

This article was downloaded by: [Consorti de Biblioteques Universitaries de Catalunya]

On: 18 December 2008

Access details: Access Details: [subscription number 789296669]

Publisher Taylor & Francis

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Grana

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpptitle-content=t713648917>

An approach to the knowledge of pollen and allergen diversity through lipid transfer protein localisation in taxonomically distant pollen grains

María Suárez-Cervera ^a; Ana Vega-Maray ^b; Teresa Castells ^a; F. Javier Rodríguez-Rajo ^c; Juan A. Asturias ^d; Annick Le Thomas ^e; Juan A. Seoane-Camba ^a

^a Department of Botany Faculty of Pharmacy, University of Barcelona, Spain ^b Department of Biodiversity and Environmental Management (Botany), Faculty of Biology, University of León, Spain ^c Department of Plant Biology and Soil Sciences, Sciences Faculty of Ourense, University of Vigo, Ourense, Spain ^d R & D Department, Bial-Arístegui S.A., Bilbao, Spain ^e Muséum National d'Histoire Naturelle, Paris, France

Online Publication Date: 01 December 2008

To cite this Article Suárez-Cervera, María, Vega-Maray, Ana, Castells, Teresa, Rodríguez-Rajo, F. Javier, Asturias, Juan A., Le Thomas, Annick and Seoane-Camba, Juan A.(2008)'An approach to the knowledge of pollen and allergen diversity through lipid transfer protein localisation in taxonomically distant pollen grains',Grana,47:4,272 — 284

To link to this Article: DOI: 10.1080/00173130802513776

URL: <http://dx.doi.org/10.1080/00173130802513776>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

An approach to the knowledge of pollen and allergen diversity through lipid transfer protein localisation in taxonomically distant pollen grains

MARÍA SUÁREZ-CERVERA¹, ANA VEGA-MARAY², TERESA CASTELLS¹,
F. JAVIER RODRÍGUEZ-RAJO³, JUAN A. ASTURIAS⁴, ANNICK LE THOMAS⁵ &
JUAN A. SEOANE-CAMBA¹

¹Department of Botany Faculty of Pharmacy, University of Barcelona, Spain, ²Department of Biodiversity and Environmental Management (Botany), Faculty of Biology, University of León, Spain, ³Department of Plant Biology and Soil Sciences, Sciences Faculty of Ourense, University of Vigo, Ourense, Spain, ⁴R & D Department, Bial-Aristegui S.A., Bilbao, Spain, and ⁵Muséum National d'Histoire Naturelle, Paris, France

Abstract

The aim of the present study is to localise non-specific lipid transfer proteins (nsLTPs), obtained from Rosaceae (*Prunus persica* (L.) Batsch) in pollen grains of taxonomically distant plants, such as Iridaceae (*Aristea latifolia* G.J. Lewis), Platanaceae (*Platanus acerifolia* (Aiton) Willd.) and Urticaceae (*Parietaria judaica* L.), in order to compare pollen and nsLTP diversity. A combination of transmission electron microscopy, immunocytochemical techniques, and rabbit specific antiserum against peach nsLTPs (Pru p 3) were used. Abundant labelling to Pru p 3-like proteins was observed in the cytoplasm, walls and pollenkitt of *A. latifolia* pollen grains. The presence of nsLTPs associated with the pollenkitt proves that it takes part in the defence mechanism of pollen grains. The labelling was less intense in the cytoplasm and walls of *P. acerifolia*. Immuno-stained gold particles were associated with the vacuoles, lipid inclusions, endoplasmic reticulum and Golgi complex. No significant labelling was found in the *P. judaica* pollen grains incubated with anti-Pru p 3 polyclonal antibodies. These results indicate important variations in the nsLTPs of different pollen species. Consequently, no taxonomic relationship between pollen grains and nsLTPs could be established.

Keywords: Allergens, lipid bodies, lipid transfer proteins, pollen grains, pollenkitt, Pru p 3

Stephen Blackmore (2007) recently published an interesting review about pollen biodiversity indicating that pollen grains are the microscopic keys to understanding the Earth's biodiversity. The genuine interest in pollen grains is due to their diverse morphological characteristics which are evident to palynologists (Figure 1). On the other hand, the current flowering plant phylogeny is supported by the application of molecular techniques (Doyle, 2005; APG, 2003). However, of the 7 868 protein families found in all plants, only 29 contain pollen allergens (Radauer & Breiteneder, 2006). It seems that the great biodiversity of pollen grains does not correspond to the same diversity in allergenic proteins. This discrepancy could be in part because the current knowledge of pollen

allergens is concentrated in major allergen sources in Europe, North America and Japan. Moreover, in these countries there are several poorly characterised pollen allergen sources. The molecular characterisation of tropical and subtropical pollen species could increase pollen allergen diversity (Weber, 2007). Another reason for the discrepancy is that pollen allergens have enzymatic activity which is essential in plant cell development, for example, carbohydrate metabolism, lipid transport, plant defence, expansins and profilins, among others (Radauer & Breiteneder, 2006). For this reason, an important number of pollen antigens in distant taxonomical plants have homology and thus have the capacity to provoke cross-reactions (Weber, 2007).

Correspondence: María Suárez-Cervera, Department of Botany, Faculty of Pharmacy, University of Barcelona, Av. Diagonal s/n, 08028 Barcelona, Spain.
E-mail: msuarez@ub.edu

(Received 30 May 2008; accepted 17 September 2008)

ISSN 0017-3134 print/ISSN 1651-2049 online © 2008 Collegium Palynologicum Scandinavicum
DOI: 10.1080/00173130802513776

