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REVIEW

Antibodies in plant science

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CONTENTS

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INTRODUCTION

Antibodies are useful for the detection, measurement and purification of intracellular and extracellular constituents of plants and of their pathogens. Their high sensitivity coupled

with a potential for high specificity, can be used to indicate the presence and amount of a given antigen in whole cells, tissues, and in crude and purified extracts of plant material. Antibodies are useful in plant biochemistry, cell and molecular biology to localize key cellular constituents (enzymes, structural proteins, etc.), to quantify developmental or other changes and to identify specific gene products. Their usage in plant pathology is also growing where antigen detection provides a powerful and specific tool for identification of disease at an early stage.

In this review, we seek to identify those areas where the use of immunological techniques can be an invaluable aid to plant scientists and to outline techniques and approaches available for dealing with a variety of problems. We do not seek to provide an exhaustive coverage of the literature but instead have included those general texts, reviews and papers which illustrate the techniques we have found to be useful in research in plant biochemistry, cell biology, molecular biology and pathology.

ANTIBODIES IN PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

Antibodies provide a sensitive and very specific method for the identification and measurement of plant constituents. They can also be used as specific inhibitors, because binding to an antigen frequently results in steric effects which inhibit the activity of the antigen of interest. Antibody–antigen binding also provides a basis for affinity purification of antigens. It is therefore not surprising that antibodies are proving to be a useful and powerful tool in both biochemistry and molecular biology. Examples of their application are presented in Table 1 and include work on enzymes, structural proteins, carbohydrates, growth regulators, signal receptors and many other cell constituents. Further discussion will deal with a consideration of the strategies which can be employed to produce and use antibodies in plant biochemistry and molecular biology, and the problems likely to be encountered in their use.

Preparation of antigens

In general, the starting point for workers in plant biochemistry and molecular biology will be a well defined (although not necessarily completely pure) antigen. Frequently, purification of antigen will be possible and (providing this does not result in prohibitively small yields) a pure antigen (determined using a number of different criteria) is the best starting point for antibody production. Antigen-enriched fractions are also a possible starting point for monoclonal antibodies if subsequent screening procedures are available to permit selection of antibodies of interest; it is also possible to purify antibodies from a mixed polyclonal antibody preparation such that a monospecific antibody is obtained.

An alternative approach to careful purification of antigen from a plant tissue is its preparation from an alternative source, perhaps from tissue from another kingdom. Where the homology of antigens between species makes it possible, it eliminates many of the problems of contaminant antigens, as (for instance) mammalian contaminants are less likely to be present in plant cells. In many instances, much higher yields of antigen are possible from alternative sources, so that higher titre antibodies can be obtained. Similarly, the use of antigens from another species may well be useful in screening and purifying antibodies raised against a purified or mixed plant antigen preparation.

Native protein, synthetic peptides or plant proteins expressed in bacterial systems?

The techniques of plant molecular biology have provided alternative routes for obtaining immunogen in addition to purification directly from plant cells. If facilities (and sufficient protein!) are available, peptide sequencing will yield sufficient information for the production of comparatively large amounts of synthetic peptides which can then be used as antigen. These will be pure, but care is needed to ensure that synthetic peptides are produced which represent a unique region of the antigen and not one which is present in a variety of proteins. The ability to identify cDNA encoding the antigen of interest permits the use of plant proteins expressed in bacterial systems. These are obtained from cDNA expression libraries, generally in *Escherichia coli*; methodologies for preparation of such libraries and identifying clones that express the protein are presented elsewhere (e.g. Sambrook *et al.* 1989) and will not be further considered here. The use of bacterially expressed protein is a useful alternative when only small amounts of native plant protein are available; the protein produced will require further purification but this is likely to be more straightforward as it will be present in excess. Drawbacks of the method are the possibilities that the tertiary structure of the expressed protein may differ from that in the plant and the cost and effort required to produce and authenticate the expressed protein. Given sufficient resources, all three methods are worth using; the last two may prove to be successful if the use of native plant protein proves impossible.

Monoclonal versus polyclonal

A good monospecific polyclonal antiserum frequently affords many advantages over other methods, at least in terms of titre, recognition of antigen in many extracted/purified forms and ease of preparation and handling. Rabbits are ideal for polyclonal antibody production as the total quantity of serum available is quite large (20–50 ml) and yet immunization doses are relatively small. However, unlike monoclonal antibodies, the total amount of antibody-containing serum available is limited and production of further antibody requires a new injection series into another rabbit and may well result in the production of antibodies with different properties and/or titre. The amounts available can be increased by using larger animals (e.g. sheep, goats) but few laboratories have the resources needed to handle such animals. Affinity purification of antibodies is possible and can yield a very specific high-titre preparation.

Monoclonal antibodies for plant biochemistry and molecular biology require a significantly greater investment in resources than equivalent polyclonal antisera. Their advantages are several: large quantities of antibody may be obtained over many years which do not vary in properties; antibodies directed to just one epitope of the antigen can be obtained, permitting study of structure/function relationships; immunogen need not be pure as a highly selective screening protocol can be used. Monoclonal antibodies have drawbacks however, in addition to cost and time in production, the fact that a given antibody preparation only recognizes one epitope may lead to lack of apparent specificity or to loss of antigen recognition if that epitope is altered, for instance by fixation or in eletrophoretic procedures. It may also lead to a titre apparently lower than for polyclonal antiserum which contains antibodies that recognize a number of sites in the antigen.

