

Localization of the two major allergens in rye-grass pollen using specific monoclonal antibodies and quantitative analysis of immunogold labelling

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Summary

The intracellular localization of the two major allergens, *Lol p I* and *Lol p IX*, in rye-grass anthers was examined using monoclonal antibodies FMCA1 (specific for *Lol p I*) and FMCA7 (specific for *Lol p IX*) with immunocytochemical techniques and quantitative analysis. A newly developed anhydrous fixation technique in a mixture of glutaraldehyde, paraformaldehyde and 2,2-dimethoxypropane followed by embedding in LR Gold resin resulted in both improved infiltration of pollen grains compared with existing techniques and the localization of these water-soluble antigens in their original sites compared with diffusion artefacts following aqueous methods. After anhydrous fixation, *Lol p I* was predominantly located in the electron-opaque regions of the cytosol of the vegetative cell of the tricellular pollen grains (24 counts μm^{-2}), whereas *Lol p IX* was detected mainly within starch granules (16 counts μm^{-2}). For both *Lol p I* and *Lol p IX*, similar labelling was detected in the cells of the endothecium and middle layer (18 counts μm^{-2}), but none was found in the tapetal cells or orbicules.

Introduction

Rye-grass pollen antigens are among the most reactive allergens in eliciting hay fever and allergic asthma (Freidhoff *et al.*, 1986; Ford & Baldo, 1986). The most clinically significant allergens of rye-grass are *Lol p I* and the newly identified *Lol p IX* (previously designated *Lol p Ib*, Singh *et al.*, 1991). *Lol p I* is an abundant acidic glycoprotein of M_r 35 kDa and pI 5.5 whereas *Lol p IX* is a 31 kDa protein with a pI of 9.0 (Griffith *et al.*, 1991; Singh *et al.*, 1991).

Plant tissues such as pollen grains, which must leave the parent plant upon maturity, are in a dormant and often dehydrated state. The low water content gives pollen grains a high osmotic value and they react rapidly when in contact with a moist environment; they swell and may even rupture. Allergens diffuse away from the pollen grain within seconds of aqueous contact (Howlett *et al.*, 1979) and thus it is a challenge to maintain allergens in their cellular sites. Of fundamental importance in understanding pollen allergenicity is the question as to whether allergens are secreted from the pollen cytoplasm or are deposited on the grain by the tapetal cells of the surrounding anther wall layer.

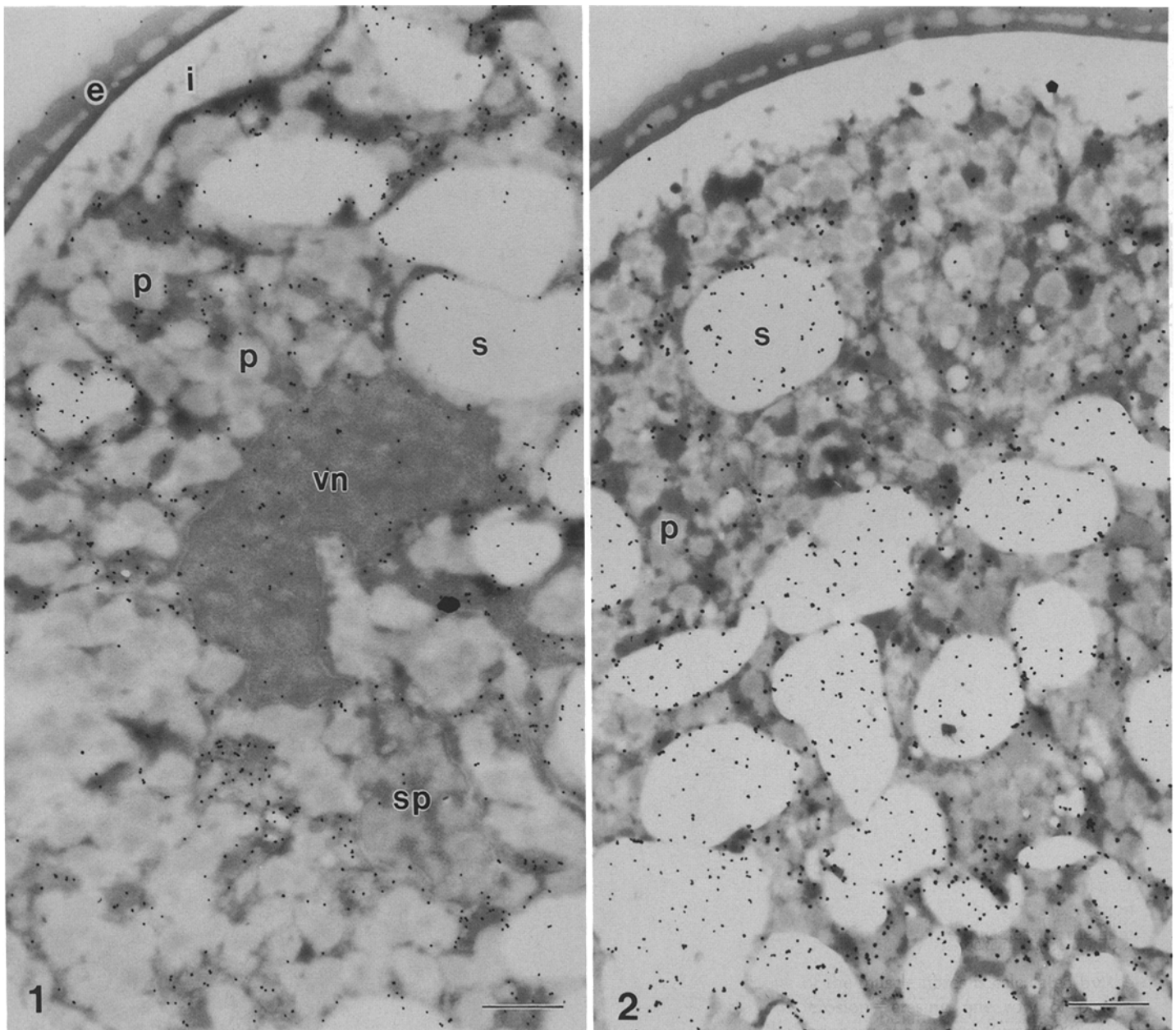
Using immunocytochemistry to localize allergenic proteins in their primary sites presents an interesting approach to understanding the release mechanism of these antigens. Allergen localization has been attempted with either precipitating agents (Grote *et al.*, 1983, 1988; Grote & Fromme, 1984a, b, 1986; Fromme *et al.*, 1985; Völker *et al.*, 1986) or with various techniques of anhydrous fixation (Knox *et al.*, 1970; Knox & Heslop-Harrison, 1971; Howlett *et al.*, 1973, 1979, 1981; Heslop-Harrison, 1979; Vithanage *et al.*, 1980, 1982; Abadie *et al.*, 1988). This often resulted in poorly preserved ultrastructure and the localization of allergens in the pollen wall. Many of the probes used were later found to have non-specific cross-reactivity (Staff *et al.*, 1990; Grote, 1991). Aqueous precipitation techniques have had limited success due to rehydration-induced structural changes and subsequent movement of antigens prior to their immobilization.