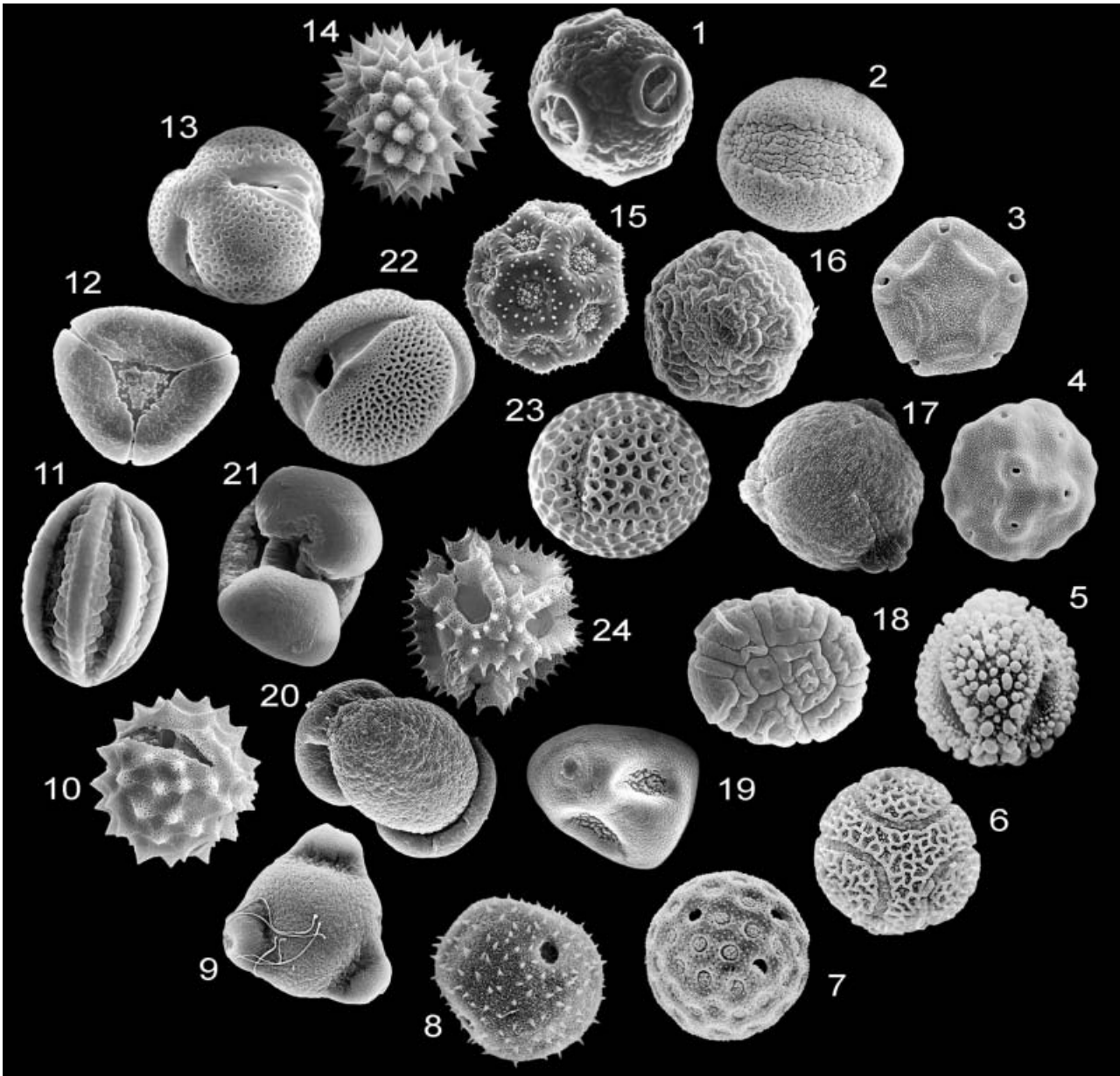


Figure 1. Pollen morphological diversity by SEM. (1). Fumariaceae, *Fumaria capreolata* (18.5 μm); (2). Arecaceae, *Phoenix canariensis* (30 $\times$ 17 μm); (3). Betulaceae, *Alnus glutinosa* (18 $\times$ 23 μm); (4). Juglandaceae, *Juglans regia* (40 $\times$ 35 μm); (5). Aquifoliaceae, *Ilex aquifolium* (25 $\times$ 21 μm); (6). Passifloraceae, *Passiflora incarnata* (49 μm); (7). Caryophyllaceae, *Silene legionensis* (33 μm); (8). Campanulaceae, *Campanula herminii* (29 μm); (9). Onagraceae, *Epilobium angustifolium* (65 μm); (10). Asteraceae, *Aster sedifolius* (30 μm); (11). Ephedraceae, *Ephedra* sp. (32.5 $\times$ 20 μm); (12). Myrtaceae, *Feijoa sellowiana* (18.5 μm); (13). Euphorbiaceae, *Neoguillauminia cleopatra* (41 $\times$ 37 μm); (14). Asteraceae, *Arnica montana* (22.5 μm); (15). Ranunculaceae, *Anemone* sp. (21.7 μm); (16). Ulmaceae, *Ulmus minor* (33.5 μm); (17). Saxifragaceae, *Saxifraga granulata* (27 $\times$ 25 μm); (18). Mimosaceae, *Acacia spinosa* (32 $\times$ 27 μm); (19). Cyperaceae, *Carex* sp. (24 $\times$ 20 μm); (20). Pinaceae, *Pinus radiata* (72 μm); (21). Pinaceae, *Pinus halepensis* (65 μm); (22). Zygophyllaceae, *Zygophyllum fabago* (14 $\times$ 16 μm); (23). Oleaceae, *Ligustrum vulgare* (29 $\times$ 25 μm); (24). Asteraceae, *Taraxacum officinalis* (26 $\times$ 30 μm).

Non-specific plant lipid transfer proteins (nsLTPs) are small proteins (9 kD) which facilitate the transfer of phospholipids and other lipids across membranes (Kader, 1996). These proteins are widely distributed throughout the plant kingdom and take part in the plant defence system (Hoffmann-Sommergruber, 2000). NsLTPs were identified as major allergens in fruits and in many

other plant foods causing severe allergenic symptoms (Zuidmeer & van Ree, 2007). Recently published reviews have provided a thorough background on nsLTPs in foods and pollen (Mills et al., 2004; Salcedo et al., 2004, 2007; Breiteneder & Mills, 2005). Major allergens of *Parietaria judaica* L. pollen grains, Par j 1 and Par 2, belong to the nsLTP family (Costa et al., 1994; Asturias et al., 2003;

Colombo et al., 2003) and are localised in the cytoplasm and walls of *P. judaica* pollen grains (Vega-Maray et al., 2004). Moreover, nsLTPs have been reported as important allergens in fruits from the Rosaceae family, such as peach, Pru p 3 (Pastorello et al., 1999a; Sánchez-Monge et al., 1999; Fernández-Rivas et al., 2003), cherry, Pru av 3 (Scheurer et al., 2001) and apple, Mal d 3 (Pastorello et al., 1999b), as well as from the Corylaceae family, such as hazelnut, Cor a 8 (Schocker et al., 2004). It is assumed that nsLTPs from different species present diverse homologies. For example, the peach nsLTP, Pru p 3, has sequence similarities with *Platanus* and *Artemisia* pollen grains, plants which are botanically distant from the Rosaceae family (Lombardero et al., 2004; Lauer et al., 2007). Nevertheless, undetectable cross-reactivity between Pru p 3 and Par j 1, the major allergen of *P. judaica*, was found in allergic patients (Lombardero et al., 2004).

The objective of this study is to localise Rosaceae nsLTPs in pollen grains of taxonomically distant plants, such Iridaceae (*Aristea latifolia* G.J. Lewis), Platanaceae, (*Platanus acerifolia* (Aiton) Willd.) and Urticaceae, (*P. judaica*), in order to compare pollen and nsLTP diversity. A combination of transmission electron microscopy, immunocytochemical techniques, and rabbit specific antiserum against peach nsLTPs were used.

Material and methods

Transmission electron microscopy

Plant materials. – Pollen from mature anthers of *P. judaica* and *P. acerifolia* was collected from plants growing in Barcelona, Spain. Pollen from mature anthers of *A. latifolia* was provided by the Botanical Garden of Cape Town in South Africa. Fresh pollen samples were used without any storage. Observations were made in mature and hydrated pollen grains.

In vitro hydration and activation. – Pollen was placed into a water germination medium (15% sucrose in distilled water, supplemented with 0.35 M sucrose, 300 mg/l CaCl₂ and 100 mg/l boric acid) for 5 and 15 min at 21°C (Suárez-Cervera et al., 2005), after which the process was interrupted and the pollen grains were fixed.

Fixation and embedding: Freezing protocol. – Mature and hydrated pollen grains of *P. acerifolia* and *P. judaica* 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 in a Leica EM CPC (Leica, Vienna, Austria) and stored in liquid nitrogen at –196°C. Ultrathin sections were prepared with an ultracryomicrotome (model Leica EM FCS; Leica, Vienna), collected in 2% methylcellulose in 2.3 M sucrose (Liou et al., 1996) and observed in a Jeol 1010 (Tokyo, Japan) TEM.

Fixation and embedding: Conventional protocol. – Mature and hydrated *A. latifolia*, *P. judaica* and *P. acerifolia* pollen grains were fixed using conventional protocols and infiltrated in Spurr resin (Suárez-Cervera et al., 2003). Ultrathin sections were prepared using a Leica ultramicrotome (Leica UCT, Vienna), followed by OTO staining (osmium tetroxide with osmiophilic thiocarbonylhydrazide) to detect total lipids (Seligman et al., 1966), and then observed in a Jeol 1010 (Tokyo, Japan) TEM.

Purification of peach nsLTPs and production of nsLTP-specific antiserum. – Peach nsLTPs was obtained as described by Pastorello et al. (1999a) with some modifications. Peach peel was used to obtain the nsLTP with Björkstén buffer, and after centrifugation the supernatant was dialyzed in 20 mM phosphate buffer pH 6.0 and subjected to cation-exchange chromatography (High Flow SP 16/10 column; GE-Healthcare, Buckinghamshire, UK). Pooled fractions of the extract were concentrated by ultrafiltration using an Amicon Ultra-4 device with a 5 kDa cut-off (Millipore Co., Bedford, Mass, USA) and then subjected to a gel filtration chromatography (Superdex SX200 16/10 column; GE-Healthcare). Positive fractions were stored at –20°C. Polyclonal antibodies were obtained in New Zealand rabbits by immunising with 5 boosts of 200 µg of purified peach nsLTP every 2 weeks, emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, USA). Sera, collected 10 days after the last injection, was titrated and stored at –80°C. Cross-reactivity of rabbit antiserum against Pru p 3 was detected by immunoblotting analysis in pollen extracts from *Artemisia vulgaris*, *Betula verrucosa*, *Chenopodium album*, *Cynodon dactylon*, *Helianthus annuus*, *Olea europaea*, *Platanus acerifolia*, and *Salsola kali*. On the other hand, no cross-reactivity was found in pollen extracts from *Lolium perenne* and *Phragmites communis*. Allergens were described using the first three letters of the genus, followed by a single letter for the species and an Arabic numeral to indicate the chronological order of allergen purification (Chapman et al., 2007).

Immunogold labelling. – Samples obtained by cryosections were incubated with anti-Pru p 3 polyclonal antibodies in 5% fetal bovine serum (FBS) in phosphate buffered saline (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. G. Posthuma, Utrecht University, 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 performed. Resin embedded sections were incubated at room temperature on drops of 5% bovine serum albumin (BSA) in PBS for 15 min, followed by anti-Pru p 3 antiserum for 2 h. After three washes with drops of 0.25% Tween© 20 (Biosciences inc., La Jolla, California, USA) 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, Wales) 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. Sections were also incubated with pre-immune serum to control the specific labelling of the primary antibody. Other sections were incubated, omitting the primary polyclonal antibody to control the non-specific binding of the colloidal gold-conjugated antibody. They were observed using a JEOL 1010 (Tokyo, Japan) TEM.

Experiments were performed using replicates of three equivalent samples of *A. latifolia*, *P. acerifolia* and *P. judaica*. Three blocks were sectioned and three replicate grids were incubated for each sample with the polyclonal antibodies. Labelling was considered significant when all grains presented the same distribution pattern.