Purification of antigens, criteria of purity and hapten conjugation

A wide variety of techniques are available to purify antigens, depending on their nature. Affinity chromatography is particularly useful as a method for rapid and efficient

Table 1. Some examples of uses of antibodies in plant biochemistry/molecular biology

Antigen	Antibody type	Reference
Plant growth substances (indole-3-acetic acid, abscisic acid, cytokinins, gibberellins)	Monoclonal and Polyclonal	See Weiler *et al.* 1986 for references
Cell wall polysaccharides	Polyclonal	Northcote *et al.* 1989
Isocitrate lyase	Polyclonal	Martin & Northcote 1982
Endopolygalacturonase	Polyclonal	Grierson *et al.* 1985
Auxin-binding protein	Monoclonal and Polyclonal	Napier *et al.* 1988
	Polyclonal	Löbler & Klambt 1985
Phytochrome	Monoclonal and Polyclonal	See refs in: Thomas *et al.* 1986
Plasma membrane H^+ transporter	Polyclonal	Oleski & Bennett 1987
Plasma membrane Ca^{2+} transporter	Monoclonal and Polyclonal	Briars *et al.* 1988; Evans *et al.* 1989

purification (possibly coupled with other preparative steps) as it usually produces a high yield of relatively pure antigen in a short time. Gel electrophoresis is also a useful final preparative step for the preparation of peptide antigens after initial purification; bands cut from polacrylamide gels are frequently strongly antigenic (especially as the acrylamide present acts to stimulate the immune system of the experimental animal) and relatively pure antigen is present. Other antigens may be purified by any of the major chromatographic techniques, which may also be used to determine purity prior to injection; here, as with peptide antigens, the experience of the investigator will indicate the best techniques to use.

Production of antibodies to small molecules ($< 5{,}000$), which are not strongly antigenic or which are rapidly degraded, requires that they are conjugated to a large, more highly antigenic molecule prior to immunization. Such small molecules are known as haptens and are generally conjugated to large proteins, such as bovine serum albumin, ovalbumin, keyhole limpet haemocyanin and thyroglobulin. The techniques used for hapten conjugation depend on the nature of the hapten; functional groups such as $—NH_2$, —COOH, —OH or —SH permit the hapten to be covalently linked to a protein (Bauminger and Wilchek 1980; Kabakoff 1980; Bodanszky & Bodanszky 1984; Tijssen 1985). If the hapten lacks such groups then derivatization procedures may be used to introduce a functional group and permit conjugation. Hapten conjugation techniques have been used in plant science to raise antisera and monoclonal antibodies to a number of plant growth substances. The plant growth substances involved and the techniques used are described extensively by Weiler *et al.* (1986).

Experimental animals should preferably be immunized using antigen dialysed into phosphate buffered saline (PBS) and in the absence of toxins (e.g. sodium azide), which may have been added during purification. Adjuvants (compounds intended to provoke a strong immune response) should be avoided if at all possible but may be necessary if the antigen is weakly immunogenic or in small quantities. Full details of immunization procedures are presented in Harlow & Lane (1988).

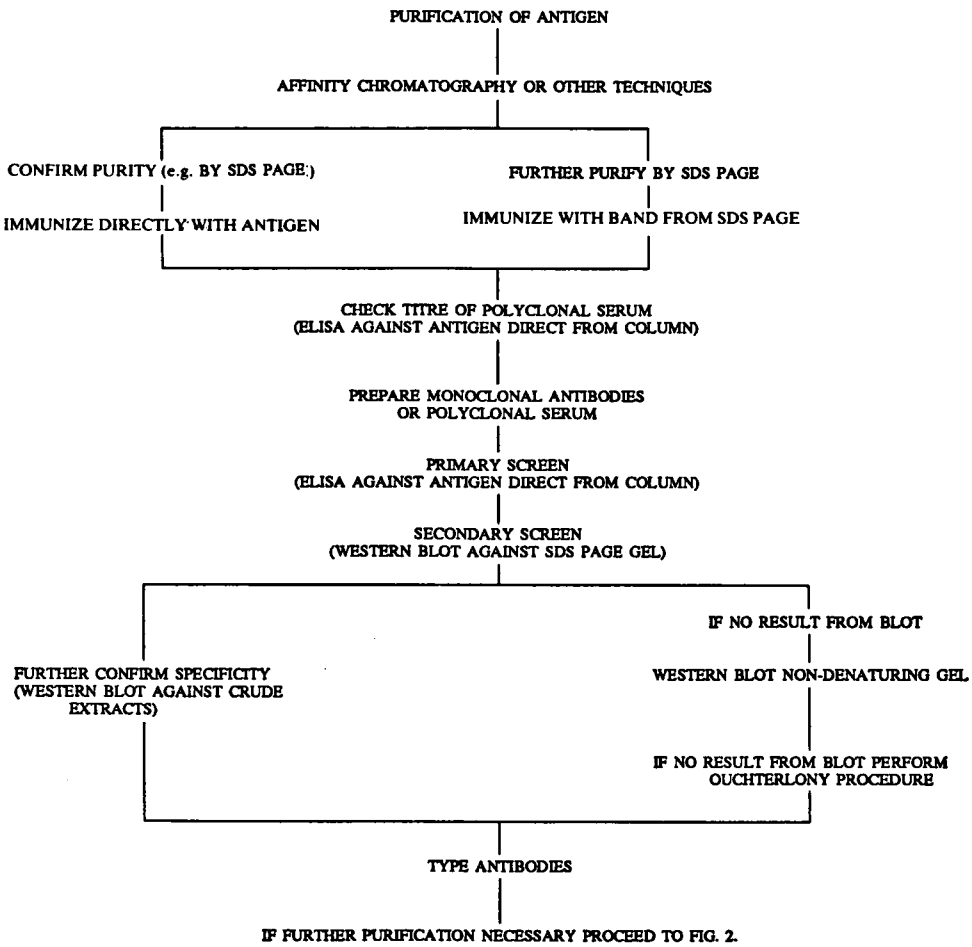

Fig. 1. Flow chart outlining the major steps in the production of antibodies.