The major allergen *Bet v I* has been localized in the cytoplasm of birch pollen (Grote, 1991). This involved cracking the pollen and air drying for three days followed by paraformaldehyde vapour fixation or ethylene glycol dehydration, embedding in Lowicryl resin and immunolabelling with a specific monoclonal antibody (mAb). This resulted in the detection of allergens exclusively in the

cytoplasmic matrix and some nuclei. Grote (1991) considered that previous studies which localized the allergen in the wall to be the result of diffusion artefacts.

Our previous investigation using glutaraldehyde vapour fixation followed by dehydration in 2,2-dimethoxypropane and incubation of sections in specific mAbs localized *Lol p I* in the cytosol, and to a much lesser extent in the outer wall and surface, of whole tricellular pollen grains of rye-grass (Staff *et al.*, 1990). This was the first report of allergens being localized in

the pollen cytoplasm. However, this method did not enable good infiltration of all pollen grains and resulted in some sectioning problems. Also, the fixative contained 30% water which may have resulted in some extraction of the allergens in the moist vapour. More recently, double immunogold labelling of allergens indicated that *Lol p IX* was located within starch granules of rye-grass pollen (Singh *et al.*, 1991). A fuller investigation of the sites of this allergen across the whole anther is required.



Figs 1, 2. Ultrathin sections of anhydrously fixed tricellular pollen grains of *Lolium perenne* were incubated with antibodies followed by gold probes and silver enhancement. Sections were viewed with a transmission electron microscope. e = exine; i = intine; s = starch granule; p = P-particle; vn = vegetative nucleus; sp = sperm cell. Bar = 1 μ m.

Fig. 1. *Lol p I* localization. Label is seen in the electron-opaque regions of the cytosol.

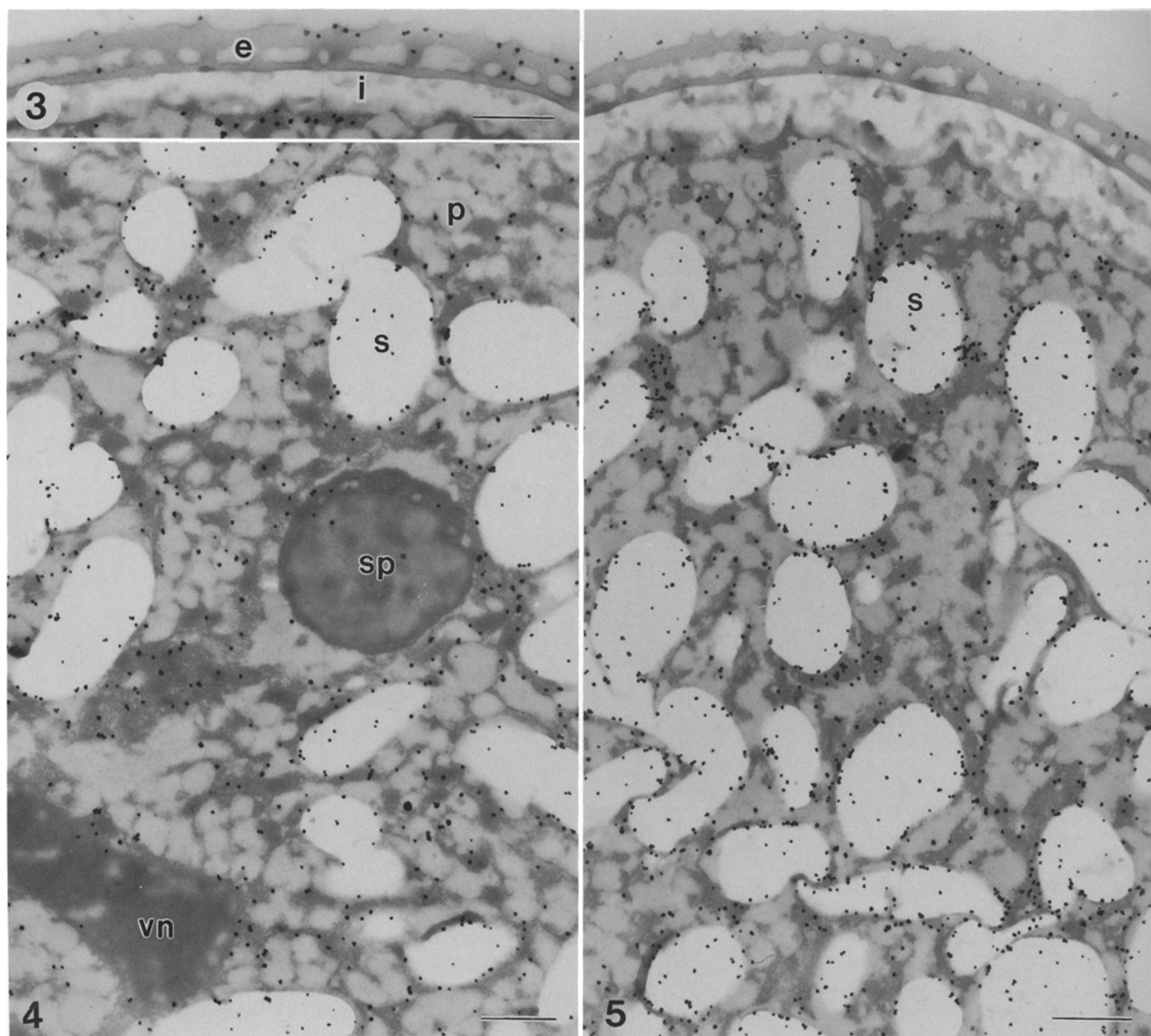
Fig. 2. *Lol p IX* localization. Label is visible within starch granules and, to a lesser extent, in the electron-opaque regions of the cytosol.