Scanning electron microscopy

Pollen grains of different species were used from the pollen collection of the Botany Laboratory of the Faculty of Pharmacy, University of Barcelona. The samples were prepared by standard acetolysis, followed by ethanol dehydration. 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 (SEM).

Results

Iridaceae, *Aristea latifolia*

The vegetative cytoplasm of the *Aristea latifolia* pollen grains showed abundant lipid inclusions

surrounded by the rough endoplasmic reticulum (RER) which was associated with vacuoles (Figures 2A and 3A, B). The pollenkitt was located on the exine surface and in the infratectal spaces (Figure 2A). When the pollen grains were incubated with anti-Pru p 3 polyclonal antibodies, intense labelling was observed in the tectum and pollenkitt (Figure 2B, C). The labelling was abundant in the vegetative cytoplasm associated with the lipid globules and RER (Figure 3A, B). Scarce gold particles were localised in the vacuoles and dictyosomes (Figure 3B). The generative cell was situated at the centre of the pollen grain, showing a lobe-shaped contour (Figure 4A). The labelling was observed forming clusters in the lobules, near the fibrillar vesicles. (Figure 4B). In the central part of the generative cell, the gold particles were mainly associated with the microtubules and small and large fibrillar vesicles (Figure 4C). No significant labelling was observed in the controls.

Platanaceae, *Platanus acerifolia*

Platanus acerifolia pollen grains also showed numerous lipid inclusions distributed throughout the vegetative cytoplasm and bordering the plasma membrane (Figure 5A). The exine labelling which localised peach nsLTP-like proteins at the aperture level was significant in the ectexine and endexine (Figure 5B). In the vegetative cytoplasm, the labelling was scarce and specifically associated with the lipid inclusions, RER and dictyosomes (Figure 5C–F). No significant labelling was observed in the controls.

Urticaceae, *Parietaria judaica*

The cytoplasm of the *Parietaria judaica* vegetative cell was characterised by large starch granules and numerous lipid inclusions (Figure 6A). These lipids were mostly associated with vacuoles (Figure 6B). After incubation with anti-Pru p 3 serum, the labelling was very scarce. Rare gold particles were located in the cytoplasm (Figure 6C, D). No significant labelling was observed in the controls.

Discussion

Immunocytochemical considerations

The use of both conventional and freezing protocols allowed us to interpret the association of nsLTPs with the lipid inclusions and the pollenkitt. Despite the fact that the preservation of lipids was better with conventional techniques, we also used freezing protocols for the immunocytochemical localisation. Unexpectedly, the labelling was comparable in both

protocols. Our results showed that the nsLTPs preserve their antigenicity in spite of the strong chemical fixation of the pollen and its inclusion in Spurr resin. NsLTP preservation is a consequence of the molecular characteristics of nsLTPs, which are resistant to heat treatment and proteolytic digestion (Asero et al., 2000; Zuidmeer & van Ree, 2007). This would explain the preservation of the nsLTP antigenicity shown in the immunocytochemical studies carried out with Spurr resin in *P. judaica* (Vega-Maray et al., 2004).

Pollen vs nsLTP diversity

The intense labelling observed in *A. latifolia* pollen grains showed a high nsLTP concurrence between the Rosaceae and Iridaceae families. Although Iridaceae nsLTPs have not been studied, the nsLTP, Zea m 14, was localised in other monocotyledons, such as *Zea mays*, (Pastorello et al., 2003). The nsLTP of *P. acerifolia*, Pla a 3, is clearly implicated in plant food and pollen sensitisation and shows an amino acid sequence identity of 58.3% with Pru p 3 (Enrique et al., 2002; Lauer et al., 2007). This is in agreement with the moderate labelling obtained in *P. acerifolia* pollen grains. It is interesting to point out that this labelling was less intense than that obtained with Pla a 1 and Pla a 2, the major allergens of *P. acerifolia* pollen grains (Suárez-Cervera et al., 2005). Regarding *P. judaica* pollen grains, their major allergens, Par j 1 and Par j 2, represent another type of nsLTP member (Ayuso et al., 1995; Costa et al., 1994; Asturias et al., 2003). Although the sequential positions for IgE epitopes of

Par j 1 and Par j 2 and Pru p 3 show similarities of 26–29%, both allergens have strong sequence divergence in most of their regions (Salcedo et al., 2004, 2007). Moreover, undetectable cross-reactivity between Pru p 3 and Par j 1 and Par j 2 was found in allergic patients (Lombardero et al., 2004). This sequence divergence also explained why no significant labelling was observed in *P. judaica* pollen grains incubated with anti-Pru p 3 polyclonal antibodies. On the other hand, in the Urticaceae family, no significant labelling was found when the anti-Par j 1 serum was incubated with *Urtica dioica* pollen grains (Vega-Maray et al., 2006), which indicates the absence of cross-reactivity between the antigens of two related genera, *Parietaria* and *Urtica*. The absence of cross-reactivity between these two genera was previously revealed in a study with patients from the Mediterranean area (Bousquet et al., 1986).

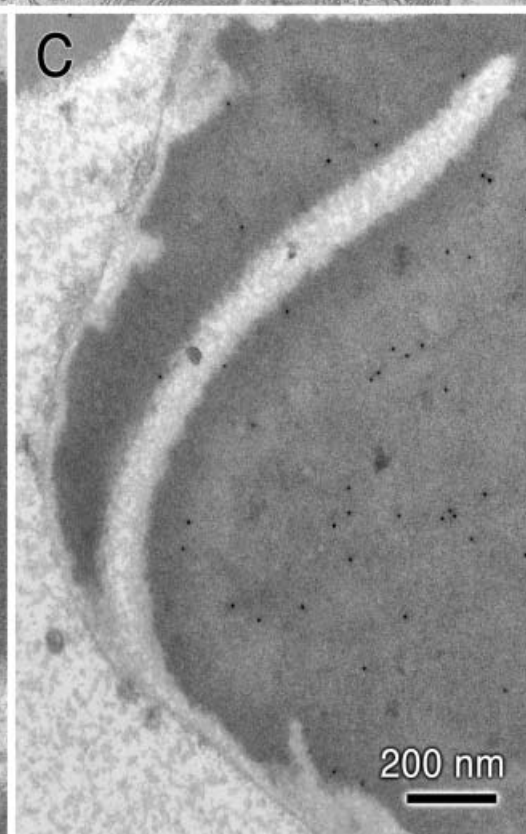
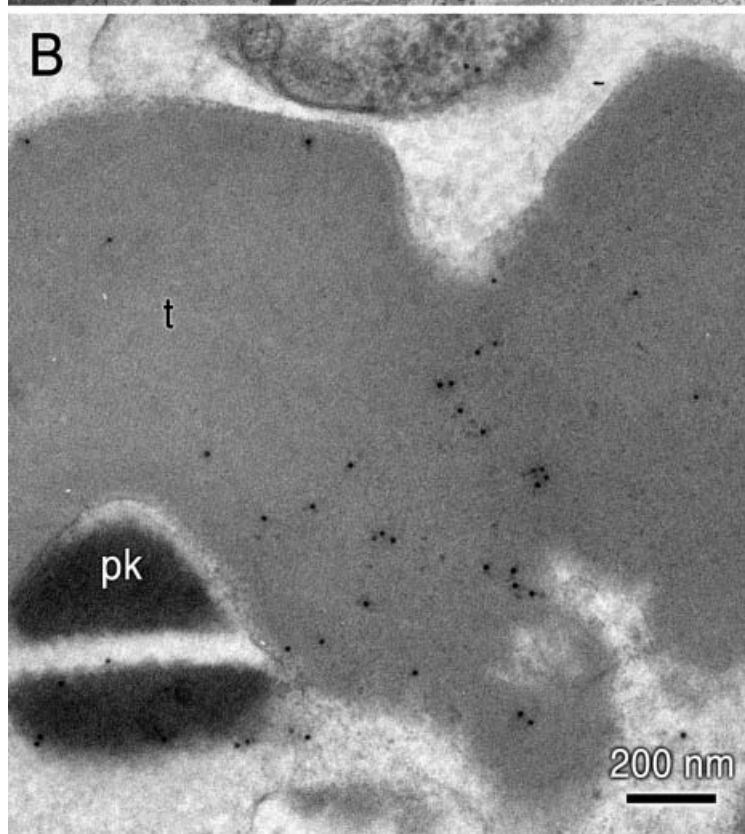
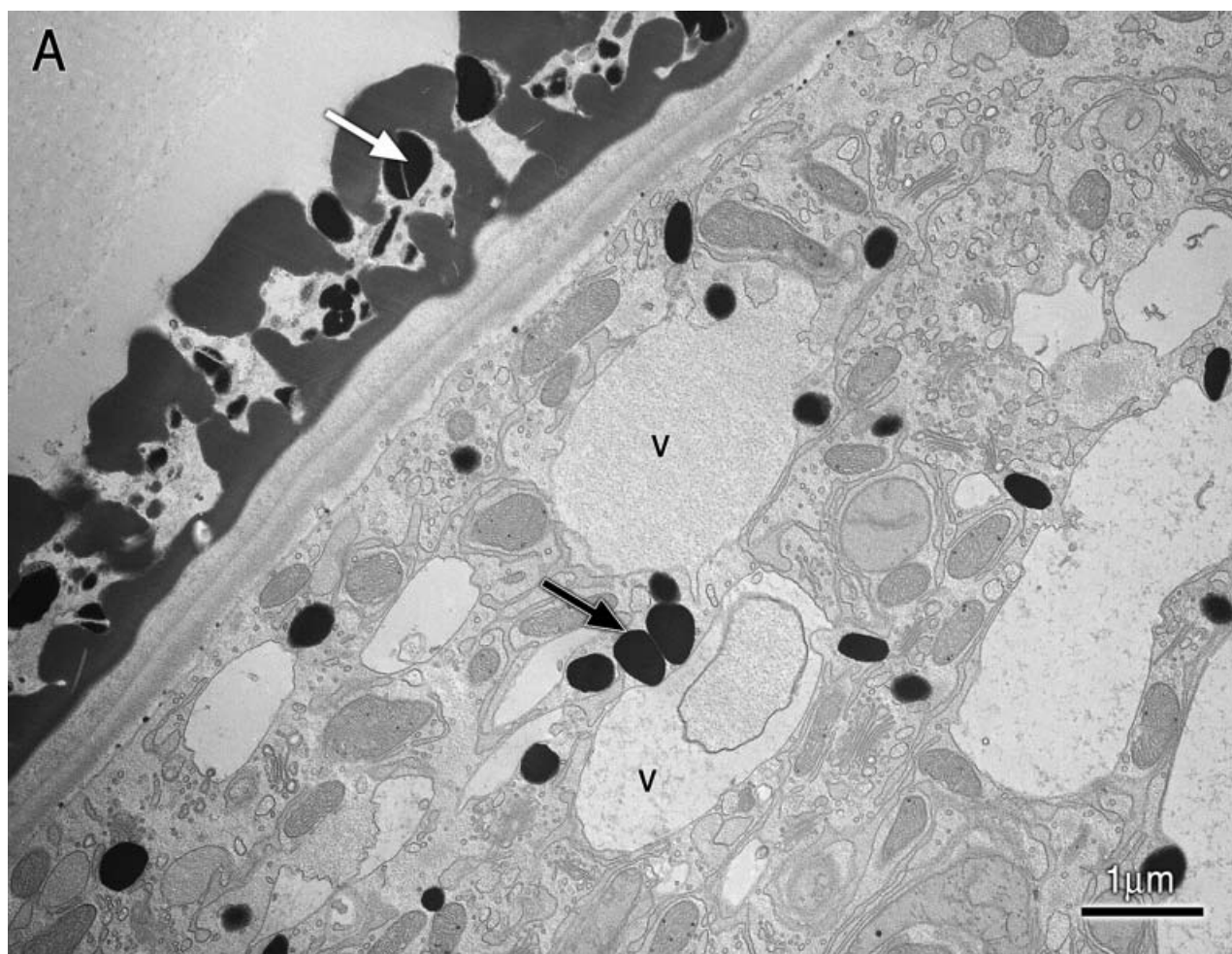
The variability of nsLTPs was also shown in the Corylaceae hazelnut nsLTP, Cor a 8, which had poor cross-reactivity with Rosaceae fruit nsLTPs (Flinterman et al., 2008). On the contrary, *Artemisia vulgaris* pollen grains have cross-reactivity with nsLTPs of Rosaceae fruits (Díaz-Perales et al., 2000; Lombardero et al., 2004). Concerning conifers, antibodies against peach nsLTPs recognised the epitopes of the nsLTPs of *Cupressus arizonica* pollen grains (Suárez-Cervera et al., 2008a). While an important number of plant families show inter-relationships between the nsLTPs of plant food and pollen grains, it is not currently possible to establish a good taxonomical correlation between pollen from different plant families and their nsLTPs.

