

## Original Article

# Cross-reactivity of Birch Anthers and Leaves With Birch Pollen Antigens and Allergens. A Fine-structural Immunocytochemical Study Using the Post-embedding Protein-A-gold Technique

MONIKA GROTE<sup>1</sup> and HANS G. FROMME

*Institute of Medical Physics, Münster University, D-4400 Münster, FRG.*

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Ultra-thin sections of vegetative tissues from birch (anthers and leaves) were labeled for pollen antigens and allergens using a commercial rabbit IgG antibody preparation directed against birch pollen antigens and allergens. Antibody binding sites were visualized using the protein A-gold technique. Specific labeling occurred in anther tissue (taperum cells, anther wall cells) as well as in the birch leaf (assimilation parenchyma). In both types of tissue, antigens and allergens were detected throughout the living protoplast (including cell organelles such as nuclei, mitochondria,

and plastids). The cellulose cell walls were always free from anti-birch-pollen IgG-binding sites. The immunological controls (normal rabbit IgG) showed a low degree of nonspecific labeling. In plant tissues belonging to genera quite different from birch (tulip anther, rhododendron leaves), after incubation with the specific IgG weak labeling was observed. The immunological basis for these results is discussed. (*J Histochem Cytochem* 34:1459, 1986)

KEY WORDS: Birch pollen; Antigens/allergens; Birch vegetative tissues; Post-embedding protein A-gold technique.

## Introduction

It is commonly known that allergic reactions to pollen are stimulated by the wind-dispersed pollen grains of a number of flowering plant species (*Spermatophyta*). On contact with moist human mucosa, pollen grains rapidly release substances (e.g., proteins, glycoproteins) that lead to the formation of specific reagenic antibody IgE and the clinical symptoms of pollinosis in hypersensitive individuals. Quite early, however, observations were made that pointed to a more generalized (i.e., not pollen-bound) occurrence of pollen allergens. Thus, by clinical (Rebhun et al., 1954) and biochemical (Shafiee and Staba, 1973; Shafiee et al., 1973) testing the presence of ragweed pollen allergens within vegetative parts of the ragweed plant was demonstrated. The rather surprising finding that ragweed pollen allergy could be triggered by airborne particles much smaller than the ragweed pollen grain (Busse et al., 1972; Lowell, 1973; Solomon et al., 1983; Agarwal et al., 1984) also pointed to a source of pollen allergens independent from the pollen grains, perhaps very small vegetative plant debris. Finally, from 1970 onwards clinical observations were published that reported correlations between certain pollen and food allergies (cf. Halmeupuro et al., 1984), indicating a more generalized distribution of pollen allergens in the plant kingdom.