In this report, we present a detailed quantitative analysis of the localization of these two allergens in anthers of rye-grass prepared for immunocytochemistry with a recently modified anhydrous method which combines a precipitating agent with anhydrous fixation, and compare the results with aqueous fixation.

Materials and methods

Plant material

Anthers were collected just prior to dehiscence from flowering spikes of rye-grass (*Lolium perenne* L.) growing in a garden in Brunswick, Victoria.



Figs 3–5. Ultrathin sections of aqueously fixed tricellular pollen grains were incubated with antibodies followed by gold probes and silver enhancement and viewed by transmission electron microscopy. e = exine; i = intine; s = starch granule; p = P-particle. Bar = 1 µm.

Fig. 3. Detail of the pollen wall showing *Lol p I* localization in the exine.

Fig. 4. *Lol p I* localization. Label is seen in the cytosol. vn = vegetative nucleus; sp = sperm cell.

Fig. 5. *Lol p IX* localization. Label is visible in the cytosol abutting the starch granules, in the exine and, to a lesser extent, within the starch granules.

Anhydrous fixation

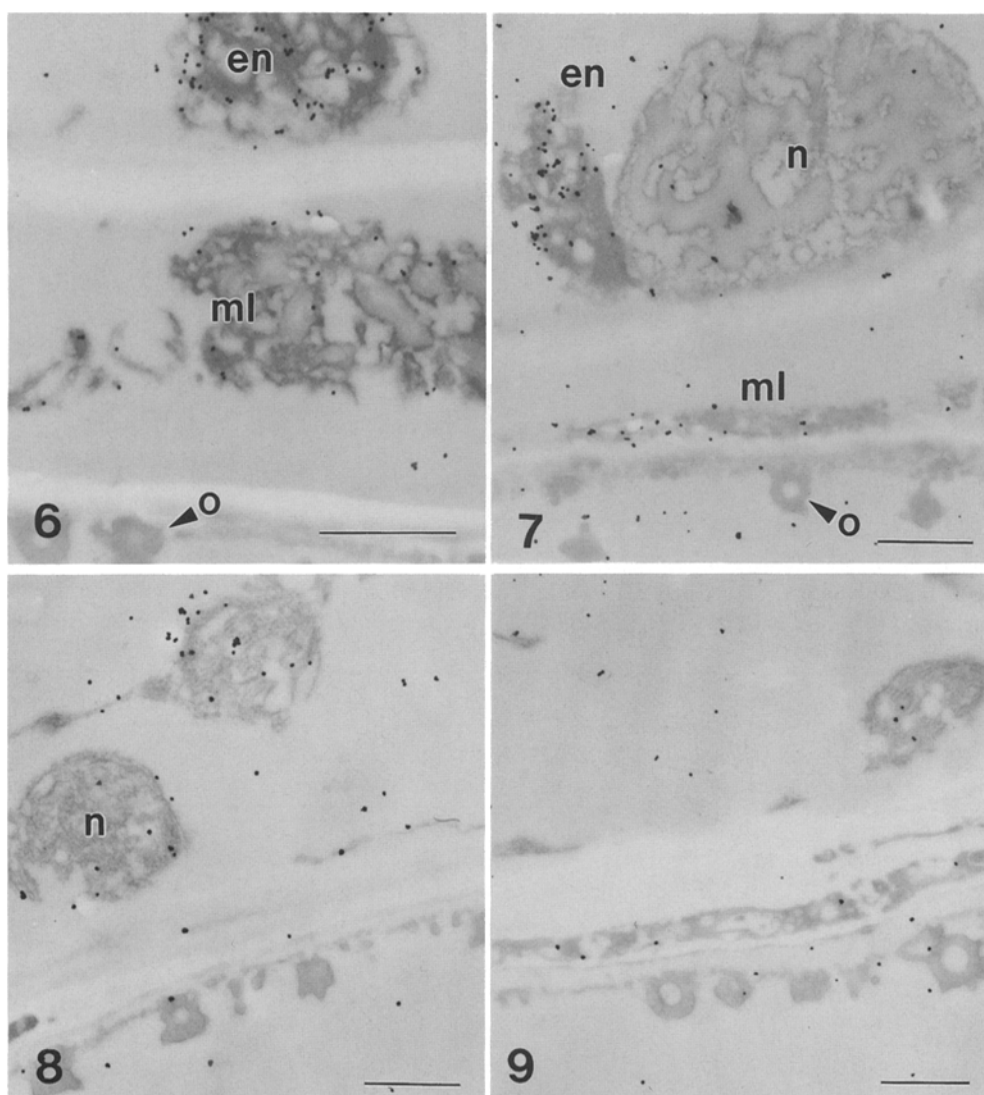
Anthers, after being lanced with a fine tungsten needle to facilitate entry of the fixative, were immersed for 24 h in one of the following fixative mixtures, pre-cooled to 4°C to slow the reaction with the tissue: (1) 0.5% glutaraldehyde in acidified 2,2-dimethoxypropane (DMP; Sigma Chemical Co., St Louis, MO, USA); (2) 0.1% glutaraldehyde and 1% paraformaldehyde in acidified DMP or (3) 0.5% glutaraldehyde and 0.5% paraformaldehyde in DMP. DMP was acidified with concentrated HCl (25 µl/100 ml), glutaraldehyde was used from 70% stock (Serva, Heidelberg, Germany) and paraformaldehyde was made as a 10% aqueous solution.