The amounts of antigen required to induce an immune response vary depending on the antigen and the animal in which antibodies are to be raised. Our own experience has indicated that three injections at monthly intervals of 0·1–1·0 mg plant protein into rabbits produced a good antibody titre, while about three injections at 2-week intervals of 6–60 μg of purified plant plasma membrane calcium pump into mice gave a weak response from which viable monoclonal antibodies could be raised. These figures and immunization schedules should only be taken as a guide and will need to be amended depending on the antigen; amounts in excess of 1 mg antigen per injection are, however, seldom required. Larger total amounts of antigen will also increase the possible effects of any contaminants; for instance 10 μg of a contaminant present as 1% of the antigen would be injected in a 1 mg boost, sufficient to evoke an immune response. Immunization schedules should be monitored carefully, by taking a pre-immune bleed and trial bleeds and carrying out primary screening (see below and Fig. 1). The injection should stop when a high titre is obtained as further injection is likely to increase the likelihood of the presence of contaminant antibodies.

Table 2. Uses of antibodies in plant biochemistry and molecular biology

Method	Label on second antibody	Suitable for measurement?
Immunosorbent assays	ELISA (enzyme conjugates)	Yes by comparison
	RIA (radiolabelled antibody or preferably antigen)	Yes (competition assay very specific)
	Fluorescent conjugate	Yes
Dot blotting/Western blotting	Enzyme conjugate	Yes by comparison
	Gold conjugate/silver	Yes
	Biotin/avidin/streptavidin	Yes
	Chemiluminescence	Yes
	Radio-isotope	Yes
Immunoprecipitation	Radiolabelled antigen or secondary antibody	Yes
	Gel electrophoresis	Yes

Table 3. Applications of antibody/antigen detection systems in plant biochemistry/molecular biology

Technique	Application
ELISA	Primary screening of antibodies. Measurement of antigens; usual to perform ELISA against dilution series of antigen; note that comparison between assays not always valid
RIA	Measurement of antigens; competition assay particularly useful where radiolabelled pure antigen available
Dot blotting	Primary screening of antibodies. Measurement of antigens by comparison of dilution series with standard set. Note that comparison between assays not always valid
Filter lift/blotting	Screening expression libraries
Western blotting	Measurement of antigens. Measurement difficult (requires scanning densitometer; values obtained not absolute and vary between experiments). Gives absolute identification of antigen
Immunoprecipitation	Measurement of antigens. Preferably requires either labelled antigen or second antibody. Use of gel electrophoresis to identify antigen advisable

Screening antibodies

Techniques for screening antibodies are detailed in a number of laboratory manuals (e.g. Johnstone and Thorpe 1982; Harlow and Lane 1988) and will not be presented here. The techniques available are summarized in Tables 2 and 3. The ELISA (enzyme-linked immuno-sorbent assay) is probably most useful as a primary screening method as most

antigens can be coated onto the wells of ELISA plates and a variety of antisera or supernatants can be screened rapidly. The results obtained are only reproducible within one assay however, so absolute comparison is difficult and standards must be included to verify results. Plates are generally coated with the same antigen preparation as used to immunize the animals and the test does not differentiate antibodies to contaminants from those of interest. An alternative to ELISA, which is also used as a rapid primary screen, is 'dot blotting', in which the antibody is assayed by its binding to antigen dotted onto nitrocellulose (or similar) paper.

After primary screening, high titre antisera or monoclonal antibody supernatants must be screened further to demonstrate specificity of the antibody. 'Western' blotting is commonly used in our laboratory for this procedure; antigen is separated by SDS PAGE and the proteins of the gel are transferred to nitrocellulose paper (or similar) by application of an electric current (full details of this technique, developed by Towbin *et al.* (1979), are given in Johnstone & Thorpe 1982). Non-specific antibody binding sites on the paper are blocked using a solution of milk powder or bovine serum albumin and the paper incubated with primary antibody; after washing unbound antibody, the presence of primary antibody bound to the antigen is indicated by incubating the paper in a secondary antibody specific to the primary which is conjugated either to an enzyme (commonly alkaline phosphatase or horse-radish peroxidase), which produce an insoluble coloured reaction product, or to gold, which can be subsequently identified using silver intensification. Other techniques, which are also available to indicate the location of secondary antibodies, include biotin/streptavidin labelling, use of radio-iodination of antibodies and chemi-luminescent assay systems. In some cases, the antibody fails to recognize any material on a Western Blot; this may be because the quantity of antigen is too small or because preparation for electrophoresis/transfer has masked the antigenic site. In the latter case, alternative procedures for studying antibody specificity are required; antigenicity may be retained if non-denaturing gels are used, but if this fails to give a clear result the use of an immunoprecipitation technique is suggested. In the Ouchterlony double diffusion assay (Ouchterlony 1968), antigen is placed into circular wells cut into agar surrounding a central well containing antibody. Other techniques that yield additional information induce immunoprecipitation followed by SDS–PAGE of the precipitated antibody–antigen complex, and preparation of an antibody affinity column followed by analysis of the antigens which bind can both demonstrate the nature of the material(s) for which the antibody has affinity without involving denaturation of the antigen prior to antibody–antigen binding.

