaceae). *Eur J Cell Biol* 70: 179–188
- Castells T, Arcalís E, Moreno-Grau S, Bayo J, Elvira-Rendueles B, Belchí J, Seoane-Camba JA, Suárez-Cervera M (2002) Immunocytochemical localization of allergenic proteins from mature to activated *Zygophyllum fabago* L. (Zygophyllaceae) pollen grains. *Eur J Cell Biol* 81: 107–115
- D'Amato G, Ruffilli A, Ortolani C (1991) Allergenic significance of *Parietaria* (pellitory of the wall) pollen. In: D'Amato G, Spiekma FTM, Bonini S (eds) *Allergenic pollen and pollinosis in Europe*. Blackwell, Oxford, pp 113–118
- Denecke J, Botterman J, Deblaere R (1990) Protein secretion in plant cells can occur via a default pathway. *Plant Cell* 2: 51–59
- Duro G, Colombo P, Costa MA, Izzo V, Porcasi R, Di Fiore R, Locorotondo G, Cocchiara R, Geraci D (1997) Isolation and characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1.0101. *Int Arch Allergy Appl Immunol* 112: 348–355
- El-Ghazaly G, Moate R, Cresti M, Walles B, Takahashi Y, Ferreira F, Obermeyer C (1999) Localization and release of allergens from tapetum and pollen grains of *Betula pendula*. *Protoplasma* 208: 37–46
- Galili G, Sengupta-Gopalan Ch, Ceriotti A (1998) The endoplasmic reticulum of plant cells and its role in protein maturation and biogenesis of oil bodies. *Plant Mol Biol* 38: 1–29
- Grote M, Vrtala S, Valenta R (1993) Monitoring of two allergens, Bet v I and profilin, in dry and rehydrated birch pollen by immunogold elec-

- tron microscopy and immunoblotting. *J Histochem Cytochem* 41: 745–750
- Grote M, Hayek B, Reichelt R, Kraft D, Valenta R (1999) Immunogold electron microscopic localization of the cross-reactive two-EF-hand calcium-binding birch pollen allergen Bet v 4 in dry and rehydrated birch pollen. *Int Arch Allergy Appl Immunol* 120: 287–294
- Grote M, Vrtala S, Niederberger V, Valenta R, Reichelt R (2000) Expulsion of allergen-containing materials from hydrated rye grass (*Lolium perenne*) pollen revealed by using immunogold field emission scanning and transmission electron microscopy. *J Allergy Clin Immunol* 105: 1140–1145
- Heslop-Harrison J (1987) Pollen germination and pollen tube growth. *Int Rev Cytol* 107: 1–78
- Heslop-Harrison J, Heslop-Harrison Y (1991) Structural and functional variation in pollen intines. *Pollen Spores* 44: 331–343
- Knox RB, Heslop-Harrison J (1970) Pollen-wall proteins: localisation and enzymic activity. *J Cell Sci* 6: 1–27
- Knox RB, Taylor PE, Ladiges P, Nelson G, Suphioglu C (1998) Pollen allergens: molecular and immunological analyses and implications for systematics. In: Owens SJ, Rudall PJ (eds) *Reproductive biology in systematics, conservation and economic botany*. Royal Botanic Gardens, Kew, pp 449–463
- Márquez J, Seoane-Camba JA, Suárez-Cervera M (1997a) Allergenic and antigenic proteins released in the apertural sporoderm during the activation process in grass pollen grains. *Sex Plant Reprod* 10: 269–278
- Márquez J, Seoane-Camba JA, Suárez-Cervera M (1997b) The role of the intine and cytoplasm in the activation and germination processes of Poaceae pollen grains. *Grana* 36: 328–342
- Miki-Hirosige H, Nakamura S, Yasueda H, Shida T, Takahashi Y (1994) Immunocytochemical localization of the allergenic proteins in the pollen of *Cryptomeria japonica*. *Sex Plant Reprod* 7: 95–100
- Parui S, Kumar-Mondal A, Mandal S (1999) Identification and partial characterization of the allergenic proteins of *Ricinus communis* L. pollen. A new approach. *Grana* 38: 311–315
- Pfeffer SR, Rothman JE (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu Rev Biochem* 56: 828–852
- Rodríguez-García MI, Fernández MC, Alché JD (1995a) Immunocytochemical localization of allergenic protein (Ole e I) in the endoplasmic reticulum of the developing pollen grain of olive (*Olea europaea*). *Planta* 196: 558–563
- Rodríguez García MI, Fernández MC, Alché JD, Olmedilla A (1995b) Endoplasmic reticulum as a storage site for allergenic proteins in pollen grains of several Oleaceae. *Protoplasma* 187: 111–116
- Rowley JR (1975) The permeability of the pollen grain wall to exogenous proteins tracers. *J Ultrastruct Res* 50: 394
- Seoane-Camba JA, Suárez-Cervera M (1986) On the ontogeny of the oncus in the pollen grain of *Parietaria officinalis* ssp. *judaica* (Urticaceae). *Can J Bot* 64: 3155–3167
- Staff IA, Taylor PE, Smith P, Singh MB, Knox RB (1990) Cellular localization of water soluble allergenic proteins in rye-grass (*Lolium perenne*) pollen using monoclonal and specific IgE antibodies with immunogold probes. *Histochem J* 22: 276–290
- Suárez-Cervera M, Le Thomas A, Goldblatt P, Márquez J, Seoane-Camba JA (2000) The channelled intine of *Aristea major*: ultrastructural modifications during development, activation and germination. In: Harley MM, Morton CM, Blackmore S (eds) *Pollen and spores: morphology and biology*. Royal Botanical Gardens, Kew, pp 57–71
- Suárez-Cervera M, Takahashi Y, Vega-Maray AM, Seoane-Camba JA (2003) Immunocytochemical localization of Cry j 1, the major allergen of *Cryptomeria japonica* (Taxodiaceae) in *Cupressus arizonica* and *Cupressus sempervirens* (Cupressaceae) pollen grains. *Sex Plant Reprod* 16: 9–15
- Taylor PE, Staff IA, Singh MB, Knox RB (1994) Localization of the two major allergens in rye-grass pollen using specific monoclonal antibodies and quantitative analysis of immunogold labelling. *J Histochem Cytochem* 26: 392–401
- Vega-Maray AM, Fernández-González D, Valencia-Barrera RM, Seoane-Camba JA, Suárez-Cervera M (2003) Ultrastructural modifications in the apertural intine of *Parietaria judaica* L. (Urticaceae) pollen during the early stages of hydration. *Grana* 42: 220–226
- Vega-Maray AM, Fernández-González D, Valencia-Barrera R, Polo F, Seoane-Camba JA, Suárez-Cervera M (2004) Lipid transfer proteins in *Parietaria judaica* L. pollen grains: immunocytochemical localization and function. *Eur J Cell Biol* 83: 493–497
- Walden DB (1993) In vitro pollen germination. In: Freeling M, Walbot V (eds) *The maize hand book*. Springer, New York, pp 723–724