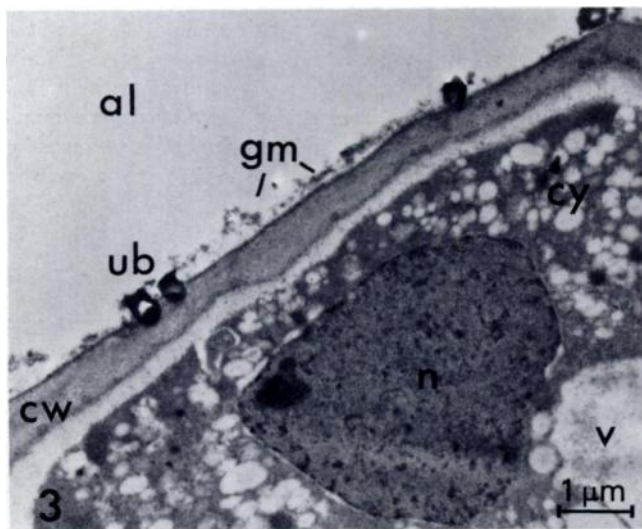
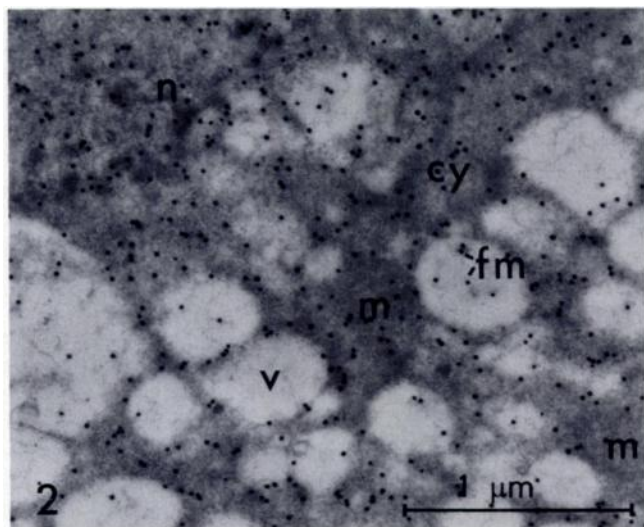
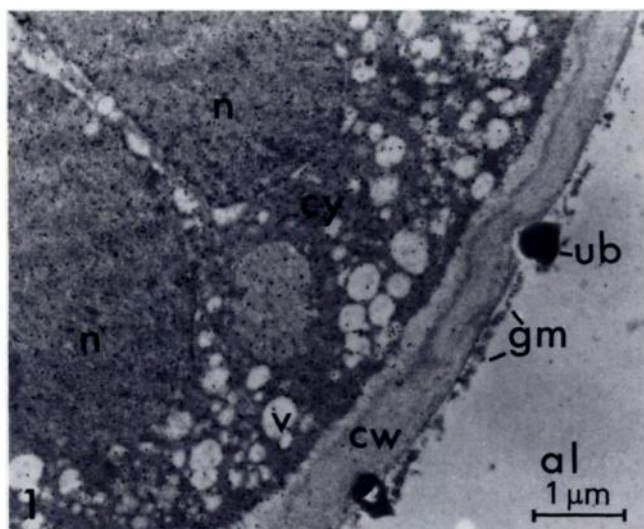
All of these reports and observations on pollen allergens outside the pollen grain were based on the results of clinical skin tests, such as the prick or scratch test, or on biochemical laboratory analyses, such as the RAST or RAST-inhibition techniques using extracts from homogenized plant material.

In the present study, we wished to investigate if birch pollen allergens, which play a major role in pollen allergies in Europe, are likewise to be found in vegetative parts of the plants. Moreover, for the first time, we attempted a cytochemical approach which will allow visualization of allergens within their native morphological sites in plant tissues and cells. For this purpose, fresh anthers and leaves from birch trees were prepared for transmission electron microscopy. Ultra-thin sections of these tissues were incubated with IgG antibodies raised in rabbits against an extract from birch pollen grains. The specifically bound IgG antibodies to birch pollen allergens and antigens were then visualized within their morphological sites by the protein A-gold (pAg) technique, established some years ago by Roth et al. (1978), which since that time has found wide acceptance among immunocytochemists. For comparison, anthers from tulip and leaves from rhododendron were included in our investigation.

## Materials and Methods

**Fixation and Embedding.** Whole anthers were removed from the im-

<sup>1</sup> To whom correspondence should be addressed.



mature catkins of birch trees (*Betula pendula*) in March, 1985, cut into two halves with a sharp razor blade, and fixed in 0.071 M cacodylate-buffered, freshly prepared 4% *p*-formaldehyde, pH 7.4. The tissue pieces were fixed overnight at 4°C, washed for 1 hr in cacodylate buffer, and then embedded in the hydrophilic resin Lowicryl K4M in the cold (for details see Grote and Fromme, 1984).

Parts from the assimilation parenchyma of birch leaves were dissected, cut into 1-mm cubes, and fixed and embedded as described above for the anthers.

Anthers from tulip (*Tulipa gesneriana* hybr.) and leaves from rhododendron (*Rhododendron katawbiense* hybr.) were treated as indicated above.

Ultrathin sections of all specimens were cut on an LKB ultratome and placed on pioloform-coated nickel grids.

**Immunocytochemical Reagents.** Rabbit IgG antibodies against an extract of *Betula verrucosa* pollen were supplied by the Allergologisk Laboratorium (Copenhagen, Denmark). According to the description provided by the manufacturer, these polyspecific antibodies are derived from long-term immunization, using a broad-spectrum allergen extract. They were tested for activity towards major allergens by column absorption of homologous allergen extracts. More than 90% of the RAST activity was removed by the specific antibodies. Normal rabbit IgG was obtained from Dynatech (Denkendorf, FRG).