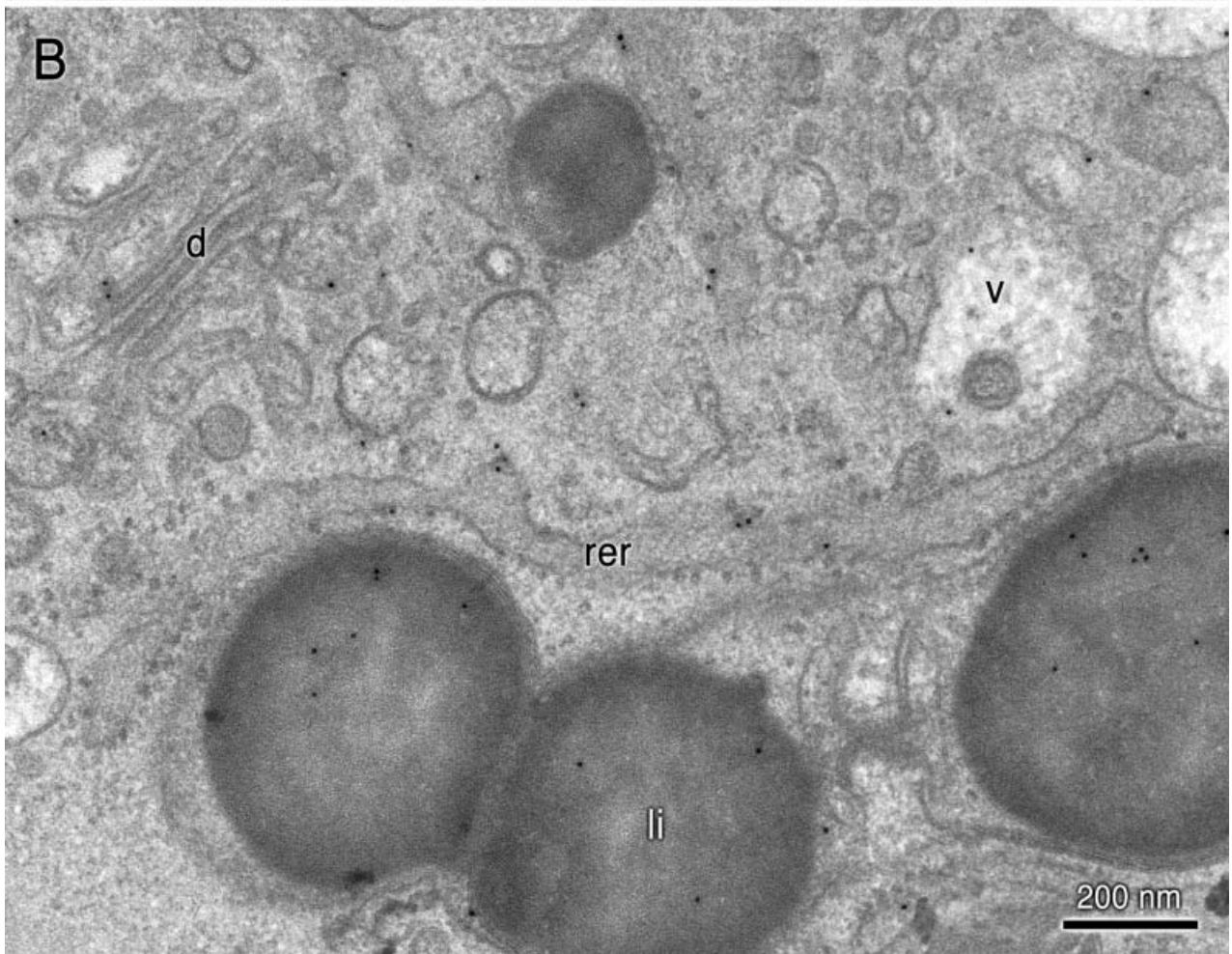
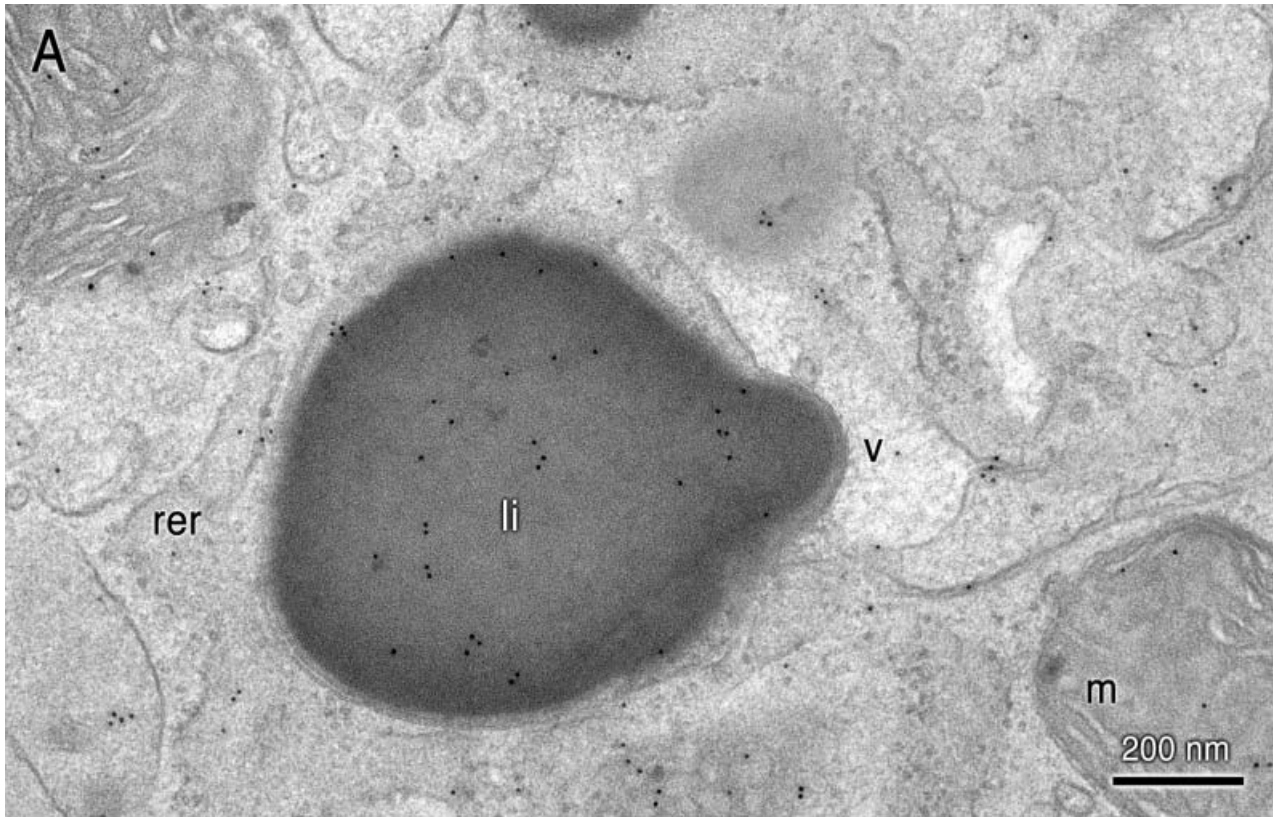
Figure 2. TEM sections of *Aristea latifolia* pollen grains. Vegetative cell. **A**. Cytoplasm with abundant electrondense lipid inclusions (black arrow) near the vacuoles (v). Note the electrondense pollenkit in the exine (white arrow). OTO staining. **B**, **C**. Sections incubated with anti-Pru p 3 polyclonal antibodies. **B**. Gold particles are abundant in the tectum (t) and in the pollenkit (pk). **C**. An enlarged pollenkit droplet showing abundant labelling. Conventional protocol fixation and Spurr resin inclusion.

