

REVIEW

Botanical immunocytochemistry: a review with special reference to pollen antigens and allergens

R. B. KNOX, H. I. M. V. VITHANAGE and B. J. HOWLETT

School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia

Received 18 September 1979

Contents

Introduction

Principles

Antigen preparation

Specificity of antibodies

Antibody markers

Tissue preparation

Applications of immunocytochemistry to plants

Case history: localization of pollen antigens and allergens

Conclusions

References

Introduction

Antigen-antibody recognition is an essential component of the immune system of defence reactions in vertebrates, but is foreign to plant cell biology. Plants exhibit some of the features of immunity: they are able to discriminate self from non-self, though they have largely lost the capacity for phagocytosis, now confined to certain algae (see review by Clarke & Knox, 1979). Plant responses appear to be largely cellular in nature, resembling allograft recognition seen in coelenterates, and there is little evidence as yet for humoral responses exemplified by the circulating antibodies of cyclostomes and higher vertebrates (Cooper, 1976). The closest analogy in plants is perhaps the production of inhibitory phytoalexins by cells of some species in

contact with potential fungal pathogens. The antigen concept is one that is basic to animal cell biology, and it is its applications that have met with success in plant cell biology since many plant proteins, glycoproteins and polysaccharides are antigens in experimental animals.

The fluorescent antibody technique developed by Coons *et al.* (1942) was ignored by botanists for more than twenty years, perhaps because of the fundamental disparity in both concept and terminology that has shrouded immunology since its inception, compared with the traditional morphological approach of plant biology. Although a preliminary report of the use of fluorescent antibodies against flowering plant antigens from birch pollen was given in 1964 (Hagman, 1964), detailed immunocytochemical studies of flowering plant antigens were not published until 1970. Two groups successfully applied the method to the localization of plant antigens, the first to extracellular antigens of pollen (Knox *et al.*, 1970), and the second to intracellular antigens of bean cotyledons (Graham & Gunning, 1970). Today, immunocytochemistry is well established as a highly specific tool for the localization of proteins, glycoproteins and polysaccharides in plant cells, tissues, organs and surfaces.

The purpose of this review is to present some of the problems that have been encountered during the past decade in applying to plants immunocytochemical methods developed for animal tissues. This is particularly important in view of the widespread use of modern high resolution plastic embedding techniques, and the availability of a wide range of fixatives in plant cytochemistry. The review concludes by focusing on a particular case history (the localization of the antigens and allergens of pollen grains) where their extreme mobility in aqueous solutions together with their extracellular location has posed special problems of fixation and antigen retention. Such studies will be of particular interest in the 1980s when attention seems likely to be focused on interactions between cells and between organisms, at cell surfaces.

Principles

Immunocytochemistry is based on the ability of antibody molecules (especially Immunoglobulin G, IgG) to recognize and bind to the antigen against which they were raised in an experimental animal. Each IgG molecule has two sites for antigen binding, so that the molecules are bivalent, and may bind to antigen or form precipitates between adjacent antigen molecules. The IgG molecules may also be cleaved enzymically to give monovalent fragments, $F(ab)_2$, of lower molecular weight, which may also be used in immunocytochemistry.

Three kinds of antigen-antibody interaction have been utilized in immunocytochemistry: the direct antibody technique, a one-step procedure in which a marker is attached to the specific antibody that detects the antigen; the indirect antibody technique, a two-step procedure in which the specific antibody is attached

to the antigen, and is detected by a marker in the anti-antibody, so that a sandwich of tissue antigen-antibody-anti-antibody marker complex is formed; and finally the mixed aggregation technique, a two-step procedure in which the tissue antigen is complexed with its specific antibody, and the complexes detected by use of labelled antigen which binds to available sites on the antibody. These methods have been elegantly reviewed and illustrated by Wachsmuth (1979). The direct method has the advantage of a short incubation period and specificity in that there is less opportunity for non-specific binding of the antibody to tissue components; it has the disadvantage of being time-consuming in that each specific antibody has to be individually conjugated to the marker by the investigator. The indirect method produces considerable amplification of the marker, since the antibody has several antigenic determinants to which the labelled anti-antibody may bind, enhancing the resolution and sensitivity of this method. An additional advantage is that this anti-antibody is commercially available. The third method is useful in cases where considerable quantities of the purified antigen, for example, an enzyme, are available for labelling with a marker, and offers the advantage of increased specificity because of the likely reduction in non-specific binding to tissue components.

Several aspects of immunocytochemistry need to be considered in the light of experience with plant cells: (1) problems in isolating macromolecules from plant tissues for use as antigens; (2) antibody preparation, partial purification and testing for specificity; (3) attachment of a marker or probe to the antibody so that it can be detected in plant tissues by light or electron microscopy. (4) tissue preparation to preserve cellular fine structure, antigenic activity and permit access of the labelled antibodies to cell sites. Immunocytochemistry offers the possibility of localizing antigens both on cell surfaces and within cells. Plant cells, of course, provide a barrier to this objective in the cell wall, which normally covers the plasma membrane except in protoplasts which have been produced by specific enzymic digestion of the wall. The primary cell wall of plants provides a potential barrier to the penetration of high molecular weight molecules. Since labelled antibodies usually have a molecular weight of 2×10^5 up to 10^6 , the problems of tissue penetration are very great.

ANTIGEN PREPARATION

Several kinds of antigen preparations have been used in botanically-oriented studies: (1) antigen extracts, which may be diffusates of whole cells or organs in aqueous buffers or detergents, or the culture filtrates of cells, containing surface macromolecules or secreted components or slurries or homogenates of organs, tissues or cells, or the soluble fraction after centrifugation; (2) antigens purified by biochemical procedures. A problem peculiar to plant tissue extracts is browning caused by polyphenol oxidases, or high levels of tyrosinases and proteases, or acidity of the sap. The released polyphenols from cell vacuoles may bind proteins and glycoproteins making them possibly inaccessible as antigens. These difficulties are especially prevalent in woody plants. They may be overcome or alleviated by

addition of polyvinyl pyrrolidone to the extraction medium (Loomis & Battaille, 1966), by extraction in an atmosphere of nitrogen at low temperature (Safonov & Safonova, 1968) or by adding small amounts of sulphhydryl reagents such as 2-mercaptoethanol (Khavkin *et al.*, 1977) or dithiothreitol (Chrispeels & Baumgartner, 1977) to inhibit or partially inhibit oxidation of polyphenols.

SPECIFICITY OF ANTIBODIES

Most work with plant antigens has been based on the use of antisera raised in rabbits, and very occasionally in sheep or goats. Rabbits are easily housed and produced adequate quantities of antiserum at a single bleed. The doses of antigen required for each injection vary from 1 μ g of a highly purified antigen to 1 mg of a mixture per kilogram of rabbit (Knox & Clarke, 1978). Multiple site injections are commonly used, and the antiserum possesses a broad specificity which will vary according to the time of collection after injection, and is usually of highest specificity about four weeks after the first injection (see Knox & Clarke, 1978, for review).

A recent development has been the production of monoclonal antibodies in mice. Minute quantities of antigen only are required and it may be present mixed with other antigens, for example, in membrane preparations. Mice are inoculated and spleen cells are hybridized with a myeloma and selected antibody-producing cell lines proliferate to provide monospecific antibodies against a narrow spectrum of antigenic determinants from the total cell population available (Herzenberg *et al.*, 1978). The technique appears to offer the possibility of detecting individual determinants within a class of antigen molecules.

Immunological specificity of the antiserum against its original antigen must be tested before use, and a wide range of methods have been used for plant antigens. Immunodiffusion in agar or agarose gel is the most common method and the precipitin patterns produced provide an indication of the nature of antigenic relationships. A unique feature of antigen-antibody interactions is that the antiserum can be absorbed with a related tissue extract to remove common determinants and enhance the specificity of the antibodies for their original antigen. This has been elegantly demonstrated by Huisman *et al.* (1977). Sepharose beads conjugated with antigens were used to absorb monovalent antisera against ferredoxin purified from chloroplasts of *Nicotiana tabacum*. These initially cross-reacted with ferredoxins from a wide range of other plants but retained determinants specific for *N. tabacum* after immunoabsorption.