Purification of antibodies

Both monoclonal supernatants and polyclonal antisera may be purified before further use. Purification methods may be divided into two areas; purification of antibodies from the serum/medium, for which ammonium sulphate precipitation, Protein A, Protein G, HPLC, gel filtration and ion-exchange chromatography are all used depending on antibody class (see Harlow & Lane 1988 for details); and purification of one antibody from others within the serum/medium by affinity chromatography. A thorough consideration of the methods available is given in Johnstone & Thorpe (1982). Alternative methods include immunoprecipitation (see above) and the technique of Olmsted (1981) in which the antibody is immunoblotted against antigen and the portion of nitrocellulose containing the antibody/antigen cut out. Antibody is then washed off the nitrocellulose using buffers which disrupt the antibody–antigen interaction. While only yielding very small

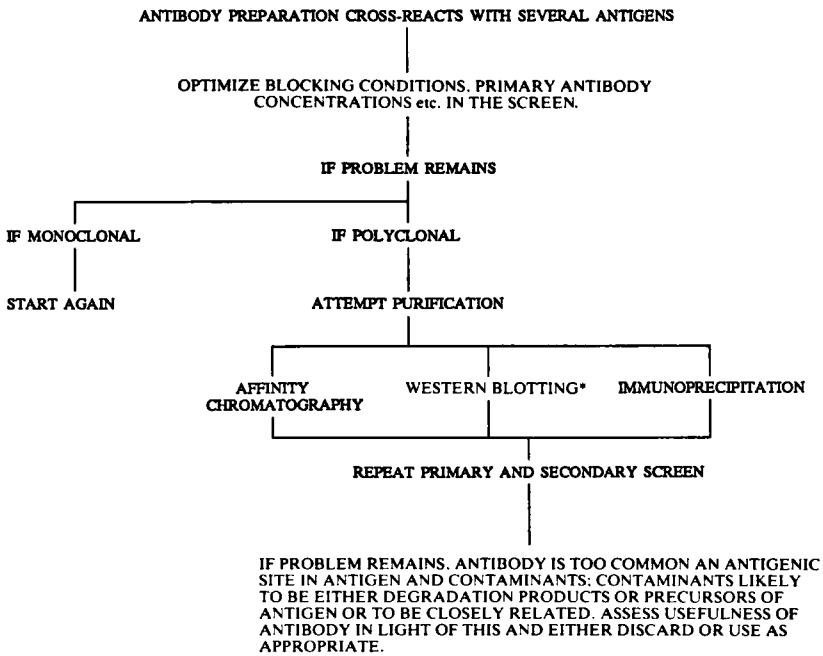

Fig. 2. Flow chart for the steps taken in the purification of antibodies. *Purification technique of Olmsted (1981). See section on Purification of Antibodies p. 7.

amounts of antibody, this procedure is rapid and effective and has been used successfully in the authors' laboratory (S. Montgomery, personal communication).

Uses of antibodies in molecular biology and biochemistry

The uses of antibodies in both biochemistry and molecular biology have essentially the same basis: the need to identify and quantify antigens. The techniques used are broadly the same and centre around three basic methods; ELISA, or similar techniques, where antigen is coated onto plastic ELISA plate wells and subsequently quantified; immunoblotting, where antigen is applied to nitrocellulose or similar paper and its presence indicated by use of an antibody; and immunoprecipitation where the antigen is precipitated using an antibody and subsequently identified or quantified. The selection of techniques depends on circumstances and will be governed by the success of the initial screening procedures used. Tables 2 and 3 indicate the the basic methodologies applicable to a variety of problems.

A number of these techniques have been employed in the authors' laboratories in the preparation of antibodies to a number of plant cell proteins, including structural proteins, ion pumps and enzymes. In several cases (antibodies to the plant plasma membrane calcium pump, to plant actin, and to plant-coated vesicles) problems resulting from antigenic homology between a plant protein and native mammalian proteins were experienced. This was used to advantage when an antibody to a mammalian protein was used for subsequent experiments. However, in general our experience indicated that larger doses of antigen were required for immunization and a low titre antibody production resulted. Raising antibodies to plant actin proved to be particularly difficult because good purification protocols for actin did not exist. Use of a very simple procedure resulting in an

impure preparation, followed by excision of a Coomassie Blue stained band from a polyacrylamide gel overcame this problem. Polyclonal antibodies were prepared for detection of the enzyme serine: glyoxylate aminotransferase; here, although the preparation was relatively pure, contaminant antibodies were produced and purification of the antiserum was required (Montgomery 1986). This was achieved by the methods described by Olmsted (1981), Talian *et al.* (1983) and Smith & Fisher (1984), in which antibody bound to nitrocellulose after Western Blotting is eluted and used (Montgomery 1986). This gave an excellent preparation for immunocytochemistry and is a method of choice when antiserum of sufficient titre is available, but antigen preparations are not pure enough to permit an affinity column to be prepared successfully. Monoclonal antibodies were prepared against the plant plasma membrane calcium pump, plant actin and plant clathrin as it was desired to raise antibodies specific to key epitopes of each protein. In the case of clathrin, they were also useful as impure antigen was used for immunization and selection after screening was required for preparation of monospecific antibodies.

IMMUNODIAGNOSTIC ASSAYS FOR PLANT PATHOGENS

Immunogdiagnostic assays are increasingly important in agriculture, horticulture and forestry. The techniques used to detect plant pathogens have been adapted from the medical diagnostics field. They are sensitive, easy to replicate and relatively inexpensive. Immunoassays provide a fast method of confirming visual symptoms and permit early detection of disease. Early detection and correct identification of pathogens have played an important role in limiting the spread of viral and bacterial diseases. Similar assays are needed for the early detection of many fungal diseases. Immunoassays are based upon methods of visualizing specific antigen–antibody complexes. These techniques are the same as those referred to in 'visualizing the antigen' on page 18 of this review. The antigen, which must be unique to the pathogen, can be either on or within the target organism, or a soluble extract. Current methods depend upon tagging the detector antibody with a fluorescent molecule such as fluorescein (immunofluorescence), an enzyme or gold particle. Enzyme–antibody conjugate assays, usually known as ELISAs, are performed in micro-titre wells linked together in strips or plates. Such assays are easy to handle and enable large numbers of samples to be assayed simultaneously. They are now used extensively for mass screening where some or all of the steps have been automated. For a general review of plant immunoassay techniques see Barbara & Clark (1986). No attempt will be made in this section of the review to cover all the aspects of plant immunoassays. Instead this review will highlight recent advances and review plant–fungal diagnostics in greater depth.