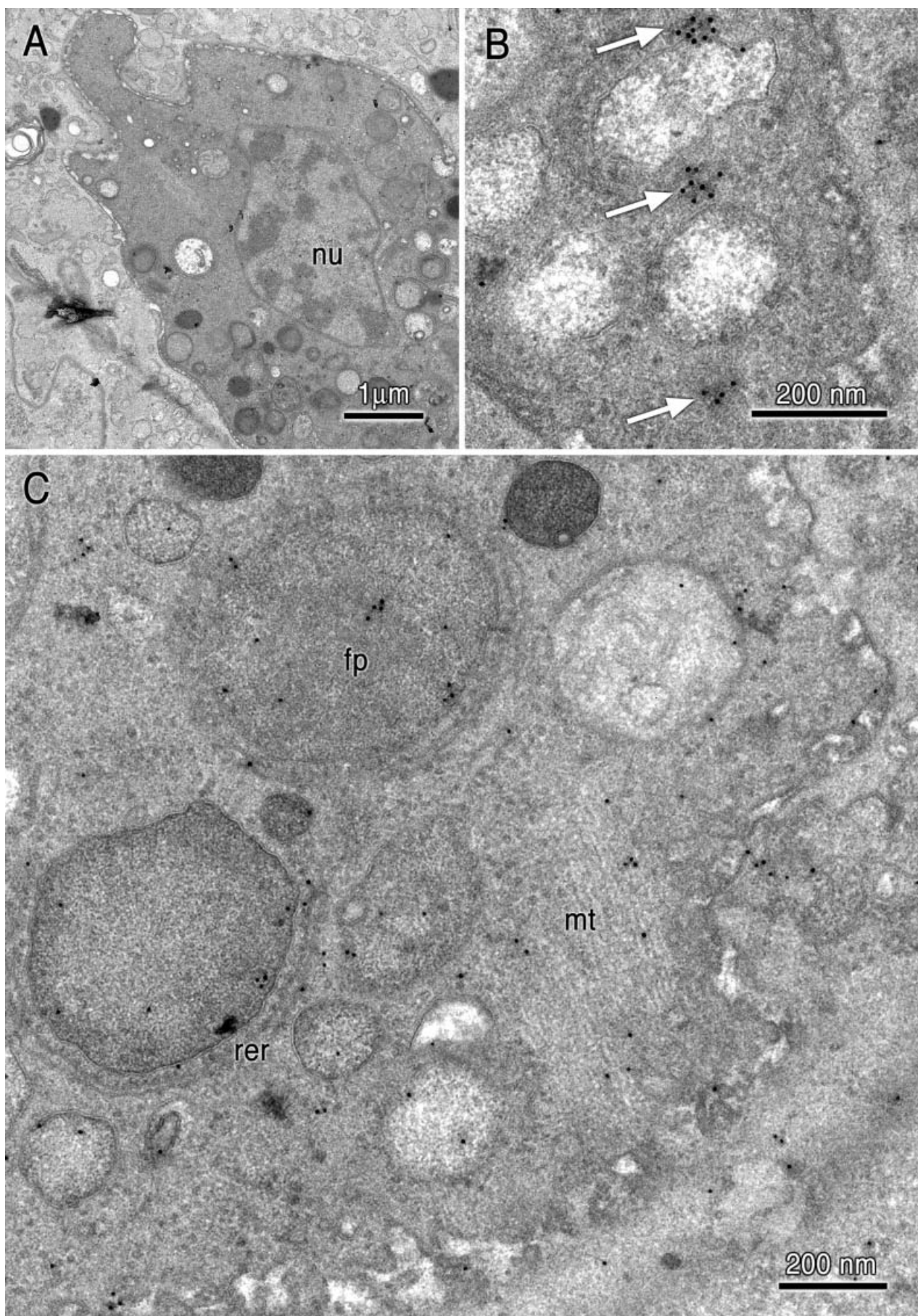
Figure 3. **A**, **B**. TEM sections of *Aristea latifolia* pollen grains. Vegetative cell. Detection of nsLTPs by incubation with anti-Pru p 3 polyclonal antibodies. Extensive labelling in the lipid inclusions (li) and in the rough endoplasmic reticulum (rer). Scarce gold particles are associated with the dictyosomes (d), vacuoles (v) and mitochondria (m). Conventional protocol fixation and Spurr resin inclusion.

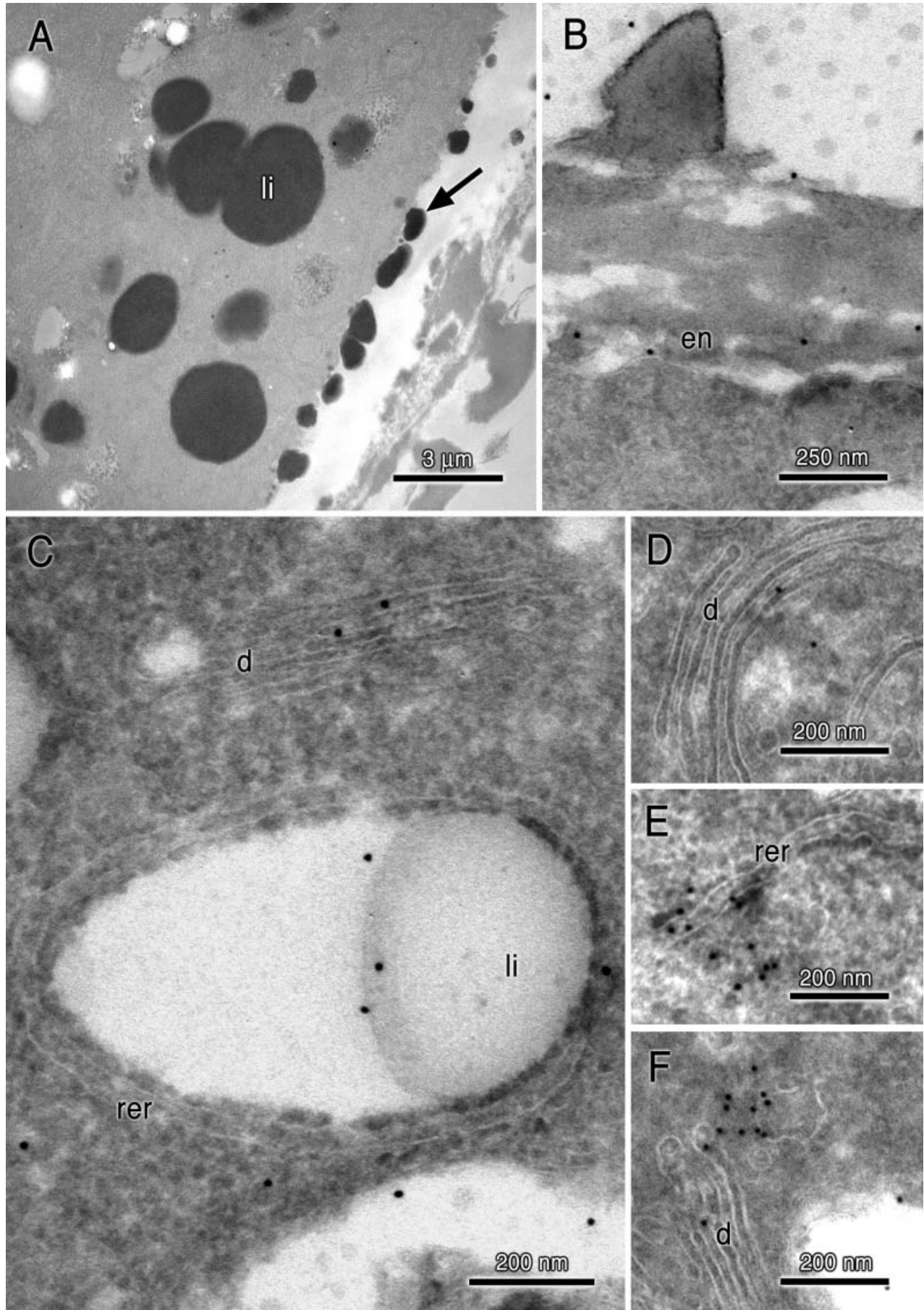
Figure 4. **A–C**. TEM sections of *Aristea latifolia* pollen grains. Generative cell. Detection of nsLTPs by incubation with anti-Pru p 3 polyclonal antibodies. **A**. Generative cell showing the lobular contour, numerous organelles and the nucleus (nu). **B**. Note the labelling forming clusters in the lobular area and near the fibrillar vesicles (arrows). **C**. Intense labelling in the fibrillar particles (fp), surrounded by the rough endoplasmic reticulum (rer). The gold particles are mostly associated with microtubules (mt). Conventional protocol fixation and Spurr resin inclusion.