The anthers, after being rinsed in DMP followed by ethanol, were infiltrated for two weeks in LR Gold resin (London Resin

Co., England) with continuous rotation and polymerized with 1% Benzil (London Resin Co., England) in gelatine capsules at -25°C with an ultra violet light for 24 h according to Singh *et al.* (1993).

Aqueous fixation

Anthers were fixed either in (1) 0.1% glutaraldehyde, 4% paraformaldehyde, 1.5 mM CaCl₂ and 30 mM PIPES buffer at pH 7.2 or (2) 0.5% glutaraldehyde, 7% sucrose and 30 mM PIPES. After fixing for 2 h under vacuum at room temperature, anthers were sequentially rinsed in 10% and 5% sucrose buffered solutions, followed by a water rinse, dehydrated in ethanol and embedded in LR Gold resin as above.



Figs 6–9. Ultrathin sections of anther wall were incubated with antibodies followed by gold probes and silver enhancement. Samples were viewed using a transmission electron microscope. o = orbicule; n = nucleus; ml = middle layer; en = endothecium. Bar = 1 µm.

Fig. 6. Anhydrous fixation and localization of *Lol p I* showing label in the cytoplasm of middle layer and endothelial cells.

Fig. 7. Anhydrous fixation and localization of *Lol p IX*. Label is visible both in the endothelial and middle layer cells.

Fig. 8. Aqueous fixation and localization of *Lol p I*. No significant label above background is seen.

Fig. 9. Aqueous fixation and localization of *Lol p IX* showing no significant label.

Immunocytochemistry

Sections, 100 nm thick, of anhydrously fixed anthers were collected on pioloform-coated gold grids (200 mesh) and incubated either with the monoclonal antibody FMCA1 or FMCA7, which were kindly supplied by Dr I. J. Smart and Dr A. Hohman (Smart *et al.*, 1983). A series of dilutions of each mAb was performed to attain optimum labelling. Sections were treated with gold goat anti-mouse IgG probe of 15 nm diameter according to Staff *et al.* (1990). Label was silver-enhanced to approximately 60 nm with the BL Intense Silver Enhancement Kit from Amersham International (Singh *et al.*, 1993). Sections were stained with 2% aqueous uranyl acetate for 8 min and lead citrate for 1 min and viewed with a Siemens (Berlin, Germany) Elmiskop 102 transmission electron microscope at 60 kV.

Controls

Controls used were: (1) antibody specificity controls where (a) the diluted mAbs, FMCA1 and FMCA7, were pre-incubated for 1 h with purified antigens, *Lol p I* and *Lol p IX*, respectively [for these experiments, *Lol p I* and *Lol p IX* were purified from crude rye-grass pollen extract by FPLC (Pharmacia, LKB; Suphioglu *et al.*, 1993)]; (b) the diluted mAb FMCA7 was pre-incubated for 1 h with a mixture of maltose, isomaltose, maltotriose, isomaltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose (Sigma Chemical Co., St Louis, MO., USA) each at either 2.5 μM or 25 μM to check for binding to α -glucans, and (c) sections were pre-treated with a saturated, aqueous solution of NaIO_4 for 30 min to digest carbohydrate epitopes (Sedgley *et al.*, 1985); (2) an isotype control where sections were incubated with a mouse mAb of IgG isotype specific for surface- and follicle-associated epithelium of bursa and medullary epithelial clusters of thymus from chicken; (3) a method control where mAbs were omitted during

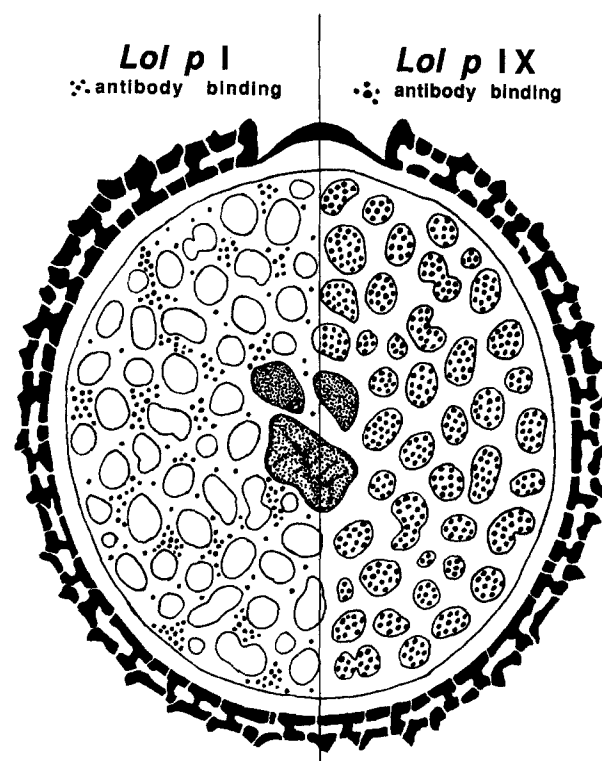


Fig. 10. Diagrammatic representation of the major antibody binding sites for both mAb FMCA1 and mAb FMCA7 in a tricolpate pollen grain of rye-grass after anhydrous fixation.

labelling, and (4) a fixation control where sections of anthers fixed either anhydrously or aqueously, as above, were immunolabelled. Each labelling procedure was repeated at least twice.