Problems may arise in assessing the specificity of monospecific antisera raised to highly purified antigens (Fig. 1). The immunogenicity of minor contaminants in the preparation may be higher than that of the purified antigen resulting in the detection of additional precipitin bands by immunodiffusion. These will often be evident when control tests are made against the original tissue extract as antigen. However, some highly purified proteins will produce two or more precipitin lines reflecting differences in the antigenic determinants present in the macromolecule; or the presence of

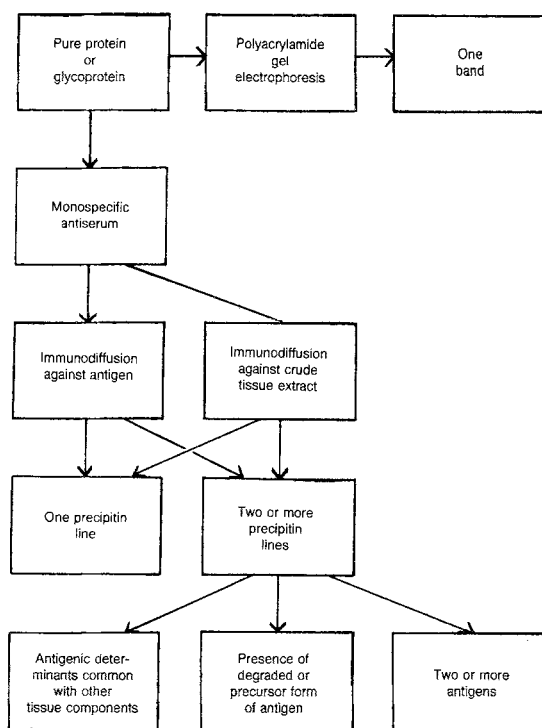


Fig. 1. Scheme of procedures for testing a purified plant antigen and the results. A protein that appears pure by polyacrylamide gel electrophoresis (native gel, SDS gel or isoelectric focusing gel, top right) may be used to produce an antiserum in rabbits. When tested by immunodiffusion, either against its original antiserum or against a crude tissue extract containing other tissue components, more than one band may be detected for the reasons given at base of diagram.

multiple forms that may be separable by electrophoresis, as found for α -amylase of barley aleurone layers (Jacobsen & Knox, 1973). SDS-PAGE revealed the presence of a single band (mol. wt. 55 000) which bound specifically to the antiserum, but two different precipitin arcs formed each associated with two of the four multiple electrophoretic forms of the enzyme revealed by starch gel electrophoresis. In another system, ferredoxin is readily denatured to apoferredoxin. This has been demonstrated both during antibody formation in the rabbit and during immunodiffusion at room temperature. In these situations, two bands were observed on immunodiffusion, whereas a single band was obtained with immunodiffusion at 2° C (Huisman *et al.*, 1977).

Some plant proteins have proved to be families of closely related macromolecules. A good example occurs in the storage globulins of legume seeds. Two major groups of proteins are present – legumin and vicilin, which were among the first antigens to be localized by immunofluorescence (Graham & Gunning, 1970). However, while

legumin has proved on purification to produce a single precipitin band by immunodiffusion, vicilin has proved to be a 'holoprotein' with four different bands (Millerd *et al.*, 1978; Thomson & Doll, 1979). Until now, very few studies have been carried out on purified plant proteins and glycoproteins, and these immunochemical tests provide a useful guide to antigen complexity.

Specificity of the antiserum may be further enhanced, especially for immunocytochemistry, by partial or complete purification of the IgG fraction of the antiserum. The total IgG fraction may be obtained by ammonium sulphate precipitation followed by DEAE chromatography (Harboe & Ingild, 1973; Baumgartner *et al.*, 1978). However, a simple one-step procedure may be preferable: affinity chromatography with staphylococcal Protein A-Sepharose according to the methods of Hjelm *et al.* (1972) and Goding (1976). The latter method has the further advantage of separating the low molecular weight Fab fragments from Fc fragments and whole antibody, in studies where tissue penetration of whole antibodies is a difficulty. Finally, monospecific antibodies directed against the specific antigen may be obtained by antigen-affinity chromatography using the method of Ternynck & Avrameas (1976). In the case of antiserum against vicilin peptidohydrolase, the specific IgG constituted about 2% of the total (Baumgartner *et al.*, 1978), representing a considerable amplification of antibody specificity.

ANTIBODY MARKERS

Suitable antibody markers for the light microscope include Fluorescein isothiocyanate (FITC), Lissamine Rhodamine B (LRB), tetramethyl rhodamine isothiocyanate (TRITC) and stilbene isothiocyanate (SITC). For fluorescence microscopy, it is essential that the best filter combinations be used. FITC gives an intense green fluorescence with blue or violet excitation, (365–405 nm) but autofluorescence of tissue components may be minimized by using short pass filters with emission at a higher wavelength between 485–500 nm; LRB providing more stable conjugates than TRITC and also having an advantage over FITC in giving less fading problems and reduced autofluorescence of tissue components at its higher excitation wavelength; stilbene a relatively new probe, gives a blue emission by excitation at 365–405 nm, and should be useful in double labelling techniques. These fluorescent markers have the advantage of giving the highest possible resolution in the light microscope, since the colours are viewed against a dark background. Following conjugation, it is important to check the affinity of the labelled antibody for its original antigen by immunodiffusion. Epi-illumination systems are now widely available for fluorescence microscopy and these provide the additional advantage of being suitable for the study of markers attached to uneven plant or cell surfaces, or in interacting cells or surfaces.

Other light microscope markers include enzymes such as horseradish peroxidase or acid phosphatase, which may be conjugated to the antibody, and their presence revealed by cytochemical simultaneous coupling procedures. Thus, the antigen is

detected by the enzyme activity as a coloured reaction product. This has the disadvantage that the colours are more difficult to detect in low concentration by bright-field microscopy and also that native enzymes present in the tissue must be inhibited prior to antigen detection. However, they have the advantage of quantitation by microdensitometry rather than the more sophisticated equipment needed for microfluorometry, and of ready adaptation for antigen detection by transmission electron microscopy.

The first antigen detected in flowering plants by TEM was the enzyme cellulase in epicotyls of pea, *Pisum sativum* (Bal *et al.*, 1976). The specific antibody was directly labelled with ferritin, a marker which is electron dense because of its high content of iron atoms. Each ferritin molecule contains approximately 3000 iron atoms, in the form of ferric hydroxide micelles of 6 nm diameter with a protein shell of 12 nm diameter, giving a molecular weight of 550 000. When covalently conjugated to IgG the molecular weight of the complex is about 10^6 .

Immunofluorescence methods have greater sensitivity in antigen detection than diffusion in gel methods because the binding of individual antibody molecules to antigen is detected, rather than extensive precipitates in a gel. On animal cell surfaces, the limit of detection has been estimated at between 100–1000 FITC molecules per cell surface by immunofluorescence and the amount of binding has been shown to be quantitatively related to the number of antigen molecules or their concentration and also to that of the antibody in the incubation medium. Soluble antigens can be effectively fixed by interaction with high concentrations of high affinity antibody – a technique that has been used to fix pollen antigens on the stigma during pollination (Knox & Heslop-Harrison, 1971a; Knox, 1973).

Quantitative estimates of the concentration of two different fluorescent markers, such as in double-labelling experiments with FITC and TRITC, may be achieved by the two-wavelength excitation method of Ploem (Cornelisse & Ploem, 1976). The fluorescence of the tissue is measured using a microscope fluorometer, first with lower wavelength blue excitation which causes both fluorochromes to fluoresce, and second with green excitation when only the red fluorescence is estimated. The values for green fluorescence are obtained by subtraction.

TISSUE PREPARATION

Immunocytochemical methods depend for their success on the integrity of the antigenic determinants in their cellular sites, which bind to the labelled antibody. These methods are basically of two types: pre-embedding staining procedures, in which whole cells, protoplasts, or tissue slices are incubated in labelled antibody; or post-embedding staining procedures, in which the cells or tissues are fixed, embedded and thin sections incubated in labelled antibody (Kraehenbuhl *et al.*, 1980). The former method suffers from the disadvantage that the antibody molecules have to diffuse into cut or whole cells, and may be unable to penetrate into uncut cells through the plasma membrane, giving false negative results. The latter method

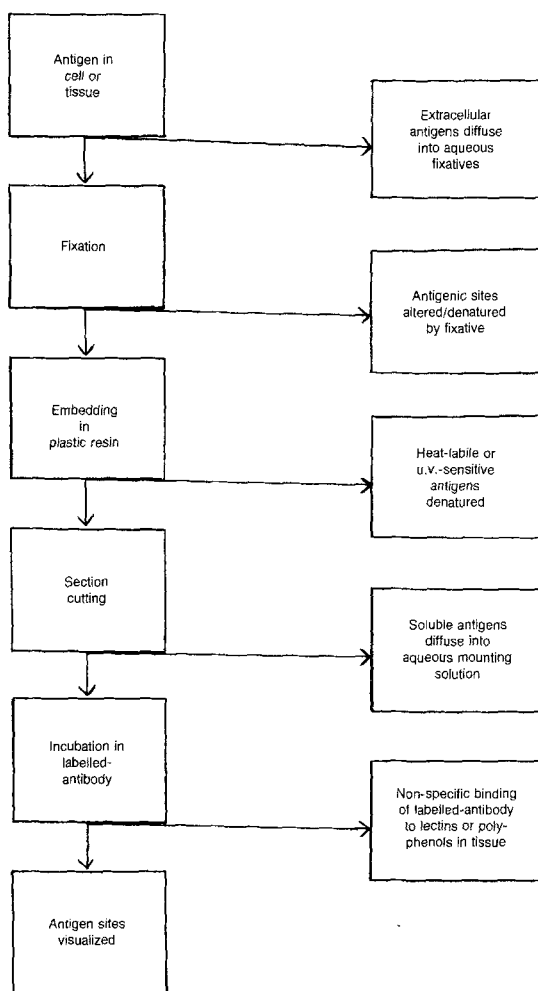


Fig. 2. Scheme showing the post-embedding staining procedure for immunofluorescence, and some of the artifacts that may occur at the various stages of processing with plant tissues.

permits access of antibody molecules to all cut surfaces, but depending on the supporting matrix, binding may occur only at the surface of the section. The problems likely to be encountered in using the post-embedding procedure in plant tissues are summarized in Fig. 2 and will now be briefly reviewed.