Figure 5. TEM sections of *Platanus acerifolia* pollen grains. Vegetative cell. **A**. Stained lipid inclusions (li) are abundant in the cytoplasm and bordering the plasma membrane (arrow). OTO staining. **B–F**. Detection of nsLTPs by incubation with anti-Pru p 3 polyclonal antibodies. **B**. Scarce labelling specifically associated with the apertural endexine (en). **C–F**. Gold particles in lipid inclusions (li), rough endoplasmic reticulum (rer) and dictyosomes (d). **A**. Conventional protocol. **B–F**. Freezing protocol.









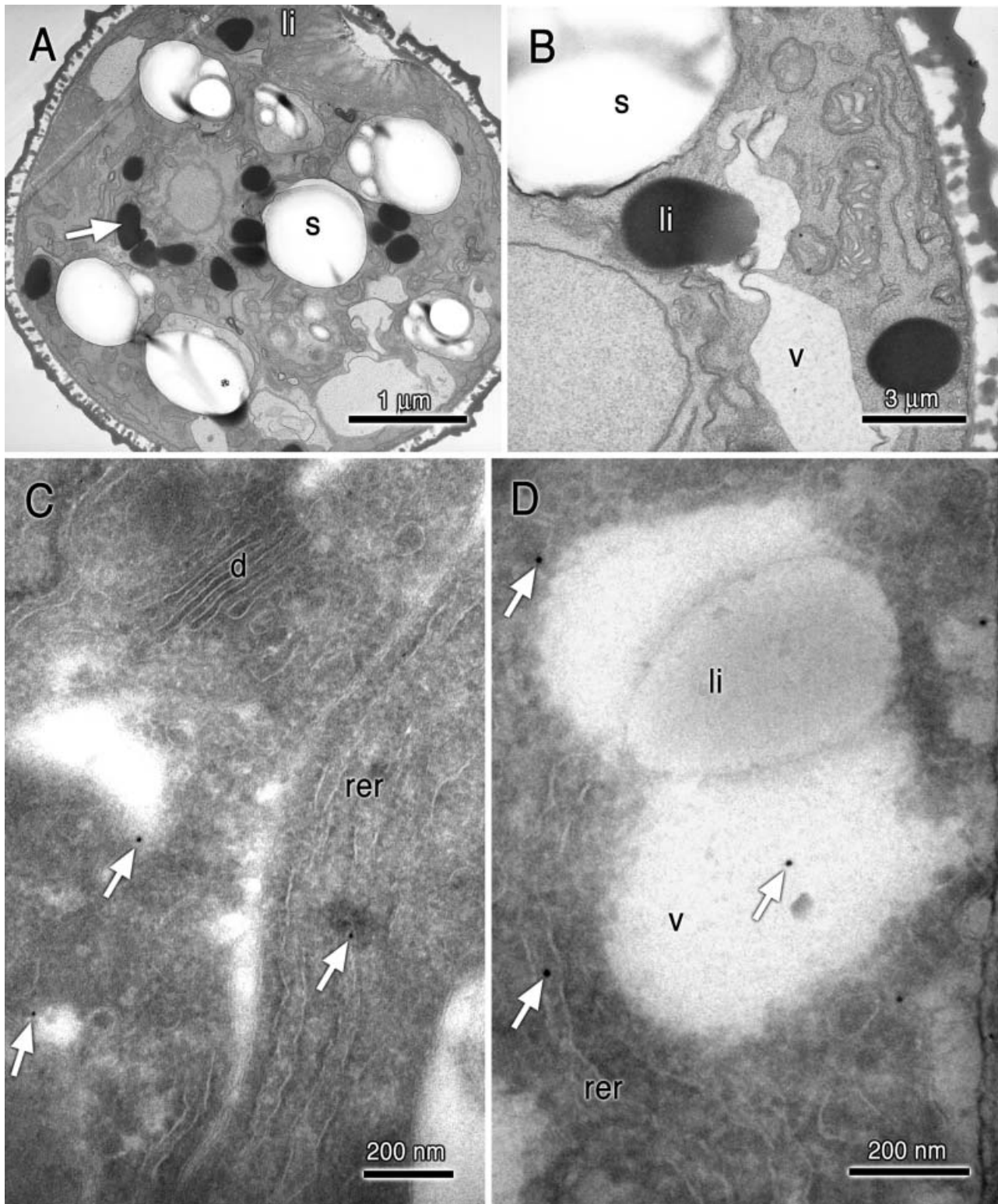


Figure 6. TEM sections of *Parietaria judaica* pollen grains. Vegetative cell. **A**. Stained lipid inclusions (arrow) in the vegetative cytoplasm. **B**. Detail of a lipid inclusion (li) associated with vacuoles (v). Starch granules (s). OTO staining. **C**, **D**. Detection of nsLTPs by incubation with anti-Pru p 3 polyclonal antibodies. Rare gold particles (arrows) were localised in the rough endoplasmic reticulum (rer), vacuoles (v) and lipid inclusions (li). No significant labelling was observed in the dictyosomes (d). **A**, **B**. Conventional protocol. **C**, **D**. Freezing protocol.

The role of nsLTPs

NsLTPs are synthesised as precursors: they enter the secretory pathway and are secreted outside the cell walls as mature polypeptides (Kader, 1996). Evidence shows that the role of nsLTPs is mainly related to the defence mechanism of plants against external attacks (Zuidmeer & van Ree, 2007). The presence of nsLTPs in the *A. latifolia* pollenkitt indicates that nsLTPs form part of the chemical composition of the pollenkitt or they are involved in the traffic of defence proteins. The pollenkitt is a hydrophobic mixture of materials, composed primarily of saturated and unsaturated lipids, carotenoids (Dobson, 1988; Hesse, 1993) and proteins and carbohydrates (Piffanelli & Murphy, 1998; Santos et al., 2003). The oleo-pollenin is considered the major protein component of the pollenkitt (Murphy & Ross, 1998; Murphy, 2001). LTP genes are specifically expressed in the tapetum and developing microspores of *Brassica napus*, *Nicotiana tabacum*, *Lilium henryi* and *Zea mays* (Koltunow et al., 1990; Foster et al., 1992; Crossley et al., 1995; Lauga et al., 2000). Therefore, the detection of nsLTPs in the *A. latifolia* pollenkitt is an expected result, because one of the many functions attributed to the pollenkitt is to protect the pollen protoplast from fungi and bacteria (Paccini & Hesse, 2005), and the localisation of nsLTPs in the pollenkitt confirms this hypothesis.