Immunodetection of plant viruses

Over the years a number of immunological methods have been used to detect plant viruses. They include immunofluorescence (IMF), immunosorption-electron microscopy (ISEM), enzyme-linked immunosorbent assays (ELISAs) and dot-blot or dip-stick assays. For reviews and practical guides on the subject consult Cooper & Edwards (1986) and Hill (1984). There is an increasing move towards development of quick 'user friendly' assay systems that can be used 'on site' by the farmer or food processor, such as dip-stick, dot-blot or agglutination assays.

For dip-stick and dot-blot assays the capture antibody is pre-coated onto a nitrocellulose surface, e.g. a flexible plastic. When sap is squeezed onto the coated surface the viral

particles, if present, are trapped and are detected by incubation successively with the antibody–enzyme conjugate, the substrate and the stopping solution, with appropriate washings in between. If positive the dip-sticks appear coloured, if negative they remain colourless. It is important that positive and negative controls are included in each test series. For an example of such an assay system see Mitchell *et al.* (1988) who developed a system, initially for the detection of potato viruses in leaves of standing crops.

The majority of viral diagnostic assays still employ antisera rather than monoclonal antibodies. In viral diagnostics the high specificity that can be achieved with monoclonal antibodies is not necessarily an advantage. Detection of individual strains is rarely desirable. In general, monospecific antisera containing many antibodies recognizing different epitopes of the same viral coat protein give a higher signal than a monoclonal antibody that only recognizes one epitope. Furthermore, polyclonal antibody assays safeguard against missing new strains where the one target epitope detected by the monoclonal antibodies may have changed. However, monoclonal antibodies to viruses have proved particularly useful in aetiological and taxonomic studies, where antisera do not discriminate between different strains that have the same physical identity but cause different symptoms, such as the closteroviruses (GLRaV I,II,III and IV) that cause grapevine leafroll disease (Gugerli *et al.* 1990), luteoviruses and others (Halk & De Boer 1985).

Immunodetection of phytopathogenic bacteria and phloem-restricted prokaryotes

Detection of bacteria, or other prokaryotes, directly from infected plant tissue is not easy. Frequently the numbers of bacteria present are too low to give strong definitive signals and cross-reactivity with contaminant bacteria and/or host molecules is common. A number of ingenious methods, as well as the use of monoclonal antibodies, have been developed to circumvent these problems. Commonly aliquots of plant extracts are incubated on/in an enrichment medium to increase the numbers of pathogenic bacteria. Van Vuurde *et al.* (1987) have developed an immunosorbent dilution–plating technique (ISDP) in which bacterial pathogens are trapped onto antibody-coated Petri dishes or inoculation rods. Unbound bacteria are removed by washing and those that remain are detected by incubating the trapped bacteria with selective media and allowing colonies to form. Colonies are detected by drying down the agar and staining by immunofluorescence. This method ensures that only viable bacteria are detected and, if desired, the bacteria can be re-isolated from the fluorescently stained colonies after rehydration.

Several workers have raised antisera to the lipopolysaccharide (LPS) present in the outer membranes of Gram-negative bacteria. LPS is highly antigenic and is easily extracted and purified. Antiserum raised against purified preparations of LPS is more specific than that to whole cells and has been used for diagnostic purposes and for differentiation of cultivar-specific strains of *Pseudomonas syringae* (Lyons & Taylor 1990). Other workers have obtained a high level of specificity with antisera raised against the extracellular polysaccharide (EPS) of bacteria (Wyatt *et al.* 1989).

Of the phloem-restricted endocellular pathogens: spiroplasmas, mycoplasma (MLO) and bacteria-like organisms (BLO), only the spirolplasmas can be grown in culture. Bove (1984) and others using antisera specific for a number of spiroplasmas have been able to detect and differentiate the organisms into various serogroups. It is very difficult, however, to raise antisera specific to mycoplasma-like organisms, partially because they are obligate pathogens, but also because they are only present in small numbers in infected plants. Preparation of the immunogen without contamination from host molecules is almost impossible. Lin & Chen (1986) and Clark *et al.* (1989) have found monoclonal antibodies

to be particularly useful in differentiating Aster Yellows and Primula Yellows MLOs respectively.

Immunodetection of plant-invading fungi

The development of immunodiagnostic techniques for the detection of fungi in diseased plants has been slow. This has been partly due to the difficulty in producing antisera that are specific. When tested by ELISA or IMF, antisera raised against mycelial fragments, extracts from lyophilized mycelia, surface washings of solid cultures or culture filtrates, cross-react widely with both related and unrelated fungi and host tissues or extracts, but appear to be species-specific when tested by immunodiffusion. Chard (1985a,b) and others have shown that non-specific antigens are present in both the insoluble and soluble fractions of fungal material and we have shown (Dewey *et al.* 1990) the converse: that specific antigens are present in both the soluble and insoluble fractions. Attempts to improve specificity by diluting out non-specific antibodies or cross–absorbing antisera with related fungi are rarely satisfactory, but there is increasing evidence that fungal antisera may be useful in competition assays (Kitagawa *et al.* 1989; N. S. Lyons *et al.* personal communication).

A number of workers have shown that reasonable levels of specificity may be obtained by using protein precipitates of culture filtrates or mycelial extracts as the immunogen. Examples include antisera raised to *Colletotrichum* in anemone corms (Barker & Pitt 1988); *Phomopsis longicolla* in soybean seeds (Gleason *et al.* 1987); *Verticillium dahliae* in cotton root tissues (Gerik *et al.* 1987) and *Phytophthora fragariae* in strawberries (Mohan 1989).

Antisera raised against specific fungal fractions, such as enzymes, toxins or soluble carbohydrates, generally have a high degree of specificity. Notermans *et al.* (1987) have raised antisera to heat-stable carbohydrates from several fungi and showed that such antisera are almost genus-specific and can be used to determine if raw food stocks are contaminated with moulds prior to processing.