Effects of fixation on antigen-antibody affinity

Aldehyde fixatives, such as formaldehyde and glutaraldehyde, are efficient cross-linkers of proteins, and may be expected to have a deleterious effect on the antigen-antibody interaction, reducing the binding of antibody in animal cell studies (Nakane, 1973). Attempts to demonstrate the effects of fixatives on the

antigen-antibody interaction are not easy; Craig *et al.* (1979) have used immunodiffusion to assess the effects of aldehyde fixatives on a plant antigen *in vitro*, and found it retained its ability to form a precipitin line after similar fixation treatment given to tissues. Such experiments may not necessarily throw light on the situation *in vivo*, where the antigen may be closely associated with other proteins or glycoproteins, and may be affected differently by fixation. Both formaldehyde and glutaraldehyde are known to alter the structure of plant glycoproteins; for example, pollen allergens are modified to 'allergoids' which are immunologically different (Marsh, 1975). It is obviously essential that the effects of fixation need to be accurately monitored in immunocytochemical reactions, just as the effects of fixation on enzyme activity are an essential feature of enzyme cytochemistry.

A quantitative approach to this problem has recently been successfully employed with animal tissues and antigens by Kraehenbuhl *et al.* (1980), who has coupled the antigen to Sepharose beads, and demonstrated the effects of fixation on the immunocytochemical reactions of fixed beads. He found that even concentrations of glutaraldehyde as low as 0.5% were almost completely inhibitory to the immunological reaction in times as short as 15 min. He recommends the use of even lower concentrations of glutaraldehyde for short periods. Paraformaldehyde has been widely used in plant studies where chemical fixation has been employed (see Tables 1 and 2), though there is a trend apparent for use of minimum incubation periods at low temperatures.

Standard electron microscopic fixatives are directed against proteins and thus denature most antigens. Possibilities exist for designing a fixative which is directed against lipid and carbohydrate components of the cell structure, with less interaction with protein components. In this regard, partial success has been achieved with a periodate/lysine/paraformaldehyde solution where relatively weak antigens have been localized in relatively well-preserved animal tissue (Nakane, 1973). *p*-Benzoquinone and di-imidoesters are chemical fixatives that have yet to be explored for plant antigens (Pearse & Polak, 1975). Some of these have been partially successful in preserving the antigenicity of peptide antigens of animal tissues but none could match the ultrastructural preservation obtained with glutaraldehyde. The possibilities offered by freeze-substitution and modern methods of freeze-drying (Edelmann, 1979) are especially important for soluble antigens, and those that are sensitive to chemical fixation.

Ensuring antibody access to cellular sites of antigen

A problem in localization of intracellular antigens is to ensure that the labelled antibodies gain access to all potential sites of the antigen within the plant cell. This has been tackled in several ways: by diffusion into intact cells after removal of the wall by enzyme treatment (protoplast) or by preparing 'footprints' of the protoplast membrane and washing away the cytoplasm (Lloyd *et al.*, 1979); or by the crude

Table 1. Selected list of intracellular antigens of plants located by immunocytochemical methods. Fixation – formaldehyde (Form); glutaraldehyde (Glut). Technique – direct (D) or indirect (I) with fluorescent markers: Fluorescein isothiocyanate (FITC); Tetramethylrhodamine isothiocyanate (TRITC); Lissamine Rhodamine B (LRB); or electron-dense markers for transmission electron microscopy [ferritin (Ferr)].

<i>Organ, tissue or cell</i>	<i>Antigen</i>	<i>Fixation</i>	<i>Sectioning</i>	<i>Technique/ probe</i>	<i>Antigen site</i>	<i>Reference</i>
Seed cotyledon						
Broad bean	Legumin vicilin	Glut	Frozen	D-FITC	Protein bodies	Graham & Gunning (1970)
Jack bean	Urease	--	Razor blade	I-FITC	Protein bodies	Murray & Knox (1977)
Mung bean	Vicilin peptidohydrolase	Form	Ultracryotomy	I-LRB	Cytoplasm, protein bodies	Baumgartner <i>et al.</i> (1978)
Pea	Legumin	Form	GMA Plastic resin	I-RITC	Protein bodies	Higgins & Spencer (1977); Craig <i>et al</i> (1979)
Cereal grain						
Wheat	Water-soluble proteins	Form	Frozen	I-FITC	Endosperm cytoplasm	Barlow <i>et al.</i> (1973)
Barley	α -Amylase	--	Frozen	I-FITC	Aleurone cytoplasm	Jacobsen & Knox (1972, 1973)
	α -Amylase	Freeze-substitution	Paraffin	I-RITC		Jones & Chen (1976)
	Phytochrome	Form	Paraffin or polyethylene glycol	I-Peroxidase	Cytoplasm	Coleman & Pratt (1974)

Seed endosperm

Leucojum aestivum Tubulin Form --- I-FITC Mitotic spindle Franke *et al.* (1977)

Stem

Pea Cellulase (buffer soln) Form and glut Tissue slices D-Ferr ER Bal *et al.* (1976)

Leaves

Grasses RUDP-carboxylase Form Razor blade I-FITC Cytoplasm Hattersley *et al.* (1978)

Roots

Soybean nodules Leghaemoglobin Form and glut Tissue slices D-Ferr Cytoplasm of soybean cells Verma *et al.* (1979)

Protoplasts

Carrot callus Tubulin --- I-FITC Fibrilin cytoplasm Lloyd *et al.* (1979)

Sweet cherry callus Tissue homogenate --- I-FITC Protoplast surface Raff *et al.* (1979)

Green alga

Chara Actin --- I-FITC Microfilament bundles in cytoplasm Williamson & Toh (1980)

Table 2. Selected list of extracellular antigens of plants located by immunocytochemical methods. Abbreviations as Table 1.

Organ, tissue or cell	Antigen	Fixation	Sectioning	Technique/ probe	Cellular site of antigen	Reference
Pollen						
<i>Gladiolus</i>	Diffusible antigens	None	Frozen or 'pollen prints' in agarose	I-RITC I-FITC	Wall sites; exine and intine	Knox <i>et al.</i> (1970)
Ragweed,						Knox (1971)
<i>Cosmos</i> ,						Knox & Heslop-Harrison
Poplar,						(1971a,b); Knox <i>et al.</i>
<i>Phalaris</i> ,						(1972); Heslop-Harrison
<i>Malvaviscus</i>						<i>et al.</i> (1973)
<i>Phalaris</i> , <i>Cosmos</i> ,	Diffusible antigens	None	None	I-FITC	Pollen surface, pollen tube, and adjacent stigma surface	Knox & Heslop-Harrison
<i>Ragweed</i> , (pollinated stigmas)						(1971); Knox (1973)
Birch	Diffusible antigens	None	Whole grains	I-FITC	Pollen surface	Belin & Rowley (1971)
Ragweed	Antigen E	Freeze- substitution	Paraffin	I-FITC	Exine and intine	Howlett <i>et al.</i> (1973)

Ryegrass	Group 1 allergen, Antigen A	Methanol or freeze-dried	JB-4 plastic resin	D-FITC I-Ferr	Exine	This paper
Stigma						
Kale	Diffusible antigens	None	None	I-FITC	Surface	Heslop-Harrison <i>et al.</i> (1975)
Stem						
Pea	Cellulose (Buffer-insoluble)	Form and glut	Razor blade sections	D-Ferr	Cell wall and periplasmic space	Bal <i>et al.</i> (1976)
Seed						
Jack bean	Urease	None	Freeze-sectioning	I-FITC	Intercellular space	Murray & Knox (1977)
Brown alga						
Fucus	Alginic acid	None	GMA plastic resin	I-FITC	Cell walls	Vreeland (1972)
Slime mould						
<i>Dictyostelium</i>	Discoidin	Glut	None	I-Ferr	Cell surface	Chang <i>et al.</i> (1975)

technique of allowing the antibodies to diffuse into sectioned cut cells or tissue injured by mechanical damage or freeze-thawing. Alternatively, a more satisfactory solution is to embed the tissue and section it to ensure entry of antibodies to cut cell surfaces though this method also presents problems (Fig. 2).