Protein A from *Staphylococcus aureus* was supplied by Medac (Hamburg, FRG). The protein A-gold complex was prepared in our laboratory closely following the instructions found in Roth (1982). As the reducing agent, we chose trisodium citrate, and a final gold particle size of approximately 18 nm.

**Immunocytochemical Labeling.** All sections were pre-incubated for 10 min at room temperature with 5% BSA (Serva, Heidelberg, FRG) in PBS buffer, pH 7.4. This procedure was performed in order to decrease the number of nonspecifically bound gold particles. The following immunocytochemical incubation steps were carried out in a moist chamber at room temperature by floating the nickel grids on drops of the following solutions: (a) rabbit anti-birch antibodies, diluted 1:30 in PBS, pH 7.4, for 2 hr. After rinsing (three times for 5 min each) in PBS, pH 7.4, the grids were placed on (b) protein A-gold solution (stock solution diluted 1:8 in PBS, pH 7.4) for 1 hr. The reaction was terminated by thoroughly washing the grids, twice (10 min each) in PBS, pH 7.4, then once (10 min) in deionized water. Finally, the sections were stained with uranyl acetate (10 min) and very briefly (10 sec, danger of overstaining!) with lead citrate.

Figures 1–3. Ultra-thin sections of birch tapetal cells.

Figure 1. Part of a cell after incubation with rabbit anti-pollen IgG and pAg at low magnification. There is dense gold labeling of the cytoplasm (cy) and the nuclei (n). Cell wall (cw) and vacuoles (v) are very rarely labeled. The seam of granular material (gm) at the luminal surface of the tapetal cell is marked by gold particles. One of the two ubisch bodies (ub) is also labeled. al, anther lumen. Original magnification  $\times 12,000$ .

Figure 2. Detail at higher magnification from cytoplasm (cy) of a cell after incubation with rabbit anti-pollen IgG and pAg. There is gold labeling of the cytoplasmic matrix, the mitochondria (m), and the nucleus (n). Vacuoles (v) show gold particles associated with fibrillar material (fm) in the interior. Original magnification  $\times 30,000$ .

Figure 3. Part of a cell after incubation with normal rabbit IgG and pAg (control to Figure 1). Very few gold particles are seen as nonspecific background staining. Abbreviations as in Figure 1. Original magnification  $\times 9000$ .

**Electron Microscopy.** The sections were examined in a Hitachi H 500 transmission electron microscope operated at 75 kV. Photographs were taken on Agfa Gevaert-23 D 56 film.

**Controls.** Controls were run by substitution of the specific rabbit anti-birch IgG antibodies by rabbit normal IgG and by omission of the rabbit sera, i.e. incubation with the protein A-gold complex alone.

## Results

Figure 1 shows part of a birch tapetal cell at low magnification. Many gold particles are found in the cytoplasm and in the large nuclei, which are, however, less densely labeled than the cytoplasm. The many vacuoles spread all over the protoplast are sparsely labeled or completely unlabeled. Within the thick tapetal cell wall almost no gold particles are found. Some labeling is observed in the so-called ubisch bodies lining the surface of the tapetal cells towards the lumen of the anther. Specific labeling is also recognizable in the narrow seam of the granular or fibrillar material which loosely covers this luminal surface.

Figure 2 gives a detail from a tapetal cell at higher magnification. The edge of the nucleus (upper left corner) is recognizable. Within the ground cytoplasm the outlines of a mitochondrion and many spherical small or large vacuolar spaces are visible. Gold particles are found in the nucleus and in the cytoplasm, including the mitochondrion. The few gold markers located within the vacuoles are mostly associated with electron-dense fibrillar or granular material occasionally occurring in the lumina of these otherwise "empty" spaces.

The control section (Figure 3) which was incubated with rabbit normal IgG shows almost no gold labeling. Incubation of the sections with the protein A-gold complex alone (micrograph not shown) was likewise negative.

Within the anther wall, cells are characterized by thickened cell walls and comparatively dense protoplasts (Figure 4 and Figure 5). As in the tapetal cell, specific labeling for pollen antigens is confined to the protoplast and its structures such as the nucleus, whereas vacuoles, intercellular spaces (not shown) and cell walls are largely free of gold particles (Figure 4). The control treated with normal rabbit IgG shows a minimal degree of background staining (Figure 5).