Table 1. Immunocytochemical detection of *Lol p I* and *Lol p IX* in tricolpate pollen grains and cells of the anther wall of rye-grass with mAbs FMCA1 and FMCA7, respectively. Counts per μm^{-2} of particles (95% confidence limits, $p = 0.05$)

	<i>Lol p I</i>			<i>Lol p IX</i>		
	Anhydrous fixation	Aqueous fixation	Control ^a	Anhydrous fixation	Aqueous fixation	Control ^b
<i>Pollen</i>						
Cytoplasm	24 (15–30)	7 (3–14)	4 (1–10)	7 (3–14)	8 (3–16)	<1 (0–5)
Starch granule	4 (1–10)	3 (1–9)	4 (1–10)	16 (9–26)	6 (2–13)	<1 (0–5)
Exine	<1 (0–5)	8 (3–16)	<1 (0–5)	2 (0–7)	8 (3–16)	<1 (0–5)
<i>Anther wall cells^c</i>						
Cytoplasm	18 (11–28)	1 (0–6)	<1 (0–5)	18 (11–28)	4 (1–10)	<1 (0–5)
Cell wall	2 (0–7)	<1 (0–5)	<1 (0–5)	2 (0–7)	<1 (0–5)	<1 (0–5)
Background	<1 (0–5)	1 (0–6)	<1 (0–5)	2 (0–7)	1 (0–6)	<1 (0–5)

^aAntibody-specificity control: mAb FMCA1 was pre-incubated with *Lol p I*.

^bAntibody-specificity control: mAb FMCA7 was pre-incubated with *Lol p IX*.

^cExcluding tapetum and epidermis.

Quantification of label

Silver-enhanced gold particles on the background resin outside the tissue, and within the pollen grains and cells of the anther wall (from three electron micrographs showing labelling of three different pollen grains) were counted using a quadrat corresponding to $0.25 \mu\text{m}^2$. At least 20 measurements were made in each pollen compartment. Label in pollen walls was counted, the lengths of the walls measured, a mean width calculated from >30 measurements, and the area calculated. Counts of pollen cytosol were made both in the electron-lucent and the electron-opaque regions of the cytosol. Results were analysed using Poisson distributions. Probability levels stated in the results were determined from statistical tables.

Results

Anhydrous fixation

The optimum infiltration of pollen grains was obtained when anthers were fixed in 0.5% glutaraldehyde and 0.5% paraformaldehyde in combination with 2,2-dimethoxypropane. Similar quality electron micrographs of pollen grains were obtained with both anhydrous (Figs 1, 2) and aqueous fixation (Figs 3–5). After anhydrous fixation, intine microchannels were not well defined, but nuclear membranes and sperm cells were intact. The P-particles could be seen clearly in the electron-lucent regions of the cytosol although their membranes were poorly preserved. The electron-opaque regions of the cytosol possessed mitochondria, golgi apparatus and ribosomes. The contents of the cells of the anther wall were vacuolate and plasmolysed, but the cytoplasm and nuclei could be distinguished in the endothecium and middle layer cells in both anhydrously fixed anthers (Figs 6, 7) and aqueously fixed anthers (Figs 8, 9). The tapetal cytoplasm had resorbed, the walls collapsed and the epidermis was highly vacuolate. The cells of the middle layer appeared to be in the early stages of collapse. Wall thickenings were apparent in the cells of the endothecium.

Within anthers anhydrously fixed and incubated with mAb FMCA1 for the localization of *Lol p I* (Figs 1, 10, Table 1), label was greatest in the electron-opaque regions of the pollen grain cytosol. There was only light

label in the starch granules, vegetative nucleus, sperm cells, P-particles, pollen wall and aperture, and it was not significantly different from the label in the background. Label was also seen in the cytoplasm of the cells of the endothecium and middle layer, however there was no significant label of the epidermis, tapetum, orbicules nor the cell walls of the anther wall layer (Fig. 6).

Anthers anhydrously fixed and incubated with mAb FMCA7 for the localization of *Lol p IX* (Figs 2, 10, Table 1), showed label was greatest in the starch granules of the pollen grain. Some label was seen in the electron-opaque regions of the cytosol abutting the starch granules. No significant label occurred in the pollen wall, vegetative nucleus, P-particles nor sperm cells. Label was seen in the cytoplasm of the cells of the endothecium and middle layer, however no significant label occurred in the tapetum, orbicules nor cell walls of the anther wall layer (Fig. 7).

Controls

For aqueous fixation, the best ultrastructure of pollen grains was maintained when anthers were fixed in a combination of glutaraldehyde and paraformaldehyde (Figs 3–5). Intine microchannels were more prominent than after anhydrous fixation, sperm cells were intact although slightly plasmolysed, nuclei were intact and P-particles were obvious although their membranes were poorly preserved. The contents of the cells of the endothecium and middle layer were intact and vacuolate with prominent nuclei (Figs 8, 9).