The potential for infiltrating and embedding plant tissues in plastic resins for immunocytochemistry has recently been realised, as it permits antibody access to all cut surfaces in thin sections. Two main problems have been encountered in animal and plant studies: (1) denaturation of antigenic determinants during embedding procedures (Striker *et al.*, 1966) has been partially overcome by use of plastic resins that do not require heat for polymerization and are more water soluble allowing increased antibody access to the tissues; (2) non-specific binding of labelled antibodies to the surface of the plastic section (Striker *et al.*, 1966; Sternberger, 1974; Shahrabadi & Yamamoto, 1971), problems that have been largely solved by purification of resin (for example, Tippet & O'Brien, 1975) or by improved washing procedures after incubation.

When all these problems have been overcome, and successful antibody binding to the tissue achieved, the specificity of binding may be questioned because of a number of non-specific interactions that may occur with certain components of plant cells. Certain lectins, common components of plant cells, especially in seeds, may bind IgG (see Clarke *et al.*, 1975), and most immunocytochemical studies of seed antigens have involved considerable antibody purification to reduce total IgG levels (Graham & Gunning, 1970; Baumgartner *et al.*, 1978; Craig *et al.*, 1979). Non-specific binding to secondary-thickened cell walls, especially those of xylem vessels of vascular tissue is often encountered in immunofluorescence studies of plant tissues, and is presumably caused by binding of the antibody or probe to phenolic components which also bind basic dyes in histochemical studies. These non-specific interactions point to the importance of an adequate set of controls in the experimental design, which should include both normal (pre-immune) IgG and another standard protein such as albumin.

Applications of immunocytochemistry to plants

An interesting feature of many immunocytochemical studies of intracellular antigens (Table 1) is that seeds have provided the experimental organs, and the antigens investigated have been involved in laying down or degradation of reserves. Immunocytochemical methods provide the sole means of identifying these antigens in tissue sections, and of demonstrating the precise cell compartment in which they are located. In a recent case, Chrispeels & Baumgartner (1977) studied the catabolism of reserve proteins in mung beans, mediated by the enzyme vicilin peptidohydrolase, and their biochemical data shows that it appears in the germinating seeds and is involved in the hydrolysis of the protein bodies. Cell fractionation studies failed to establish conclusively the cellular location of the enzyme, but indirect rhodamine

immunofluorescence showed it to be present in cytoplasmic foci in the cotyledon parenchyma cells on day 3 after germination, finally appearing in the protein bodies on day 4. No fluorescent precipitins could be detected in these sites on day 1, in agreement with biochemical data. A unique feature of this study is the use of ultracryotome frozen sections cut at a thickness of 0.2–0.5 μm , of formaldehyde-fixed material, together with the use of monospecific antibodies obtained by antigen affinity chromatography of the IgG fraction of the antiserum, enabling reduction of incubation times in IgG to a total of 17 min. This was necessary because the IgG fraction and whole antiserum showed a high degree of non-specific binding, presumably to lectins in the tissue (Clarke *et al.*, 1975).

Several conclusions emerge from the compilation given in Table 1, of intracellular antigens of plants. Immunocytochemistry has now been employed successfully for a wide variety of plant cells and tissues, largely for the localization of a particular antigen that could not be readily localized by other cytochemical methods. This has necessitated the use of purified antigen preparations. Both fresh or frozen unfixed tissues and tissues briefly fixed in formaldehyde have been used. In only one case has the pre-embedding staining procedure been used with the plastic resin glycol methacrylate (Craig *et al.*, 1979). The favoured method has been indirect immunofluorescence with FITC as the antibody marker, although more recent studies have employed TRITC and LRB successfully with modern excitation filter systems.

A list of extracellular antigens is given in Table 2, and the pollen grain of flowering plants has been the most popular object of study. Extracellular sites of antigens include the cell wall, intercellular spaces and cell or organ surfaces. Results have been obtained with fresh or frozen sections, whole cells or organs and the post-embedding staining technique. In contrast to the studies of intracellular antigens (Table 1), aldehyde fixatives have only rarely been employed. These differences are explained by the nature of extracellular antigens, most of which are secretory products and show remarkable mobility. As in Table 1, the favoured immunocytochemical method has been the indirect method with FITC as marker.

CASE HISTORY: LOCALIZATION OF POLLEN ANTIGENS AND ALLERGENS

The first immunocytochemical studies of antigens of flowering plants were carried out on the proteins of pollen grains (Hagman, 1964; Knox *et al.*, 1970). These are the structures that house the male gametes of plants during the processes of pollination in their journey from the anther to the receptive stigma of the female pistil (Stanley & Linskens, 1974; Knox, 1979). In about one third of the families of flowering plants, the pollen is dispersed by air currents, and so is able to make contact with the upper respiratory tract of man, and allergens are released causing seasonal asthma and hay fever. During the past decade, immunocytochemical studies have revealed that the allergens of ragweed are located in the outer wall of the pollen

grain (Table 2). Immunofluorescence studies of pollen freeze-sectioned within the anther to minimize diffusion of antigens, has revealed that Antigen E, the principal allergen, a protein of molecular weight 38 000 (King *et al.*, 1974) is concentrated in the inner polysaccharide layer, the intine, and also in the exine, the outer surface layer where it occurs in both cavities in the wall and at the surface (Knox & Heslop-Harrison, 1971b). Freeze-substitution followed by paraffin-embedding with minimum exposure to high temperatures produced sections which gave the same localization in mature dry pollen as had been obtained with freeze-sectioned pollen within the anther (Howlett *et al.*, 1973) but without the hazard of diffusion artifacts.

When ragweed pollen was freeze-sectioned by preparing a slurry in 15% gelatine that is frozen rapidly within 30 s of pollen contact, all the antigens were found in the gelatine surrounding the grains. This extreme mobility of the antigens has been confirmed by making pollen prints of fresh pollen, to observe the routes and times of antigen emission. The pollen was sprinkled on adhesive tape, and placed in contact with a thin agarose gel on a microscope slide, and removed after varying intervals of time, the film dried rapidly and processed for Antigen E activity by indirect immunofluorescence. The pollen prints revealed that Antigen E is present in both the exine, from which it is immediately released, and intine at the apertures from which it is released more slowly during germination (Howlett *et al.*, 1973).

These observations on the kinetics of allergen release suggest that the antigens play a role in pollination: in male-female recognition at the stigma surface. An interesting adaptation of immunofluorescence has provided circumstantial evidence for this in both ragweed and grasses: pollinated stigmas were placed into specific anti-pollen diffusate sera in order to fix the antigens in their sites during the pollination process. The antigens were shown to be present both on the surface of pollen grains and tubes, but also bound to the stigma surface (Knox & Heslop-Harrison, 1971a; Knox, 1973).

Attempts to locate the allergens of grass pollen until now have been unsuccessful, although the antigens of developing pollen of *Phalaris* were found in both intine and exine sites (Knox & Heslop-Harrison, 1971a). Grass pollen is also dispersed in air currents, and is the major source of seasonal asthma and hay fever in spring and early summer in temperate climates. The pollen of ryegrass, *Lolium perenne*, contains four groups of allergens, named Groups I-IV (Johnson & Marsh, 1965; Marsh, 1975). Group I allergen is the major allergenic fraction: it is a glycoprotein, molecular weight 32 000, existing in multiple electrophoretic forms which differ only slightly in amino acid composition. In timothy and cocksfoot grasses, Augustin & co-workers (1963, 1971) isolated the I series of pollen antigens, with I₂₃ being apparently similar to Group I allergen of ryegrass. We have recently isolated a major allergenic fraction from ryegrass pollen, using ion exchange chromatography and gel filtration. This fraction has all the properties of Group I allergen including similar behaviour by starch gel electrophoresis, antigenicity and allergenic activity (Smart & Knox, 1980). Purified Group I allergen which gave a single protein band by SDS-PAGE was used

as an antigen for antiserum production in rabbits, and gave at least two precipitin bands when specific antiserum was tested against both the purified antigen and whole pollen extract by immunodiffusion and immunoelectrophoresis (Howlett *et al.*, 1980), thus confirming the observations of Lowenstein (1978).

The major antigen of ryegrass pollen, Antigen A, is noted for its low allergenic activity (Smart & Knox, 1980). It has been possible to isolate Antigen A by making use of its high isoelectric point (Howlett & Clarke, unpublished data), and it has proved to be a basic glycoprotein, mol. wt. 68 000. It exists as a single band by SDS polyacrylamide gel electrophoresis and antisera raised in rabbits gave a single precipitin line.