Among the diverse structures of the birch leaf, typical cells of the mesophyll (i.e., the assimilation parenchyma tissue) were chosen for photographic documentation of their reactivity towards anti-pollen and normal rabbit IgG. The findings, however, were the same in other living morphological structures or tissues of the leaf, such as the sieve tubes and their companion cells or the epidermal tissues. Dead cell or tissue structures, such as wood vessels within the xylem of the leaf bundle or sclerenchyma fibers, were usually not reactive towards the IgG antibodies.

Figure 6 demonstrates specific IgG binding in a cell of the assimilation parenchyma. There are gold particles in the cytoplasmic matrix, the mitochondria, and the nucleus. Even the big chloroplasts with their thylakoidal membranes are densely labeled, except for their elliptical starch inclusions. Again, there are almost no gold markers in the cell wall and the big central vacuolar spaces. The corresponding control (Figure 7), treated as those above, is unlabeled.

Anthers from tulip (Figure 8) and leaves from rhododendron (Figure 9) show a much reduced degree of labeling after incubation with antibodies against birch pollen extract. The controls (micrographs not shown) were unlabeled.

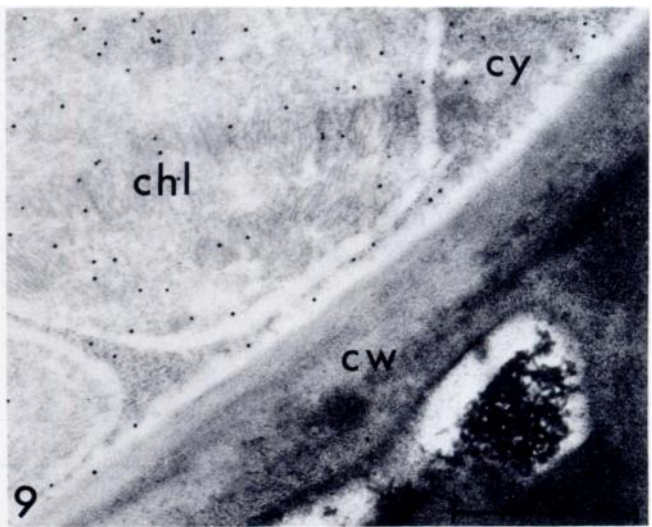
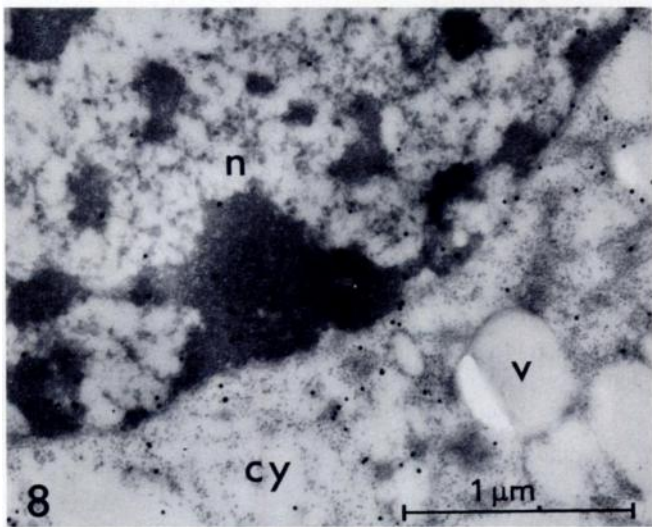
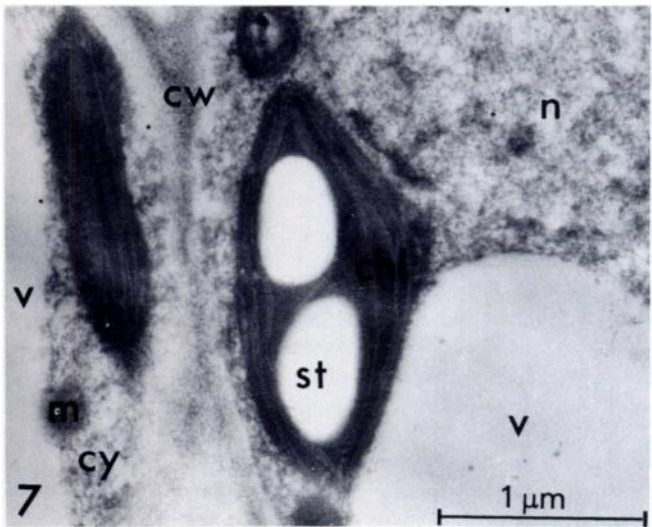
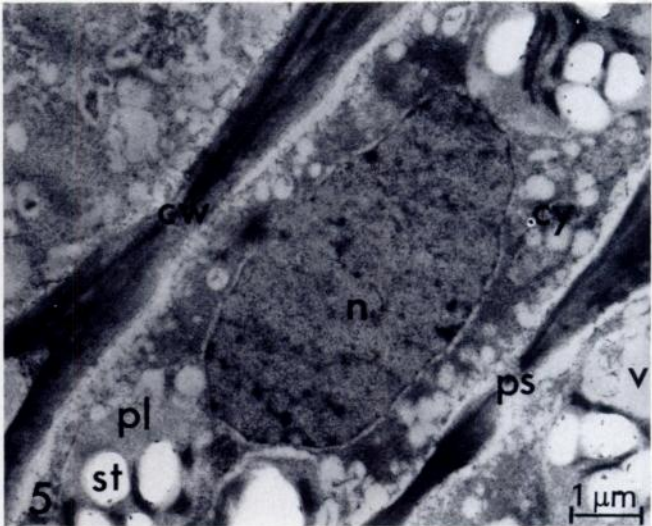
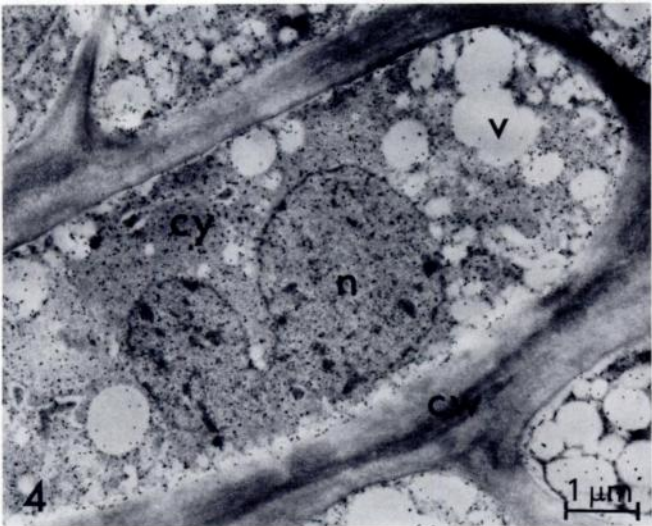
## Discussion