So far, relatively few monoclonal antibodies have been raised against fungi. Disappointing results with fungal antisera, costs and the need for animal house facilities have undoubtedly been discouraging factors. Many of the monoclonal antibodies raised against fungi have not been raised for diagnostic purposes. However, some, such as the species-specific monoclonal antibodies raised against *Phytophthora cinnamomi* (Hardham *et al.* 1986), *P. megasperma* var. *glycinea* (Wycoff *et al.* 1987), and *Pythium aphanidermatum* (Estradia-Garcia *et al.* 1989) clearly have diagnostic potential. It is unfortunate that the monoclonal antibodies for *P. cinnamomi* and *P. aphanidermatum* only differentiate species on the basis of zoospores. Specific assays for the detection of mycelia in infected plants or soil would be more useful.

Mitchell & Sutherland (1986) and Wright *et al.* (1987) have successfully raised and used monoclonal antibodies in ELISA and membrane (dot-blot) assays for the detection of the seedborne fungus *Sirococcus strobilinus* and sporocarps of the vesicular-arbuscular fungus *Glomus occultum*. Wong *et al.* (1988) have raised a monoclonal antibody to the banana wilt fungus, *Fusarium oxysporum* f.sp. *cubense* that will differentiate, by immunfluorescence, the thick-walled chlamydospores of strain 4 from those of strains 1 and 2.

Our own experiences in raising monoclonal antibodies that are specific to various species of fungi have been varied. Development of diagnostic assays for the pathogen that causes Dutch Elm disease, *Ophiostoma ulmi* (Dewey *et al.* 1989a) and three fungi, which are involved in post-harvest spoilage of rice grains and copra, *Humicola lanuginosa*

(Dewey *et al.* 1989b), *Pencillium islandicum* (Dewey *et al.* 1990) and *Aspergillus flavus* (F.M. Dewey unpublished) has been relatively straight forward and quick. In contrast, we have had difficulty in raising monoclonal antibodies that are specific, even at the genus level, for the Eyespot pathogen of cereals *Pseudocerosporella herpotrichoides* (Dewey 1988). The degree of difficulty appears to be related to the immunogenicity of the different fungi, which in turn probably reflects both the levels of soluble proteins and the presence of non-specific carbohydrates or glycoproteins that induce a non-T cell stimulated response. The site and nature of species- and sub-species-specific antigens is not generally known but in the case of *P. islandicum* we have shown (Fig. 5) that the antigens recognized by our specific monoclonal antibody are present in the walls and cross walls of the hyphae but not the spores and that some of this same antigen can be removed by gently washing the surface of a solid slant culture with PBS. There is no consensus about the best source of immunogen or the effectiveness of Freund's adjuvant in stimulating a specific response. See Table 4 for a comparison of the various schedules used by different workers and the levels of specificity attained.

Of the panel of monoclonal antibodies that we raised to *Ophiostoma ulmi* using mycelial homogenates with Freund's adjuvant as the immunogen (Dewey *et al.* 1989a), we found that most were non-specific, about one-third were genus-specific and three were species-specific. The monoclonal antibodies that were species-specific were all IgGs belonging to the sub-class IgG_1 and IgG_{2a} whereas the genus-specific antibodies were mostly IgM antibodies. When all the monoclonal antibodies were tested by ELISA against microtitre wells coated with extracts from diseased tissue (1 in 30 w/v), no correlation was found between fungal specificity and the ability to distinguish infected from non-infected plant material. For example one of the species-specific monoclonal antibodies clearly distinguished between extracts from diseased and healthy tissue whereas another species-specific monoclonal antibody cross-reacted with healthy tissue to give higher readings with these extracts than with diseased extracts. Another monoclonal antibody that recognized species of both *Ophiostoma* and *Ceratocystis* gave low absorbance values when tested against antigens produced *in vitro* but high readings when tested against extracts from diseased plants. It did not cross-react with extracts from healthy tissue.

We have successfully used cell-free surface washings of the fungi directly as the immunogen without concentration or freezing or the addition of Freund's adjuvant to raise monclonal antibodies specific to *P. islandicum, H. lanuginosa* and *A. flavus* (Dewey *et al.* 1989b/1990 and unpublished). This simple method of antigen preparation was very effective. In both cases antisera titres were high and only one or two fusions were needed to identify cell lines that secrete monoclonal antibodies with the specificity needed to develop diagnostic assays.

In trying to raise a monoclonal antibody suitable for the detection of the Eyespot pathogen we have used several different immunogens including surface washings, mycelial fragments, soluble extracts and protein and carbohydrate fractions (F.M. Dewey 1988, unpublished). All these immunogens were tried with and without Freund's adjuvant. The general response to these different immunogens has been weak and non-specific. Antisera from mice immunized with surface washings or hyphal fragments had low titres; dilution end points for antisera tested by ELISA against surface washings were 1 in 50,000 compared with 1 in 200,000 for *H. lanuginosa*. Higher titres and cell lines secreting monoclonal antibodies specific to the pathogen were only obtained when a specific protein fraction was used (Dewey *et al.* 1990).