In addition, nsLTPs have a potential role in plant growth and development (Kader, 1996), including pollen germination (Vega-Maray et al., 2004) and pollen-pistil interaction (Park et al., 2000). In plants, cytoplasm lipid bodies were detected in almost all cell types, including anthers and pollen grains (Murphy, 2001). In the different pollen grains studied here, the lipid inclusions observed were associated with nsLTPs, suggesting a role in the intracellular metabolism of lipids. Recently, an LTP has been caught in the act of transferring lipids *in vivo* from a donor membrane to an acceptor membrane (Halter et al., 2007; D'Angelo et al., 2007). These studies confirmed that LTPs do sometimes transfer lipids and showed different pathways of lipid traffic (Levine, 2007). On the other hand, the localisation of nsLTPs associated with the RER and Golgi complex in *Aristea* and *Platanus* showed that the pollen nsLTP role is most likely similar to that of other cell tissues (Hernandez-Pinzon et al., 2001; De Matteis et al., 2007).

NsLTPs in plant food and pollen grains

The large clinical evidence accumulated has defined nsLTPs as a relevant panallergen family of both plant foods and pollen grains (Breiteneder

& Mills, 2005). Thaumatin, other plant defence proteins, have been found that are more intensely expressed in response to environmental attacks (Cortegano et al., 2004; Suárez-Cervera et al., 2008b). The effect of air-pollution on pollen grains as well the effects of diverse treatments and other environmental aggressions on fruits explain the increased expression of nsLTPs, which can affect the health of the inhabitants in developed countries (Salcedo et al., 2007).

Conclusions

Pollen and plant food allergens show distinct patterns of distribution in different species. No taxonomical relationship between pollen grains and nsLTPs could be established. This should be kept in mind regarding the cross-reactivity processes and the allergen extract preparation in terms of botanical groupings. The localisation of nsLTPs in the pollenkitt provides evidence that they are involved in the traffic of plant defence proteins. The proposed role of LTPs in the lipid traffic pathways for other cellular tissues could be applied to the living cells of pollen grains.

Acknowledgements

The authors are grateful to the Scientific Technical Services of the University of Barcelona for their careful preparation of samples for TEM and SEM. The authors also thank T. Grace Johnson for her valuable help in reviewing the English text. This study was supported by grant CGL2006-15103-C04-01, Department of Science and Technology, Spain.

References

- APG [Angiosperm Phylogeny Group]. (2003). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Bot. J. Linn. Soc.*, 141, 399–436.
- Asero, R., Mistrello, G., Roncarolo, D., de Vries, S. C., Gautier, M. F., Ciurana, C. L. F., Verbeek, E., Mohammadi, T., Knul-Brettlova, V., Akkerdaas, J. H., Bulder, I., Aalberse, R. C. & van Ree, R. (2000). Lipid transfer protein: A pan-allergen in plant derived foods that is highly resistant to pepsin digestion. *Int. Arch. Allergy Immunol.*, 122, 20–32.
- Asturias, J. A., Gómez-Bayón, N., Eseverri, J. L. & Martínez, A. (2003). Par j 1 and Par j 2, the major allergens from *Parietaria judaica* pollen have similar immunoglobulin E epitopes. *Clin. Exp. Allergy*, 33, 518–524.
- Ayuso, R., Carreira, J. & Polo, F. (1995). Quantization of the major allergen of several *Parietaria* pollens by anti Par 1 monoclonal antibody-based ELISA. Analysis of cross-reactivity among purified Par j 1, Par o 1 and Par m 1 allergens. *Clin. Exp. Allergy*, 25, 993–999.

- Blackmore, S. (2007). Pollen and spores: Microscopic keys to understanding the earth's biodiversity. *Plant Syst. Evol.*, 263, 3–12.
- Bousquet, J., Hewitt, B., Guerin, B., Dhivert, H. & Michel, F. B. (1986). Allergy in the Mediterranean area II: Cross-allergenicity among Urticaceae pollens (*Parietaria* and *Urtica*). *Clin. Allergy*, 16, 57–64.
- Breiteneder, H. & Mills, C. (2005). Nonspecific lipid-transfer proteins in plant foods and pollens: An important allergen class. *Curr. Opin. Allergy Clin. Immunol.*, 5, 275–279.
- Chapman, M., Pomés, A., Breiteneder, H. & Ferreira, F. (2007). Nomenclature and structural biology of allergens. *J. Allergy Clin. Immunol.*, 119, 414–420.
- Colombo, P., Bonura, A., Costa, M. A., Izzo, V., Passantino, R., Locorotondo, G., Amoroso, S. & Geraci, D. (2003). The allergens of *Parietaria*. *Int. Arch. Allergy Immunol.*, 130, 173–179.
- Cortegano, I., Civantos, E., Aceituno, E., Moral, A., López, E., Lombardero, M., del Pozo, V. & Lahoz, C. (2004). Cloning and expression of a major allergen from *Cupressus arizonica* pollen, Cup a 3, a PR-5 protein expressed under polluted environment. *Allergy*, 59, 485–490.
- Costa, M. A., Colombo, P., Izzo, V., Kennedy, H., Venturella, S., Cocchiara, R., Mistrello, G., Falagiani, P. & Geraci, D. (1994). cDNA cloning, expression and primary structure of Par j 1, a major allergen of *Parietaria judaica* pollen. *FEBS Lett.*, 341, 182–186.
- Crossley, S. J., Greenland, A. J. & Dickinson, H. G. (1995). The characterisation of tapetum-specific cDNAs isolated from a *Lilium henryi* L. meiocyte subtractive cDNA library. *Planta*, 196, 523–529.
- D'Angelo, G., Polishchuk, E., Tullio, G. D., Santoro, M., Campli, A. D., Godi, A., West, G., Bielawski, J., Chuang, C. C., van der Spoel, A. C., Platt, F. M., Hannun, Y. A., Polishchuk, R., Mattjus, P. & De Matteis, M. A. (2007). Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. *Nature*, 449, 62–67.
- De Matteis, M. A., Di Campli, A. & D'Angelo, G. (2007). Lipid-transfer proteins in membrane trafficking at the Golgi complex. *Biochim. Biophys. Acta*, 1771, 761–768.
- Díaz-Perales, A., Lombardero, M., Sánchez-Monge, R., García-Sellés, F. J., Pernas, M., Fernández-Rivas, M., Barber, D. & Salcedo, G. (2000). Lipid-transfer proteins as potential plant panallergens: Cross-reactivity among proteins of *Artemisia pollen*, *Castanea* nut and Rosaceae fruits, with different IgE-binding capacities. *Clin. Exp. Allergy*, 30, 1403–1410.
- Dobson, H. E. M. (1988). Survey of pollen and pollenkit lipids – chemical cues to flower visitors? *Am. J. Bot.*, 75, 170–182.
- Doyle, J. A. (2005). Early evolution of angiosperm pollen as inferred from molecular and morphological phylogenetic analyses. *Grana*, 44, 227–251.
- Enrique, E., Cisteró-Bahima, A., Bartolomé, B., Alonso, R., San Miguel-Moncin, M., Bartra, J. & Martínez, A. (2002). *Platanus acerifolia* pollinosis and food allergy. *Allergy*, 57, 351–356.
- Fernández-Rivas, M., González-Mancebo, E., Rodríguez-Pérez, R., Benito, C., Sánchez-Monje, R., Salcedo, G., Alonso, D., Rosado, A., Tejedor, M. A., Vila, C. & Casas, L. (2003). Clinically relevant peach allergy is related to peach lipid transfer protein, Pru p 3, in the Spanish population. *J. Allergy Clin. Immunol.*, 112, 789–795.
- Flinterman, A. E., Akkerdaas, J. H., den Hartog Jager, C. F., Rigby, N. M., Fernandez-Rivas, M., Hoekstra, M. O., Bruijnzeel-Koomen, C. A., Knulst, A. C., van Ree, R. & Pasmans, S. G. (2008). Lipid transfer protein-linked hazelnut allergy in children from a non-Mediterranean birch-endemic area. *J. Allergy Clin. Immunol.*, 121, 423–428.
- Foster, G. D., Robinson, S. W., Blundell, R. P., Roberts, M. R., Hodge, R., Draper, J. & Scott, R. (1992). A *Brassica napus* mRNA encoding a protein homologous to phospholipid transfer proteins is expressed specifically in the tapetum and developing microspores. *Plant Sci.*, 84, 187–192.
- Halter, D., Neumann, S., van Dijk, S. M., Wolthoorn, J., de Maziere, A. M., Vieira, O. V., Mattjus, P., Klumperman, J., van Meer, G. & Sprong, H. (2007). Pre and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. *J. Cell Biol.*, 179, 101–115.
- Hernandez-Pinzon, I., Patel, K. & Murphy, D. J. (2001). The *Brassica napus* calcium-binding protein, caleosin, has distinct endoplasmic reticulum- and lipid body-associated isoforms. *Plant Physiol. Biochem.*, 39, 615–622.
- Hesse, M. (1993). Pollenkitt development and composition in *Tilia platyphyllos* (Tiliaceae) analysed by conventional and energy filtering TEM. *Plant Syst. Evol.*, 7, 39–52.
- Hoffmann-Sommergruber, K. (2000). Plant allergens and pathogenesis-related proteins: What do they have in common? *Int. Arch. Allergy Immunol.*, 122, 155–166.
- Kader, J. C. (1996). Lipid transfer proteins in plants. *Plant Mol. Biol.*, 47, 627–654.
- Koltunow, A. M., Truettner, J., Cox, K. H., Wallroth, M. & Goldberg, R. B. (1990). Different temporal and spatial gene expression patterns occur during anther development. *Plant Cell*, 2, 1201–1224.
- Lauer, I., Miguel-Moncin, M. S., Abel, T., Foetisch, K., Hartz, C., Fortunato, D., Cistero-Bahima, A., Vieths, S. & Scheurer, S. (2007). Identification of a plane pollen lipid transfer protein (Pla a 3) and its immunological relation to the peach lipid-transfer protein, Pru p 3. *Clin. Exp. Allergy*, 37, 261–269.
- Lauga, B., Charbonnel-Campaa, L. & Combes, D. (2000). Characterization of MZm3- 3, a *Zea mays* tapetum-specific transcript. *Plant Sci.*, 157, 65–75.
- Levine, T. P. (2007). A lipid transfer protein that transfers lipid. *J. Cell Biol.*, 179, 11–13.
- Liou, W., Geuze, H. J. & Slot, J. W. (1996). Improving structural integrity of cryosections for immunogold labelling. *Histochem. Cell Biol.*, 106, 41–58.
- Lombardero, M., García-Sellés, F. J., Polo, F., Chamorro, M. J., García-Casado, G., Sánchez-Monge, R., Díaz-Perales, A. & Barber, A. (2004). Prevalence of sensitization to *Artemisia* allergens Art v 1, Art v 3 and Art v 60 kDa. Cross-reactivity among Art v 3 and other relevant lipid-transfer protein allergens. *Clin. Exp. Allergy*, 34, 1415–1421.
- Mills, E. N. C., Jenkins, J. A., Alcocer, M. J. C. & Shewry, P. R. (2004). Structural, biological, and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Crit. Rev. Food Sci. Nutr.*, 44, 379–407.
- Murphy, D. J. (2001). The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog. Lipid Res.*, 40, 325–438.
- Murphy, D. J. & Ross, J. H. E. (1998). Biosynthesis, targeting and processing of oleosin-like proteins, which are major pollen coat components in *Brassica napus*. *Plant J.*, 13, 1–16.
- Pacini, E. & Hesse, M. (2005). Pollenkitt – its composition, forms and functions. *Flora*, 200, 399–415.
- Park, S. Y., Jauh, G. Y., Mollet, J. C., Eckard, K. J., Nothnagel, E. A., Walling, L. L. & Lord, E. M. (2000). A lipid transfer like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix. *Plant Cell*, 12, 151–163.
- Pastorello, E. A., Farioli, L., Pravettoni, V., Ortolani, C., Ispano, M., Monza, M., Baroglio, C., Scibola, E., Ansaloni, R., Incorvaia, C. & Conti, A. (1999a). The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J. Allergy Clin. Immunol.*, 103, 520–526.