The results of the present study have clearly demonstrated that in birch trees, antigens and allergens located in the pollen grain are also found in other, vegetative parts of the plant. Whereas this statement agrees in principle with clinical and biochemical observations (see above), the cytochemical approach applied in this study for the first time makes possible exact morphological coordination of these antigens/allergens with their specific tissue or cell sites. The uniformity of the reaction pattern in different tissues towards specific antibodies has its basis in a remarkable uniformity of the antigen/allergen distribution on the cellular level: binding of antibodies occurs indiscriminantly throughout the living protoplast and even in its organelles, such as the nucleus, the mitochondria, or the plastids. On the other hand, the cellulose or pectin/cellulose envelope of the protoplast (i.e., the cell wall), is essentially free of antigens and allergens, a finding that confirms the results previously published by the present authors for the (equally cellulosic) intine of the pollen grain (Grote and Fromme, 1984; Fromme et al., 1985). In starch grains (amyloplasts), the central carbohydrate mass does not give positive reaction with rabbit anti-pollen IgG. Those gold particles sometimes observed in the peripheral zone of a cross-sectioned amyloplast are probably bound by the thin residual plastidal envelope which, however, may not always be visible in high-contrast photographic prints.

If labeling occurs within vacuolar spaces, as was sometimes the case within the tapetal cells, antibodies are mostly bound to traces of fibrillar material which may have been precipitated during the process of fixation and preparation for electron microscopy. Ubisch bodies, regarded as small parcels of pollen exine material secreted by the tapetum (Heslop-Harrison, 1968) react exactly as the pollen exine, in that they form a rather homogeneous electron-dense mass interspersed with IgG-IgE binding sites (Grote and Fromme, 1984; Fromme et al., 1985).

The pollen surface coat material (Grote et al., 1983; Grote and Fromme, 1984) shows structural similarity to the granular/fibrillar material which in the present study was observed on the luminal surface of the tapetal cells. The reactivity of both materials towards rabbit anti-pollen IgG is the same. This finding is supported by the observation that this material—secreted by the tapetum cells—is injected into and deposited on the exine of the pollen grains (Knox et al., 1975).

The immunological basis for the observed binding of anti-pollen IgG to vegetative parts of birch plants may be explained by the assumption that both pollen and vegetative tissue antigens and allergens have common antigenic determinants. Moreover, as the results from tulip anthers and rhododendron leaves show, there is even a certain degree of crossreactivity of the anti-birch-pollen IgG with tissues from plants that belong to different botanical genera. The postulation of structural (molecular) similarity of pollen and tissue allergens has been verified in the case of birch pollen and certain fruits and vegetables by biochemical tests such



as RAST-inhibition and CLIE (Crossed Line Immuno Electrophoresis) (Halmepuro et al., 1984; Halmepuro and Löwenstein, 1985). Aalberse et al. (1981) suggested that antigenic determinants are represented by oligosaccharide portions of identical chemical structure in glycoproteins. In the case of some birch pollen allergens, their glycoprotein nature has already been elucidated (Vik et al., 1983), but rye grass pollen allergen was also shown to be a protein with a carbohydrate part (Howlett et al., 1981).

For further investigations in the field of antigen/allergen cytochemical localization in pollen grains and other plant tissues, the next step seems to be the production of rabbit antibodies directed against individual antigens (e.g., Vik et al., 1983) and their subsequent localization in the tissue. However, monoclonal antibodies, which have already been developed against some pollen allergens (cf. Smart et al., 1983; Mecheri et al., 1985) would certainly offer the best tool for obtaining the highest degree of specificity in the differentiation of pollen and tissue antigenic and allergenic structures.

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Figures 4–5. Ultra-thin sections of anther wall tissue.

Figure 4. Cells after incubation with rabbit anti-pollen IgG and pAg. Dense gold labeling is found within the cytoplasm (cy) and the nucleus (n). Vacuoles (v) and the cell wall (cw) are almost free of gold particles. Original magnification  $\times 9000$ .

Figure 5. Cells after incubation with normal rabbit IgG and pAg (control to figure 4). There is a low degree of nonspecific labeling. Abbreviations as in Figure 4. Additionally plastids (pl) containing many starch grains (st) and a plasmodium (ps) are seen. Original magnification  $\times 9000$ .

Figures 6–7. Ultra-thin sections of cells from the mesophyll of the birch leaf.

Figure 6. Detail from a cell after incubation with rabbit anti-pollen IgG and pAg. There is heavy gold labeling of the cytoplasm (cy), the nuclei (n), the mitochondria (m), and the chloroplasts (chl). Starch grains (st) included in the chloroplast show a few gold particles near the margin of the grain. The cell wall (cw) and the big central vacuole (v) are almost free of labeling. Original magnification  $\times 24,000$ .

Figure 7. Detail from a cell after incubation with normal rabbit IgG and pAg (control to Figure 6). Very few gold particles are observed as background labeling. Abbreviations as in Figure 6. Original magnification  $\times 27,000$ .

Figures 8–9. Ultra-thin sections from plant tissues other than birch after incubation with rabbit anti-pollen IgG and pAg.

Figure 8. Detail from a tapetal cell of tulip. There are some gold particles associated with cytoplasmic structures (cy) and with the nucleus (n). Vacuoles (v) are free of labeling. Original magnification  $\times 30,000$ .

Figure 9. Detail from a cell of the assimilation parenchyma of a rhododendron leaf. There is sparse labeling by gold particles of chloroplast (chl) and cytoplasm (cy). There is no labeling of the cell wall (cw). Original magnification  $\times 24,000$ .

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