Anthers fixed aqueously and incubated with FMCA1 (Figs 3–4, Table 1), showed heaviest label both in the cytoplasm and exine. The sperm cells and vegetative nucleus were not labelled. In the starch granules label was not significantly different from background counts. In aqueous fixation, there is a loss of more than 70% of the gold particles (and thus water-soluble proteins) compared with the number of gold particles seen in anhydrous fixation. In the cytoplasm of the endothelial and middle layer cells, aqueous fixation results in an almost total loss of label compared with anhydrous fixation (Fig. 8).

Within anthers fixed aqueously and incubated with mAb FMCA7 (Fig. 5, Table 1), label occurred in both

Figs 11–16. Controls for immunolabelling of allergens in tricellular pollen grains viewed by transmission electron microscopy. e = exine; i = intine; s = starch granule; p = P-particle; vn = vegetative nucleus. Bar = $1 \mu\text{m}$.

Fig. 11. mAb FMCA1 pre-incubated with *Lol p I*. Some label is still seen in the cytosol.

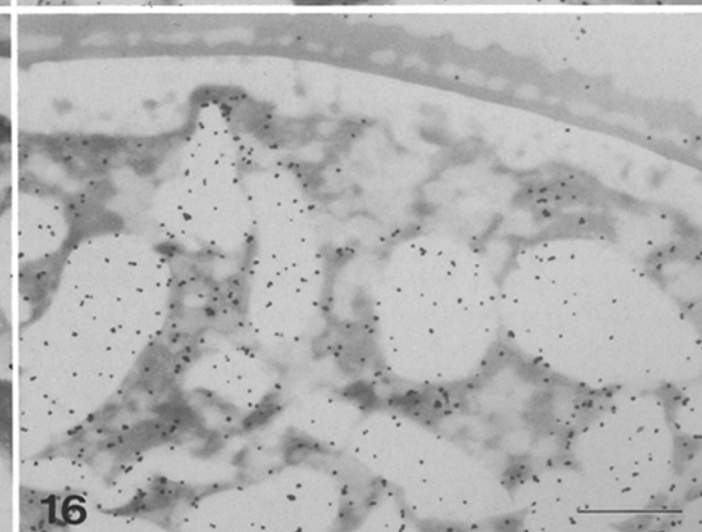
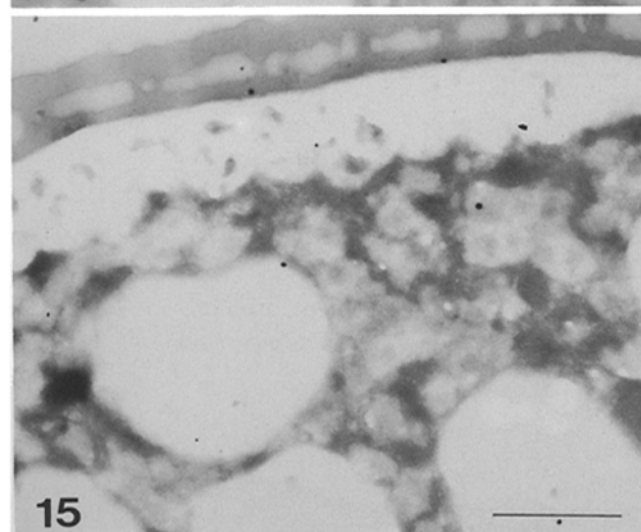
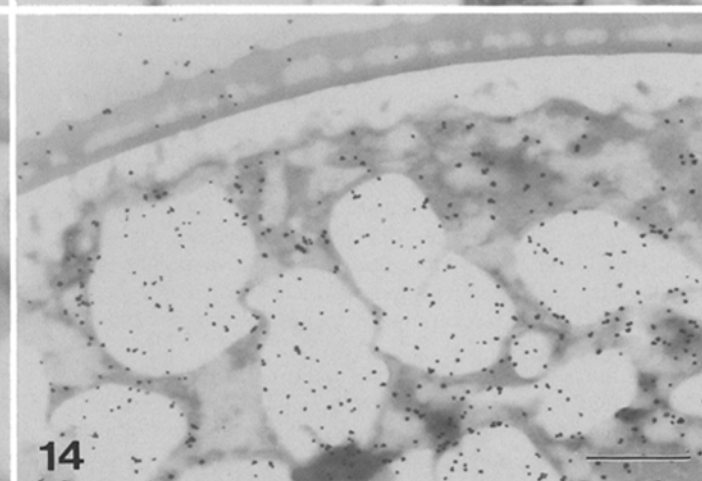
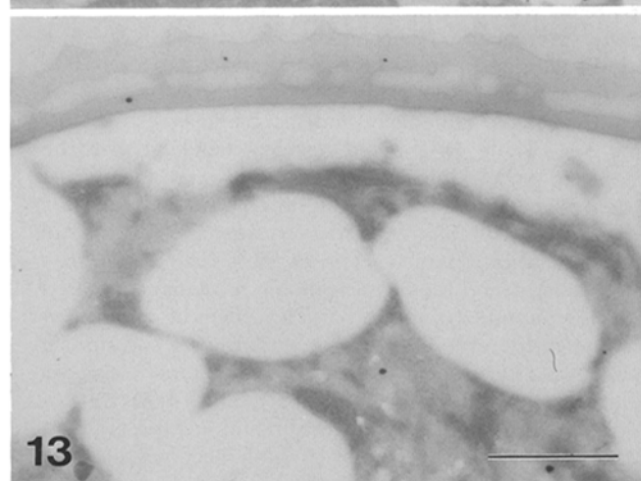
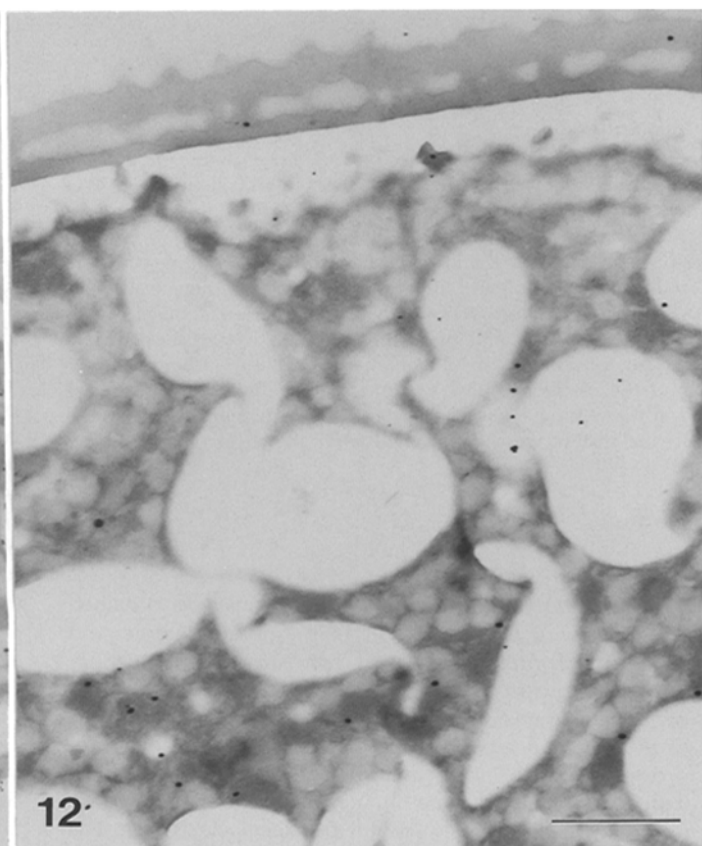
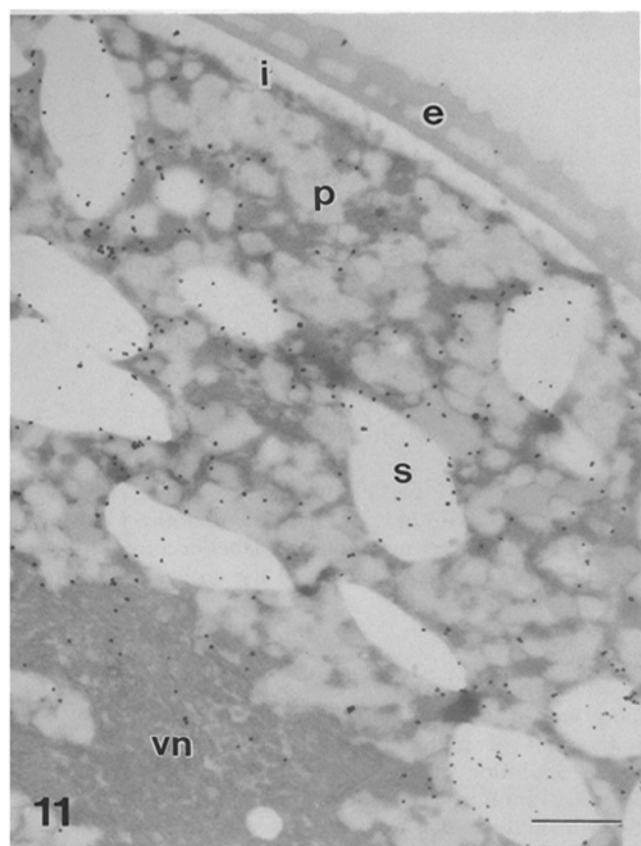
Fig. 12. mAb FMCA7 pre-incubated with *Lol p IX*. Only a few gold particles are visible.