We have used both these antisera to locate these antigens within pollen grains by both immunofluorescence and immunoelectron microscopy. In the present study, a post-embedding staining technique was adopted to overcome the difficulties of antigen mobility in aqueous media. Anthers of ryegrass just prior to anthesis were either briefly fixed in cold methanol (2° C, 30 min) or freeze-dried and placed directly in JB-4 plastic resin (Polysciences, Inc), a water-soluble methacrylate, which has the advantage of short infiltration and embedding time at 2° C and of being anhydrous so that there should be little possibility of the antigens showing diffusion artifacts. Its greater water solubility presumably permitted better access to the labelled antibodies to the antigens, and it was known that the method was suitable for localization of hydrolytic enzymes in pollen grains (V. Vithanage & J. Heslop-Harrison, personal communication).

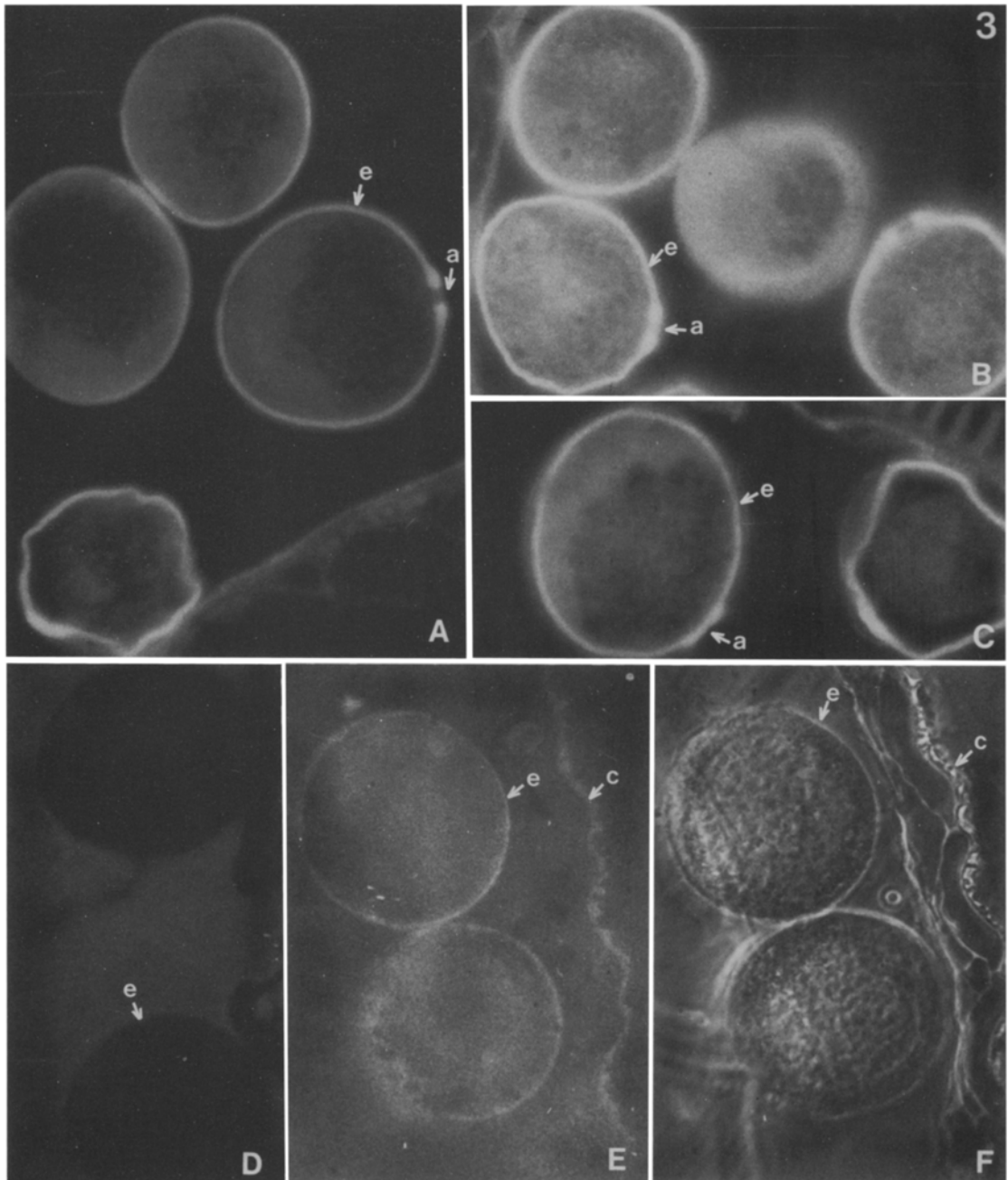
Specificity of the immunocytochemical reactions was enhanced by partial purification of the IgC fraction of the antisera using Protein A-Sepharose affinity chromatography, and by the use of direct immunofluorescence with FITC-labelled IgG, which reduced incubation times to 30 min. First results were disappointing with only low levels of fluorescence in both test and control sections. These sections had been floated in water in order to flatten them on the slides; when the dry sections were flattened onto dried 1% gelatin-coated slides by finger pressure or using methanol, some specificity of staining was obtained. In control sections, intense yellow-orange fluorescence was evident (Fig. 3A) which was identical with autofluorescence observed in unstained sections. This was present both using long-pass u.v. and blue exciter filters and KP500 short pass filters specifically designed for FITC use. After green excitation, the exine gave a bright red autofluorescence, so that substitution of Rhodamine-labelled antibody would not diminish the problem of autofluorescence. The autofluorescence appeared as two parallel lines, presumably representing the outer sexine (roof) and inner nexine (floor) layers of the exine. The intensity of the fluorescence was greatest at the germinal apertures. The autofluorescence is a common feature of pollen exines and is probably due to phenolic residues of the sporopollen wall polymer.

After treatment with specific FITC-labelled antibody, by both direct and indirect methods, some specific fluorescence was detectable in both the exine layer and in

cytoplasmic sites. In the outer exine, the intensity of the fluorescence was such that it appeared as a solid line, suggesting that the wall cavities were the sites of the antigen. Cytoplasmic fluorescence was also present, especially with anti-Antigen A IgG, although this was reduced with anti-Group I allergen IgG. The indirect method gave the greatest difference between test and control sections (Figs. 3B, C), presumably because it is known to amplify antigen detection. In order to determine the precise distribution pattern of the fluorescence from FITC-labelled antigen, cytoplasmic methods were tested to diminish or block autofluorescence of the exine. In a preliminary approach, a basophilic polychromatic dye of the thiazine series, Toluidine Blue, was used. It stains the phenolic compounds of the exine a dark green colour, presumably because of their negative charges. It was hoped that binding of the dye to the exine would diminish the level of autofluorescence. This was in fact achieved. Fig. 3D shows a normal IgG control section stained by the indirect immunofluorescence technique, then counterstained in Toluidine Blue at pH 5.0 in which all exine autofluorescence has been blocked. Unfortunately, background autofluorescence due to binding of the dye to the plastic resin has been increased. However, FITC-fluorescence is evident in test sections (Fig. 3E, F). This section was incubated with anti-Antigen A IgG and the specific FITC labelled antibody is seen bound to the exine and cytoplasmic sites. In a few grains, staining of the inner intine layer was also evident.

In order to determine precisely the sites of these antigens within the outer exine layer, immunoelectron microscopic methods were developed. The indirect method was used, with the anti-antibody labelled with ferritin (Capell Laboratories, Pennsylvania). Thick (40 μm) JB-4 sections were processed by the indirect method of immunoferritin labelling, allowing 30 min incubation in each IgG preparation. The sections were osmicated and embedded in Spurr's resin by standard methods, and ultrathin sections were cut at right angles to the original section. The results were spectacular. Using anti-Antigen A IgG, ferritin molecules were bound to spherical masses within the exine cavities and to wisps of material at the exine surface (Figs. 4A, B, 5). Sections incubated in normal (pre-immune) IgG followed by ferritin-labelled anti-rabbit IgG showed no binding in these wall sites (Figs. 4C, D). Post-staining of sections in lead citrate to enhance contrast resulted in the staining of particulate material in the micro-pores and micro-channels which cross the exine to both the surface and the inner intine layer. This staining occurred in both test and

Fig. 3. Indirect immunofluorescent localization of Group 1 allergen and Antigen A in cold methanol-fixed sections of ryegrass pollen mounted and flattened on slides using methanol. (A) Control incubated in normal rabbit IgG for 30 min, washed in phosphate-buffered saline (PBS) for 2×10 min and incubated in FITC-labelled goat anti-rabbit IgG (Behringwerke, Marburg, Germany) for 30 min, washed in PBS as before, distilled water, air-dried, mounted in Eukitt (Kindler, Freiburg) and viewed by incident fluorescence microscopy with Carl Zeiss KP500 excitor filter. The autofluorescence of the exine (e) is evident, especially at aperture (a) of grain. (B) Test incubated in anti-Antigen A IgG instead of normal rabbit IgG and with



anti-antibody as in (A). Specific fluorescence present in exine and cytoplasmic sites. (C) Test incubated in anti-Group 1 allergen and with anti-antibody as in (A). Specific fluorescence present largely in exine. (D)–(F) Observations of same sections after counter-staining in Toluidine Blue for 1 min to diminish autofluorescence. (D) as (A); (E) as (B) showing specific fluorescence in exine layer, fluorescence in outer cuticle (c) of anther was also present in control section; (F) fluorescence phase contrast image of (E) showing structural features of section. All photographs made by exposing Ilford HP4 film for 5 s, and printing negative on same grade of paper for same exposure time.