- Pastorello, E. A., Pravettoni, V., Farioli, L., Ispano, M., Fortunato, D., Monza, M., Giuffrida, M. G., Rivolta, F., Scibola, E., Ansaloni, R., Incorvaia, C., Conti, A. & Ortolani, C. (1999b). Clinical role of a lipid transfer protein that acts as a new apple-specific allergen. *J. Allergy Clin. Immunol.*, **104**, 1099–1106.
- Pastorello, E. A., Pompei, C., Pravettoni, V., Farioli, L., Calamari, A. M., Scibilia, J., Robino, A. M., Conti, A., Iametti, S., Fortunato, D., Bonomi, S. & Ortolani, C. (2003). Lipid-transfer protein is the major maize allergen maintaining IgE-binding activity after cooking at 100°C, as demonstrated in anaphylactic patients and patients with positive double-blind, placebo-controlled food challenge results. *J. Allergy Clin. Immunol.*, **112**, 775–783.
- Piffanelli, P. & Murphy, D. J. (1998). Novel organelles and targeting mechanisms in the anther tapetum. *Trends in Plant Sci.*, **3**, 250–253.
- Radauer, C. & Breiteneder, H. (2006). Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. *J. Allergy Clin. Immunol.*, **117**, 141–147.
- Raposo, G., Kleijmeer, M. J., Posthuma, G., Slot, J. W. & Geuze, H. J. (1997). Immunolabelling of ultrathin cryosections: Application in immunology. In L. A. Herzenberg, D. Weir & C. Blackwell (Eds), *Handbook of experimental immunology*, vol. 4 (pp. 1–11). Cambridge: Blackwell Sci. Inc.
- Salcedo, G., Sánchez-Monge, R., Díaz-Perales, A., García-Casado, G. & Barber, D. (2004). Plant non-specific lipid transfer proteins as food and pollen allergens. *Clin. Exp. Allergy*, **34**, 1336–1341.
- Salcedo, G., Sánchez-Monge, R., Barber, D. & Díaz-Perales, A. (2007). Plant non-specific lipid transfer proteins: An interface between plant defence and human allergy. *Biochim. Biophys. Acta*, **1771**, 781–791.
- Sánchez-Monge, R., Lombardero, M., García-Sellés, F. J., Barber, D. & Salcedo, G. (1999). Lipid-transfer proteins are relevant allergens in fruit allergy. *J. Allergy Clin. Immunol.*, **103**, 514–519.
- Santos, R. P., Mariath, J. E. A. & Hesse, M. (2003). Pollenkitt formation in *Ilex paraguariensis* A.St.Hil. (Aquifoliaceae). *Plant Syst. Evol.*, **237**, 185–198.
- Scheurer, S., Pastorello, E. A., Wangorsch, A., Kastner, M., Hausteiner, D. & Vieths, S. (2001). Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the in vitro diagnosis of cherry allergy. *J. Allergy Clin. Immunol.*, **107**, 724–731.
- Schocker, F., Luttkopf, D., Scheurer, S., Petersen, A., Cisteró-Bahima, A., Enrique, E., Miguel-Moncin, M. S., Akkerdaas, J., van Ree, R., Vieths, S. & Becker, W.-M. (2004). Recombinant lipid transfer protein Cor a 8 from hazelnut: A new tool for in vitro diagnosis of potentially severe hazelnut allergy. *J. Allergy Clin. Immunol.*, **113**, 141–147.
- Seligman, A. M., Wasserkug, D. H. & Hanker, J. S. (1966). A new staining method (OTO) for enhancing contrast of lipid containing membranes and droplets in osmium tetroxide fixed tissue with osmiophilic thiocarbonylhydrazide (TCH). *J. Cell Biol.*, **30**, 424–432.
- Suárez-Cervera, M., Takahashi, Y., Vega-Maray, A. & Seoane-Camba, J. A. (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.
- Suárez-Cervera, M., Asturias, J. A., Vega-Maray, A., Castells, T., López-Iglesias, C., Ibarrola, I., Arilla, M. C., Gabarayeva, N. & Seoane-Camba, J. A. (2005). The role of allergenic proteins Pla a 1 and Pla a 2 in the germination of *Platanus acerifolia* pollen grains. *Sex. Plant Reprod.*, **18**, 101–112.
- Suárez-Cervera, M., Vega-Maray, A., Asturias, J. A., Castells, T., Rodríguez-Rajo, F. J., Moreno-Grau, S., Fernández-González, D., Jato, V. & Seoane-Camba, J. A. (2008a). Immunocytochemical localization of Pru p 3, a lipid transfer protein, in *Cupressus arizonica* pollen grains. In Org. Comm. (Eds), *27th Congr. Eur. Acad. Allergol. Clin. Immunol. Barcelona 2008. Abstracts Vol.* (No 1820, p. 655). *Allergy*, **63**, Sp. Issue.
- Suárez-Cervera, M., Castells, T., Vega-Maray, A., Civantos, E., del Pozo, V., Fernández-González, D., Moreno-Grau, S., Moral, A., López-Iglesias, C., Lahoz, C. & Seoane-Camba, J. A. (2008b). Effects of air pollution on Cup a 3 allergen in *Cupressus arizonica* pollen grains. *Ann. Allergy Asthma Immunol.*, **101**, 57–66.
- Vega-Maray, A., Fernández-González, D., Valencia-Barrera, R. & Suárez-Cervera, M. (2006). Allergenic proteins in *Urtica dioica*, a member of the Urticaceae allergenic family. *Ann. Allergy Asthma Immunol.*, **97**, 343–349.
- Vega-Maray, A. M., Fernández-González, D., Valencia-Barrera, R. M., Seoane-Camba, J. A. & Suárez-Cervera, M. (2004). Lipid transfer proteins in *Parietaria judaica* L. pollen grains: Immunocytochemical localization and function. *Eur. J. Cell Biol.*, **83**, 493–497.
- Weber, R. W. (2007). Cross-reactivity of pollen allergens: Impact on allergen immunotherapy. *Ann. Allergy Asthma Immunol.*, **99**, 203–212.
- Zuidmeer, L. & van Ree, R. (2007). Lipid transfer protein allergy: Primary food allergy or pollen/food syndrome in some cases. *Curr. Opin. Allergy Clin. Immunol.*, **7**, 269–273.