Fig. 13. Primary mAb omitted from the immunolabelling procedure. Only a few gold particles are seen on the section.

Fig. 14. mAb FMCA7 pre-incubated with a mixture of maltose sugars, each at $25 \mu\text{M}$, to test for cross-reactivity with carbohydrate epitopes in the starch granules. Label of the starch granules is seen similar to *Lol p IX* localization.

Fig. 15. Section incubated with IgG mAb specific for chicken epithelial cells. Only a few gold particles are visible.

Fig. 16. Section pre-treated with NaIO_4 solution to denature carbohydrate epitopes followed by incubation with mAb FMCA7. Label of the starch granules is seen, similar to *Lol p IX* localization.



Figs 11-16.

the cytoplasm and exine. It was significantly different ($p < 0.05$) from the background count. Some gold particles occurred in the starch granules but the count was not significantly different from background. If the counts in the starch granules from the two treatments are compared, their totals show more than 60% loss during aqueous fixation. In the cytoplasm of the endothelial and middle layer cells, aqueous fixation results in more than 75% loss of label compared with anhydrous fixation (Fig. 9).

In the antibody specificity controls, pre-incubation of FMCA1 with *Lol p I* reduced the label in the pollen grain cytoplasm by more than 80% (Fig. 11, Table 1) and by more than 90% in the cytoplasm of the endothelial and middle layer cells. Pre-incubation of mAb FMCA7 with *Lol p IX* reduced the label in the starch granules by more than 90% (Fig. 12, Table 1) and by more than 90% in the cytoplasm of the endothelial and middle layer cells. Omission of the primary mAb resulted in no appreciable labelling (Fig. 13). With anhydrously fixed samples, serial dilution of mAb FMCA7 resulted in lowered counts across both the starch granules and the cytoplasm of the endothelial and middle layer cells.