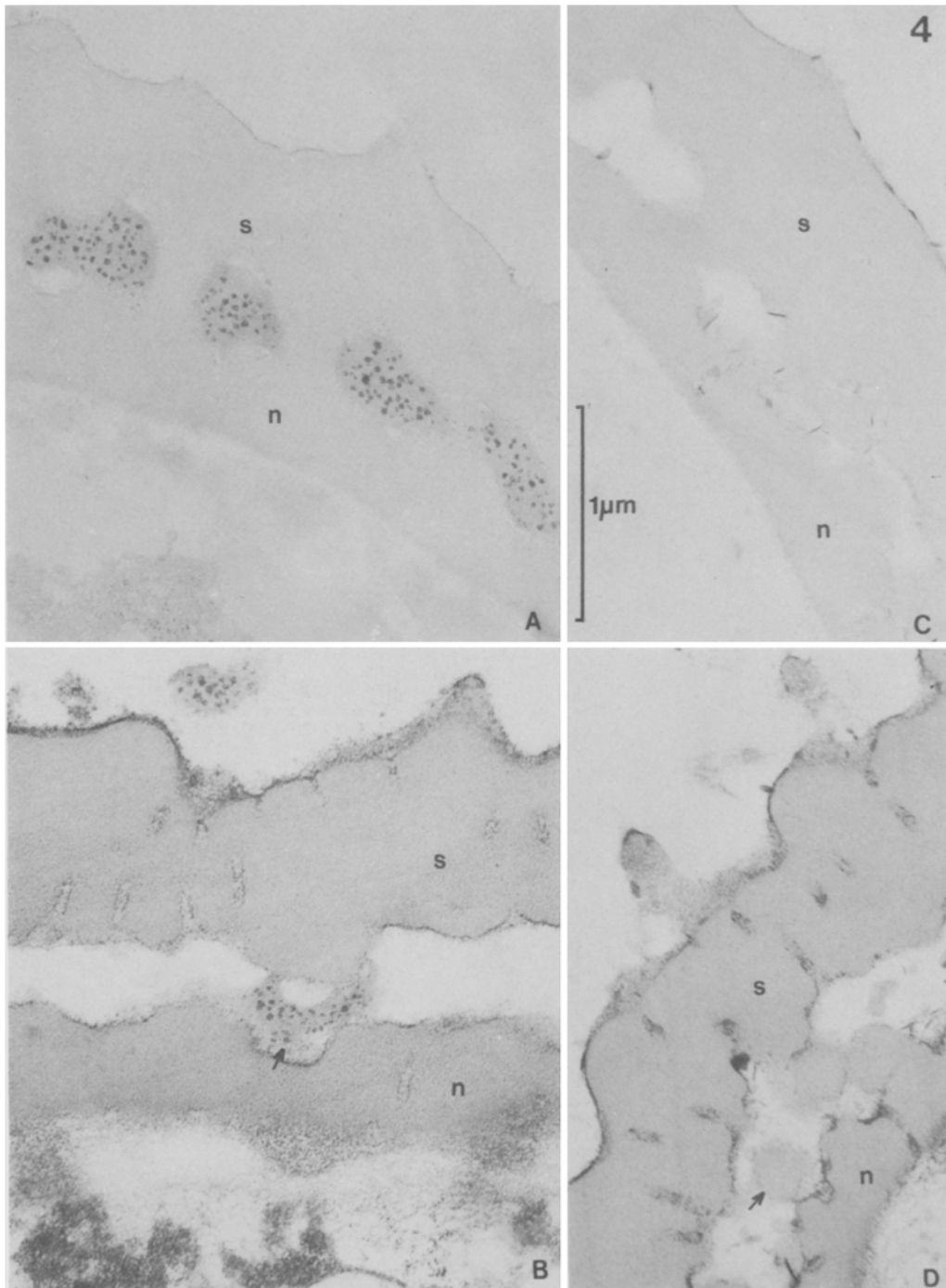


Fig. 4. Localization of Antigen A in ryegrass pollen by indirect immunoelectron microscopy using the post-embedding staining method. Ultrathin sections stained with uranyl acetate (A,C) and uranyl acetate and lead citrate (B,D). (A) and (B) Test incubated in anti-Antigen A IgG followed by ferritin-labelled anti-rabbit IgG (Capell Laboratories, Pennsylvania, U.S.A.) for 30 min each, with two washes of PBS for 10 min each between and after antibody incubations. Ferritin labelling is confined to spherical masses present in the cavities of the exine and on the surface of the grain; n, floor (nexine) of exine layer; s, roof (sexine) of exine layer. Ferritin appears to be absent from the inner wall layer, the intine, electron-lucent zone below nexine. (C) and (D) Control incubated in normal IgG followed by ferritin-labelled anti-rabbit IgG. No ferritin labelling is detectable.

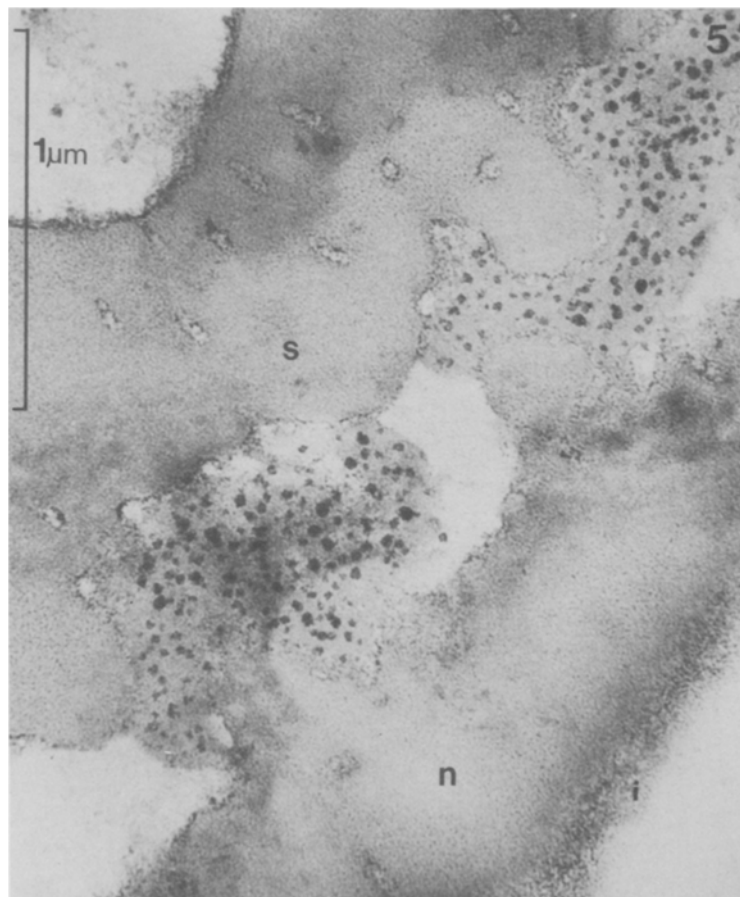


Fig. 5. Detail of section treated as Fig. 4B, showing ferritin labelling of spherical material within the exine cavities.

control sections (Figs. 4B, D, 5). These observations by immunoelectron microscopy confirm the antigen localization within the exine cavities that was suggested from results of immunofluorescence studies, and provides a precision in localization not possible by light microscopy in such small structures as the ryegrass pollen wall.

We have now demonstrated that the Group I allergen and Antigen A molecules are concentrated in the exine at the surface of mature pollen, presumably ready for a role in the early events of pollination, perhaps in recognition reactions at the stigma surface – or in the case of Group I allergen, in inducing allergic reactions in the upper respiratory tract of susceptible humans. The latter response is presumably an unfortunate accident of evolution. Developmental studies are now needed to determine the origin of these glycoproteins and to explain their apparent absence from the intine. Antigens have been detected in the intine in a number of pollen

types (see reviews by Knox *et al.*, 1975; Howlett *et al.*, 1979). Perhaps in mature grass pollen they have been transferred from the intine to the exine during development, or have originated by transfer from the diploid tapetal cells, as have the exine proteins of other pollen types. Certainly there seems to be no technical reason why antigens should not be detected in the intine in the JB-4 sections, as the intine marker enzyme, acid phosphatase, can be demonstrated cytochemically in the intine of these sections, just as in freeze-sectioned grains (Knox & Heslop-Harrison, 1971a).