Table 4. Monoclonal antibodies raised against plant-invading fungi

Fungus	Nature	Immunogen: Dose per Injection	F	Route: Early	Route: Booster	Screening method	Specificity	mAb class	Reference
Fusarium oxysporum f. sp. *lyucopersici*	Spores	10^7	+	i.p.	i.p.	ELISA-S	Genus	NG	Ianelli *et al.* (1983)
Tilletia	Spores	3 mg	−	i.p.	i.v.	ELISA-S	NSG	IgM	Banowetz *et al.* (1984)
	Spore extracts		−	i.p.	i.v.	ELISA-S			
Phytophthora cinnamomi	Zoosp.	2×10^7	−	i.p.	i.p.	IMF-zoosp.	NSG	NG	Hardham *et al.* (1986)
Sirococcus strobilinus	Hyphal fragments	1 mg	+	i.p.	i.v.	ELISA-sol.Ag	NS genus	IgM IgG	Mitchell & Sutherland 1986)
Glomus occultum	Spores	50,000	−	i.p.	i.p.	ELISA-S	Species	IgG	Wright *et al.* (1987)
Pythophtora meqasperma f. sp. *glycinea*	Culture filtrate	0·15 mg protein	+	i.p.	NG	ELISA-sol.Ag western blot	NSG	IgM IgG IgA	Wycoff *et al.* (1987)
Fusarium oxysporum f. sp. *cubense*	Conidia hyphal fragments	8×10^7 40 μg	−	Intrasplenic		IMF	NSG Race-specific	IgM	Wong *et al.* (1988)
Armillaria mellea	Hyphal fragments	NG	±	i.p.	NG	ELISA-h.f.	N.S.G.	ND	Fox & Hahne (1988)
Ophiostoma ulmi	Hyphal fragments	3 mg	+	i.p.	i.v.	ELISA-sol.Ag	Species & genus	IgG IgM	Dewey *et al.* (1989a)
Humicola lanuginosa	SW	150 μg protein	−	i.p.	i.p.	ELISA-SW	Genus*	IgM	Dewey *et al.* (1989b)
Penicillium islandicum	SW	500 μg protein	−	i.p.	i.p.	ELISA-SW	Species	IgG	Dewey *et al.* (1990)
Pythium	Zoosp.	1×10^7	−	i.p.	i.v.	IMF-zoosp.	Zoospores & Cyst: NS Species	IgM IgG IgM	Estrada-Garcia *et al.* (1990)

F = Freunds adjuvent, i.p. = intraperitoneal, i.v. = intravenous, NG = not given, ND = not determined, SW = surface washings, NSG = not tested against other species or genera, NS = not tested against other species, sol.Ag = soluble antigens, zoosp. = zoospores, h.f. = hyphal fragments.
*Tested against species from 18 related genera, cross-reacts with 2.

Most of the monoclonal antibody diagnostic assays developed for fungi are, with the exception of the few commercial assays, all indirect assays. They involve direct coating of micro-titre wells or membranes with the mixture of antigens present in extracts from the infected plants followed by incubation with the specific monoclonal antibody and the use of a commercial secondary antibody–enzyme or gold conjugate as the reporter antibody. We have found, as did Gleason *et al.* (1987) using polyclonal antisera, that these assays work particularly well where the fungus is present on or near the surface of the infected tissue and where passive release, by overnight soaking, is sufficient to enable detection at very low levels. This method has proved invaluable in the detection of *H. lanuginosa* and *P. islandicum* in rice grains (Dewey *et al.* 1989b/1990). Grains are soaked, individually, overnight in micro-titre wells and removed the next day. The wells are then processed as usual. This method has enabled us to determine if there is a low level of infection in all grains or if only a few individual grains are infected. We have also developed a 'user friendly' dip-stick assay that has proved successful under field conditions. It has enabled detection of significant growth of *H. lanuginosa* in Paddy heaps *in situ* in the Philippines, within 5 days of harvest. The dip-sticks were made of a new membrane 'Immobilon P' (Millipore, UK). Fungal diffusates from grains soaked individually, in PBS, in Eppendorfs were allowed to coat the surface of the dip-sticks overnight. The dip-sticks were then dried, re-wetted incubated with hybridoma supernatants followed by commercial immunogold conjugates. The signal was amplified by exposure to a commercial silver enhancing solution.

The specific fungal antigens that we have worked with bind strongly to both Immobilon P and to micro-titre wells. We have also found that the specific antigens bind rapidly to their respective antibodies. More than 95% of the total monoclonal antibody that binds to the antigen is bound within 30 s. The nature of most specific antigens is not known but we have shown that in the case of *P. islandicum* and *H. lanuginosa* that the specific antigens are glycoproteins (Dewey *et al.* 1989b/1990).

Only a few double antibody sandwich assays (DAS-ELISA) have been developed for detection purposes. Most of these are commercial and have been developed for detection of turf grass diseases by Miller *et al.* (1988, 1990) for Agri-Diagnostics. They employ both monoclonal antibodies and polyclonal antisera.

Commercial potential

The use of antibodies to detect plant pathogens, for both research and commercial purposes, is now well established. The commercial value of plant immunodiagnostic assays was estimated to be £3 million in 1985 and is projected to be about £8.5 million in 1995 (Klausner 1987). At the moment this market is mainly concerned with the detection of viruses but important advances are likely to be made in the production of commercial assays to detect spiroplasmas, mycoplasma-like organisms, bacteria and fungi. Most of these assays will necessitate the use of monoclonal antibodies in order to attain the level of specificity needed. Use of immunoassays by growers and importers will depend on the development of low-cost, on-site, user friendly assay kits such as dip-stick assays. Good methods of sampling and interpretation of the results to asses the levels of infection, will be critical.

IMMUNOCYTOCHEMISTRY

Over the last decade the use of antibodies as probes for macromolecules within cells and tissues has emerged as one of the most powerful techniques in cell biology. There are,

therefore, many excellent reviews and texts outlining the numerous techniques and protocols that have been developed (See Bullock & Petrusz, 1982, 1983, 1985, 1989; Polak & Varndell 1984). The application of immunocytochemical techniques to plant cells has somewhat lagged behind the rapid developments in animal cell biology. However, it can now be said that the technique has finally come of age and can be used routinely at both the light and electron microscope levels. Most of the problems of inaccessibility of antibody probes to antigens, due to the cell wall and the nature of plant tissues, have been overcome by the development of a variety of tissue preparative techniques.

In this section it is our intention to review some of the methodologies that have been developed to permit successful immunocytochemical labelling of plant antigens with an emphasis on the initial fixation and preparation of the cell/tissues prior to light or electron microscope immunocytochemistry.