To check the specificity of labelling of starch granules, mAb FMCA7 was pre-incubated with a mixture of disaccharides and oligosaccharides at both 2.5 and 25 μM without any significant decrease in labelling of the starch granules; after correcting for background (2 counts μm^{-2}) results were 18 and 15 counts μm^{-2} , respectively (Fig. 14). An isotype control showed no appreciable labelling (Fig. 15). Washing the fixed section with NaIO_4 solution before incubation with mAb FMCA7 did not show a significant reduction in labelling of the starch granules; after correcting for background (1 count μm^{-2}), the result was 14 counts μm^{-2} (Fig. 16). Replicate runs of each of these labelling procedures gave consistent results.

Discussion

Allergen diffusion artefacts in pollen immunocytochemistry

The anhydrous fixation method now presented allows immunogold labelling of whole anthers, minimizing diffusion artefacts. These immunolabelling results with anhydrous fixation confirm the localization of *Lol p I* in the cytosol of rye-grass pollen grains (Staff *et al.*, 1990), except that we found no significant label at the surface of the pollen wall. The modifications to the anhydrous fixation schedule that we have developed here resulted in much improved infiltration, sectioning properties and retention of allergens at their original sites within the whole pollen grains. We did not find any labelling of the nucleus. Grote (1991) suggests that the labelling of the pollen nucleus, found in her study and by Staff *et al.* (1990), may be a diffusion artefact. This appears to be the case.

With our anhydrous method it appears that the mixture of 2,2-dimethoxypropane with aldehydes is able to both precipitate and cross-link allergens in their original sites as the fixative penetrates through the anther. The DMP is able to precipitate water-soluble proteins and prevent their extraction. Water reacts with DMP in an equimolar equation to produce acetone and methanol (Kaesler, 1989). After adding the fixatives, sufficient unreacted DMP remains to convert the water in the tissue into acetone and methanol, and these rye-grass allergens are not soluble in organic solvents.

Shivanna & Heslop-Harrison (1981) suggested that, in the partly dehydrated pollen grain at the time of dispersal, the membranes are largely dissociated and do not form an osmotic barrier. It seems that considerable structural changes occur in the plasma membrane during imbibition (Smith, 1991) and leakage from imbibing tissues may be the result. We have found that with aqueous fixation methods, the pollen grains are able to imbibe water which results in the leaching of allergens from their sites prior to cross-linking with the aldehydes. The diffusion artefacts of aqueous fixation that we have observed are probably similar to the movement of allergens that occurs in the early stages of pollen grains in contact with the human mucosa or the stigma surface. Thus allergens would be quickly available to trigger allergenic responses upon contact with the mucosa of susceptible individuals.

The specialized technique of rapid-freeze freeze-substitution enables processing of tissue in an anhydrous medium for retention of water-soluble substances. However, to achieve labelling, the omission of osmium or other highly cross-linking fixatives is often necessary and results in poor infiltration of pollen grains (Taylor *et al.*, 1993a). Martinez & Wick (1991) have described the ultrastructure of freeze-substituted rye-grass pollen. They used *en bloc* staining in uranyl acetate prior to embedding in LR Gold resin. Preliminary work on freeze-substitution and immunolabelling of allergens (Singh *et al.*, 1993) showed that pollen grains that were most fixed by the uranium, and thus gave superior quality sections, were much reduced in labelling intensity. Uranyl acetate masks the antigenic sites of allergens when probed with a mAb specific for *Lol p I*. Our anhydrous method is currently being adapted for use with freeze-substitution to optimally combine labelling intensity with structural integrity.

Cellular sites of allergens in anthers

This is the first report of the localization of allergens in specific cells of the anther wall. We have detected both *Lol p I* and *Lol p IX* within the cells of the endothecium and middle layer prior to anther dehiscence. This suggests that allergens are the product of not only haploid gene expression but also of the diploid sporophytic endothecium and middle layer. However, in the pollen grain, we found that allergens were only expressed in the

vegetative cell and were not present in the haploid sperm cells. Allergens are also absent from the degenerating tapetum. This suggests that the sporophytic tissue does not provide the pollen grain surface with an allergenic coating prior to dehiscence. Previous reports on the detection of allergens on the surface of pollen grains are most likely diffusion artefacts originating from the pollen cytoplasm. Further work is currently in progress on the immunolabelling of allergens during anther development to further investigate the presence of these antigens in the cells of the endothecium and middle layer and the timing of expression in the pollen grain.

Our immunolabelling results support the findings of Singh *et al.*, (1991) on the localization of *Lol p IX* in the starch granules of tricellular pollen grains. We consider it unlikely that our results are due to cross-reactivity of the antibodies to starch itself because the labelling is reduced by more than 90% after pre-incubation with purified *Lol p IX*, and pre-treating the sections with NaIO_4 solution did not significantly alter the binding to the starch granules in sections from anhydrously fixed pollen. Pre-incubation of antibodies with micromolar concentrations of appropriate disaccharides and oligosaccharides also provides useful controls (Taylor *et al.*, 1993b). No significant decrease in counts was observed with such a control, supporting our conclusion that the label we observed does not reflect binding to α -glucans. We have previously shown that the contents of the amyloplasts were retained during sectioning (Taylor *et al.*, 1993b), so the labelling pattern was not due to non-specific binding to the underlying grid coating. Further work in support of our observations comes from the finding that *Lol p IX* has a transit peptide targeting the allergen to amyloplasts (Singh *et al.*, 1991).

Each rye-grass pollen grain contains approximately 650 starch granules (Pacini *et al.*, 1992), and pollen grains rupture following rainfall, releasing the contents into the air (Suphioglu *et al.*, 1992). *Lol p IX* has been detected in starch granules filtered from the air (Suphioglu *et al.*, 1992). These starch granules act as micronic particles, which are respirable, and hence able to trigger an attack of asthma (Bellomo *et al.*, 1992). Thus, immunocytochemical studies on sections of intact pollen grains corroborate clinical evidence from studies on isolated starch granules.

Acknowledgements

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