The use of JB-4 plastic resin maximizes the exposure of all cellular sites to labelled antibodies, so that both intracellular and extracellular antigens may be readily detected, provided due care is taken to ensure that during fixation and embedding the procedures used minimize diffusion artifacts or loss of antigenic activity. These difficulties can now be assessed quantitatively *in vitro* using immobilized antigen attached covalently to sepharose beads (Kraehenbuhl *et al.*, 1980).

Conclusions

Immunocytochemistry often provides the sole means of localizing the cellular sites of an antigenic plant protein, glycoprotein or polysaccharide. It is essential that the antiserum used be tested for its immunological specificity by diffusion in gel techniques. This is especially necessary with antisera directed against a purified protein which may, when tested, comprise more than one antigen.

Improved specificity of antigen-antibody binding has been achieved both by fractionation of the total IgG and by isolation of specific IgG by antigen affinity chromatography. Such methods both shorten the incubation time and improve the specificity of staining and reduce non-specific binding to other plant cell components. In the future, specificity of antibodies will be further enhanced by the availability of monoclonal antibodies against plant antigens, which will provide a means of detecting specific antigenic determinants.

It is essential to use markers offering the highest possible resolution either for light or electron microscopy. Fluorescent probes offer highest resolution by light microscopy, with green (FITC), red (LRB, TRITC) or blue (SITC) markers viewed against a dark background, and may be assessed quantitatively by the two-wave length excitation method of Ploem (Cornelisse & Ploem, 1976). Ferritin is the sole probe that has been used to date for immunoelectron microscopy of plant tissues. Penetration of labelled antibodies into plant cells presents difficulties which have been partly solved by the use of (1) cut or damaged cells; (2) cultured callus cells in which the cell wall is degraded by treatment with appropriate enzymes; (3) techniques which reveal the internal face of the plasma membrane of protoplasts; and (4) sectioning procedures, especially freeze-sectioning and post-embedding staining methods with water-soluble plastic resins at low temperatures.

Extracellular antigens of plants, especially the antigens and allergens of pollen grains, have proved particularly difficult to detect by immunofluorescence without

diffusion artifacts. Use of anhydrous processing and the post-embedding staining method have successfully overcome artifacts caused by antigen solubility in aqueous media, and provided a means of antigen visualization by both light and transmission electron microscopy.

Acknowledgements

It is a pleasure to thank Professor J. Heslop-Harrison, FRS and Dr Adrienne Clarke for valued discussions; and the Australian Research Grants Committee, the Sir A. E. Rowden-White Foundation and Graduate Studies Committee of the University of Melbourne for financial support.

References

- AUGUSTIN, R. (1963) Antigens and other allergens of grass pollens. *Proc. 5th Eur. Congr Allergy, Basle* (Sellostverlag der Schweizerschen Allergie-Gesellschaft), pp. 137–46.
- AUGUSTIN, R., O'SULLIVAN, S. & DAVIES, E. (1971) Isolation of grass pollen antigens failing to induce IgE reagin formation though capable of inducing IgG antibody formation. *Int. Archs Allergy appl. Immun.* **41**, 144–7.
- BAL, A. K., VERMA, D. P. S., BYRNE, H. & MACLACHLAN, G. A. (1976) Subcellular localization of celluloses in auxin-treated pea. *J. Cell Biol.* **69**, 97–105.
- BARLOW, K. K., SIMMONDS, D. H. & KENRICK, K. G. (1973) The localization of water-soluble proteins in the wheat endosperm as revealed by fluorescent antibody techniques. *Experientia* **29**, 229–31.
- BAUMGARTNER, B., TOKUYASU, K. T. & CHRISPEELS, M. J. (1978) Localization of vicilin peptidohydrolase in the cotyledons of mung bean seedlings by immunofluorescence microscopy. *J. Cell Biol.* **79**, 10–9.
- BELIN, L. & ROWLEY, J. R. (1971) Demonstration of birch pollen allergen from isolated pollen grains using immunofluorescence and a single radial immunodiffusion technique. *Int. Archs. Allergy appl. Immun.* **40**, 754–69.
- CHANG, C. M., REITHERMAN, R. W., ROSEN, S. D. & BARONDES, S. H. (1975) Cell surface location of discoidin. A developmentally regulated carbohydrate-binding protein from *Dictyostelium discoideum*. *Expl Cell Res.* **95**, 136–42.
- CHRISPEELS, M. J. & BAUMGARTNER, B. (1977) Trypsin inhibitor in mung bean cotyledons, purification; characteristics, sub-cellular localization and metabolism. *Plant Physiol.* **61**, 617–23.
- CLARKE, A. E. & KNOX, R. B. (1979) Plants and immunity. *Devl comp. Immun.* (in press).
- CLARKE, A. E., KNOX, R. B. & JERMYN, M. A. (1975) Localization of lectins in legume cotyledons. *J. Cell Sci.* **19**, 157–67.
- COLEMAN, R. A. & PRATT, L. H. (1974) Electron microscopic localization of phytochrome in plants using an indirect antibody-labelling technique. *J. Histochem. Cytochem.* **22**, 1039–46.
- COONS, A. H., CREECH, H. J., JONES, R. N. & BERLINER, E. (1942). The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J. Immun.* **45**, 159–70.
- COOPER, E. (1976) *Comparative Immunology*. Prentice-Hall, New Jersey.
- CORNELISSE, C. J. & PLOEM, J. S. (1976) A new type of two-color fluorescence staining for cytology specimens. *J. Histochem. Cytochem.* **24**, 72–81.

- CRAIG, S., GOODCHILD, D. & MILLER, A. (1979) Immunofluorescent localization of pea storage proteins in glycol-methacrylate embedded tissue. *J. Histochem. Cytochem.* (in press).
- EDELMANN, L. (1979) Gefriertrocknung von chemisch nicht fixiertem biologischem Material für die elektronenmikroskopie. *Mikroskopie* **35**, 31–6.
- FRANKE, W. W., SEIB, E., OSBORN, M., WEBER, K., HERTH, W. & FALK, H. (1977) Tubulin-containing structures in the anastral mitotic apparatus of endosperm cells of the plant *Leucojum aestivum* as revealed by immunofluorescence microscopy. *Cytobiologie* **15**, 24–48.
- GODING, J. (1976) Conjugation of antibodies with fluorochromes: modification to the standard methods. *J. Immun. Meth.* **13**, 215–26.
- GRAHAM, T. A. & GUNNING, B. E. S. (1970) Localization of legumin and vicilin in bean cotyledon cells using fluorescent antibodies. *Nature* **228**, 81–2.
- HAGMAN, R. (1964) The use of disc electrophoresis and serological reactions in the study of pollen and style relationships. In *Pollen Physiology and Fertilization* (edited by LINSKENS, H. F.), pp. 244–250. Amsterdam: North Holland.
- HARBOE, N. & INGILD, A. (1973) Immunization, isolation of immunoglobulins, estimation of antibody titre. *Scand. J. Immunol.* **2**, Suppl. 1, 161–4.
- HATTERSLEY, P., WATSON, L. & OSMOND, C. B. (1977) *In situ* immunofluorescent labelling of Ribulose -1, 5- biphosphate carboxylase in leaves of C₃ and C₄ plants. *Aust. J. plant Physiol.* **4**, 523–39.
- HERZENBERG, L. A., HERZENBERG, L. A. & MILSTEIN, C. (1978) Cell hybrids, of myelomas with antibody forming cells and T-lymphomas with T-cells. In *Handbook of Experimental Immunology* (edited by WEIR, D. M.), Vol. 22. pp. 1–7. 3rd edn. Oxford: Blackwell.
- HESLOP-HARRISON, J., HESLOP-HARRISON, Y., KNOX, R. B. & HOWLETT, B. (1973) Pollen-wall proteins: 'gametophytic' and 'sporophytic' fractions in the pollen walls of Malvaceae. *Ann. Bot.* **37**, 402–12.
- HESLOP-HARRISON, J., KNOX, R. B., HESLOP-HARRISON, Y. & MATTSSUN, O. (1975) Pollen-wall proteins: emission and role in incompatibility responses. In *Biology of the Male Gamete* (edited by DUCKETT, J. G. & RACEY, P. A.), pp. 189–204. London: Academic Press.
- HIGGINS, T. J. & SPENCER, D. (1977) Cell free synthesis of pea seed proteins. *Plant Physiol.* **60**, 655–70.
- HJELM, H., HJELM, K. & SJOQUIST, J. (1972) Protein A from *Staphylococcus aureus*. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. *FEBS Lett.* **28**, 73–6.
- HOWLETT, B. J., KNOX, R. B. & HESLOP-HARRISON, J. (1973) Pollen wall proteins: release of the allergen Antigen E from intine and exine sites in the pollen grains of ragweed and *Cosmos*. *J. Cell Sci.* **13**, 603–19.
- HOWLETT, B. J., VITHANAGE, H. I. M. V. & KNOX, R. B. (1979) Pollen antigens, allergens and enzymes. *Curr. Adv. plant Sci.* **35**, 1–17.
- HUISMAN, J. G., BERNARDS, A., LIEBREGTS, P., BEGGINK, M. G. T. & STEGWEE, D. (1977) Qualitative and quantitative immunofluorescence studies of chloroplast ferredoxin. *Planta* **137**, 279–86.
- JACOBSEN, J. V. & KNOX, R. B. (1972) Cytochemical localization of gibberellic acid-induced enzymes in the barley aleurone layer. In *Plant Growth Substances 1970* (edited by CARR, D. J.), pp. 344–351. Berlin, Heidelberg, New York: Springer-Verlag.
- JACOBSEN, J. V. & KNOX, R. B. (1973) Cytochemical localization and antigenicity of α -amylase in barley aleurone tissue. *Planta* **112**, 213–4.
- JOHNSON, P. & MARSH, D. G. (1965) The isolation and characterisation of allergens from the pollen of rye grass (*Lolium perenne*). *Eur. Polymer J.* **1**, 63–77.

- JONES, R. L. & CHEN, R. F. (1976) Immunohistochemical localization of α -amylase in barley aleurone cells. *J. Cell Sci.* **20**, 183–96.
- KHAVKIN, E. E., MISHARIN, S. I., IVANOV, Y. N. & DANOVICH, K. N. (1977) Embryonal antigens in maize caryopses: the temporal order of antigen accumulation during embryogenesis. *Planta* **135**, 225–31.
- KING, T. P., NORMAN, P. S. & TAO, N. (1974) Chemical modifications of the major allergen of ragweed pollen, Antigen E. *Immunochemistry* **11**, 83–92.
- KNOX, R. B. (1971) Pollen-wall proteins: localization, enzymic and antigenic activity during development in *Gladiolus* (Iridaceae). *J. Cell Sci.* **9**, 209–37.
- KNOX, R. B. (1973a) Pollen proteins: cytochemical observations of pollen-stigma interactions in ragweed and *Cosmos* (Compositae). *J. Cell Sci.* **12**, 421–43.
- KNOX, R. B. (1973b) Localization of proteins in plant cells by immunofluorescence. *Zeiss Information, Oberkoehe* **20**, 52–5.
- KNOX, R. B. (1979) *Pollen and Allergy*. Studies in Biology no. 107. London: Edward Arnold.
- KNOX, R. B. & CLARKE, A. E. (1978) Localization of proteins and glycoproteins by binding to labelled antibodies and lectins. In *Electron Microscopy and Cytochemistry of Plant Cells* (edited by HALL, J. L.), pp. 149–185. Amsterdam: Elsevier-North Holland.
- KNOX, R. B. & HESLOP-HARRISON, J. (1971a) Pollen-wall proteins: fate of intine-held antigens in compatible and incompatible pollinations of *Phalaris tuberosa* L. *J. Cell. Sci.* **9**, 239–51.
- KNOX, R. B. & HESLOP-HARRISON, J. (1971b) Pollen-wall proteins: localization of antigenic and allergenic proteins in the pollen grain walls of *Ambrosia* spp. (ragweeds). *Cytobios* **4**, 49–54.
- KNOX, R. B., HESLOP-HARRISON, J. & REED, C. E. (1970) Localization of antigens associated with the pollen grain wall by immunofluorescence. *Nature* **225**, 1066–8.
- KNOX, R. B., HESLOP-HARRISON, J. & HESLOP-HARRISON, Y. (1975) Pollen-wall proteins. In *The Biology of the Male Gamete* (edited by DUCKETT, J. G. and RACEY, P. A.), pp. 177–87. London, New York: Academic Press.
- KRAEHNBUHL, J. P., RACINE, L. & GRIFFITHS, P. W. (1980) Attempts to quantitate immunocytochemistry at the electron microscope level. *Histochem. J.* **12**, 317–32.
- LLOYD, C. W., SLABAS, A. R., POWELL, A. J., MACDONALD, G. & BADLEY, R. A. (1979) Cytoplasmic microtubules of higher plant cells visualized with anti-tubulin antibodies. *Nature* **279**, 239–41.
- LOOMIS, W. D. & BATAILLE, J. (1966) Plant phenolic compounds and the isolation of plant enzymes. *Phytochem.* **5**, 423–8.
- LOWENSTEIN, H. (1978) Quantitative immunoelectrophoretic methods as a tool for the analysis and isolation of allergens. *Prog. Allergy* **25**, 1–62.
- MARSH, D. G. (1975) Allergens and the genetics of allergy. In *The Antigens* (edited by SELA, M.), Vol. 3, 271–359. London: Academic Press.
- MILLERD, A., THOMSON, J. A. & SCHROEDER, H. E. (1978) Cotyledonary storage proteins in *Pisum sativum*. III. Patterns of accumulation during development. *Aus. J. plant Physiol.* **5**, 281–94.
- MURRAY, D. R. & KNOX, R. B. (1977) Localization and antigenicity of urease in the cotyledons of jack bean, *Canavalia ensiformis*. *J. Cell Sci.* **26**, 9–18.
- NAKANE, P. K. (1973) Ultrastructural localization of tissue antigens, with the peroxidase-labelled antibody method. In *Electron Microscopy and Cytochemistry* (edited by WISSE, E., DAEMS, W. Th., MOCENAAR, I. & VAN DUIJN, P.), pp. 129–143. Amsterdam: North-Holland.
- PEARSE, A. G. E. & POLAK, J. M. (1975) Bifunctional reagents as vapour and liquid-phase fixatives for immunohistochemistry. *Histochem. J.* **7**, 179–86.

- RAFF, J. W., HUTCHINSON, J. F., KNOX, R. B. & CLARKE, A. E. (1979) Cell recognition: antigenic determinants of plant organs and their cultured callus cells. *Differentiation* **12**, 179–86.
- SAFONOV, V. I. & SAFONOVA, M. (1968) Isolation of soluble protein preparations from the vegetative organs of plants for electrophoretic investigation (in Russian). *Fiziol. Rastenii* **16**, 161–6.
- SHAHRABADI, M. S. & YAMAMOTO, T. (1971) A method for staining intracellular antigens in thin sections with ferritin-labelled antibody. *J. Cell Biol.* **50**, 246–50.
- SMART, I. J. & KNOX, R. B. (1980) Rapid batch fractionation of ryegrass pollen allergens. *Int. Archs Allergy appl. Immun.* (in press).
- STANLEY, R. G. & LINSKENS, H. F. (1974) Pollen: biology, biochemistry and management. Berlin, Heidelberg, New York: Springer-Verlag.
- STERNBERGER, L. A. (1974) *Immunocytochemistry*. Englewood Cliffs, New Jersey: Prentice-Hall.
- STRIKER, G. E., DONATI, E. J., PETRALI, J. P. & STERNBERGER, L. A. (1966) Postembedding staining for electron microscopy with ferritin-antibody conjugates. *Expl Mol. Pathol. Suppl.* **3**, 52.
- TERNYNCK, T. & AVRAMEAS, S. (1976) Polymerization and immobilization of proteins using ethylchloroformate and glutaraldehyde. *Scand. J. Immun. Suppl.* **3**, 29–36.
- THOMSON, J. A. & DOLL, H. (1979) Genetics and evolution of seed storage proteins. In *Seed Protein Improvement in Cereals and Grain Legumes*. Vol. 1. pp. 109–124. Vienna: I. A. E. A.
- TIPPETT, J. T. & O'BRIEN, T. P. (1975) A simple procedure for purifying 2-hydroxy ethyl methacrylate and some methods for using it in plant histology. *Lab. Practice* **24**, 239–45.
- VERMA, D. P. S., KAZAZIAN, V., ZOGBI, V. & BAL, A. K. (1978) Isolation and characterization of the membrane envelope enclosing the bacteroids in soybean root nodules. *J. Cell Biol.* **78**, 919–36.
- VREELAND, V. (1972) Immunocytochemical localization of the extracellular polysaccharide alginic acid in the brown seaweed, *Fucus distichus*. *J. Histochem. Cytochem.* **20**, 358–64.
- WACHSMUTH, E. D. (1979) Principles of immunocytochemical assays. *Proc. R. Microsc. Soc.* **14**, 252–5.
- WILLIAMSON, R. E. & TOH, B. H. (1980) Motile models of plant cells and the immunofluorescent localization of actin in a motile *Chara* cell model. In *Cell Motility: Molecules and Organization* (edited by HATARO, S., ISHIKAWA, H. and SATO, H.). Tokyo: University of Tokyo Press.