Light microscope immunocytochemistry

When preparing plant tissues or cells for immunocytochemistry three main factors have to be taken into consideration. First, is the likelihood that the antigen to be localized is denatured by fixation and the other steps in the preparative procedures. This can often adversely affect the cross-reactivity with the antibody, especially if a monoclonal antibody recognizing a single epitope is used. Second, is the fact that the cells or tissues have to be prepared in a manner which will permit access of both the antibody and the attached probe to the antigen in question. In consequence, a decision has to be taken as to whether whole cells, made permeable, or sectioning techniques are to be employed. Finally, the probe to examine primary antibody binding has to be selected. At the light microscope (LM) level fluorescent probes are still the most popular. However, the use of colloidal gold as an LM marker is gaining in popularity in combination with silver enhancement. At the electron microscope level colloidal gold has become the marker of choice (Hyatt 1990). However, enzyme-linked secondary antibodies can still be useful with sectioned material.

Whole cell techniques. The cell wall and the plasma membrane form the two major barriers to the entry of antibodies and associated probes into the cell. To overcome these barriers, the specimen may be sectioned or the cell made *permeable* to the antibodies. This latter approach has proved very popular, especially in the study of the plant cytoskeleton, the three-dimensional organization of which can withstand the rigours of the various steps in staining protocols involving permeabilization (Lloyd *et al.* 1980; Wick *et al.* 1981; Roberts *et al.* 1985; Goodbody *et al.* 1989). Initially such work was restricted to the use of suspension culture cells or protoplasts (Lloyd *et al.* 1980). In the former, mild treatment with enzymes was used to loosen the cellulose and the matrix of the wall. However, this technique was soon extended to tissues when it was shown that formaldehyde-fixed roots, if washed adequately, could be digested with cellulase and pectinase under controlled conditions to release whole cells which retained their original morphology, the so-called 'square protoplasts' (Wick *et al.* 1981). These cells adhered to poly-L-lysine coated slides or coverslips prior to staining. The major disadvantage of this technique is the fact that on release of cells from the tissue all information on the spatial organization of cell types within the tissue is lost.

Various approaches have been used to make the plasma membrane permeable. In many cases fixation with paraformaldehyde (3–8%) will prove sufficient. However, it is normal practice to further treat the cells with a mild detergent such a Triton X-100 or Nonidet P40

(0·5–0·01%). Air drying and cold methanol treatments (−10°C for 6–8 min) have also been used (Wick *et al.* 1981; Goodbody *et al.* 1989).

To study antigens associated with the plasma membrane (PM) the use of burst protoplasts has proved popular. By sticking protoplasts to poly-L-lysine coated slides and osmotically bursting them with buffer or distilled water, circular patches or 'footprints' of PM are left adhering to the glass. Any antigens associated with the cytoplasmic face of the PM can then be localized (Van der Valk *et al.* 1980). This technique has again proved popular for the examination of structural proteins such as clathrin (Hawes *et al.* 1989), microtubules, microtubule-associated proteins and intermediate filament antigens (Cyr & Palevitz 1989; Goodbody *et al.* 1989).

Sectioning techniques. Cryosections: Probably the most satisfactory technique for immunocytochemical labelling of sectioned material is the use of cryo-sections because tissue integrity is maintained and the whole cell is accessible to antibodies and labels. These can be cut either by a conventional cryostat or by an ultramicrotome with cryo-attachment, although with the latter system section thickness is likely to be restricted to 1 or 2 μm. Sections are then stuck onto either poly-L-lysine treated or gelatin-subbed slides prior to immunostaining (Hawes 1988).

Using a very simple fixation procedure and freezing the specimens in a drop of water on the cryo-stub, Sakaguchi *et al.* (1988) obtained excellent longitudinal, 6–8 μm cryosections of shoot tips of *Vinca major* within which they labelled the complete microtubule system of the apical dome. It is rare, however, to be able to produce such good cryosections of plant material without the infusion of some supporting matrix into the tissue. It is now common practice to perfuse tissue with sucrose up to a concentration of 2 M prior to sectioning, this then acts as both a cryoprotectant giving better freezing and as a support for the tissue during sectioning (Hawes 1988).

In a recent report from one of the authors' laboratories (Hush *et al.* 1990) it was found that following incubation of paraformaldehyde-fixed pea roots in 2 M sucrose, a further infusion of the roots with 10% gelatine in 1·5 M sucrose markedly improved the sectioning characteristics of the tissue. This enabled highly vacuolated regions of the root to be cryosectioned up to 10 μm in thickness with very little fragmentation of the tissues (Fig. 3). Sections were then transferred to glass slides by picking them up on the surface of a 1·5 M sucrose droplet held in a wire loop and touching this onto the surface of the slide.

In the same report (Hush *et al.* 1990) the effectiveness of poly-L-lysine coating of slides was also enhanced in the following manner. Cleaned multiwell slides were placed face down onto drops of 5 mg/ml 390,000 kD poly-L-lysine on a strip of laboratory film and incubated for at least 2 h. The slides were then rinsed in ultra-pure water and heated to 70°C for 2 h. It was found that this treatment of slides markedly strengthened the adhesion of thick cryosections to the slide and this will probably be a useful technique for adhering problematical plant specimens to glass slides for subsequent processing. Figure 3 demonstrates the staining of microtubules with a monoclonal antibody to yeast tubulin in thick cryosection of pea roots prepared by the methods described above.

Wax sections: Although a popular technique in histological studies of animal tissues, it has not been common practice to use paraffin wax-embedded material in plant immunocytochemistry. However, high molecular weight polyethylene glycol (PEG) can be used as an embedding matrix, the PEG being dissolved out of the section prior to staining (Van Lammeren *et al.* 1985, Hawes 1988). Sections as thin as 0·5 μm, with this technique can be

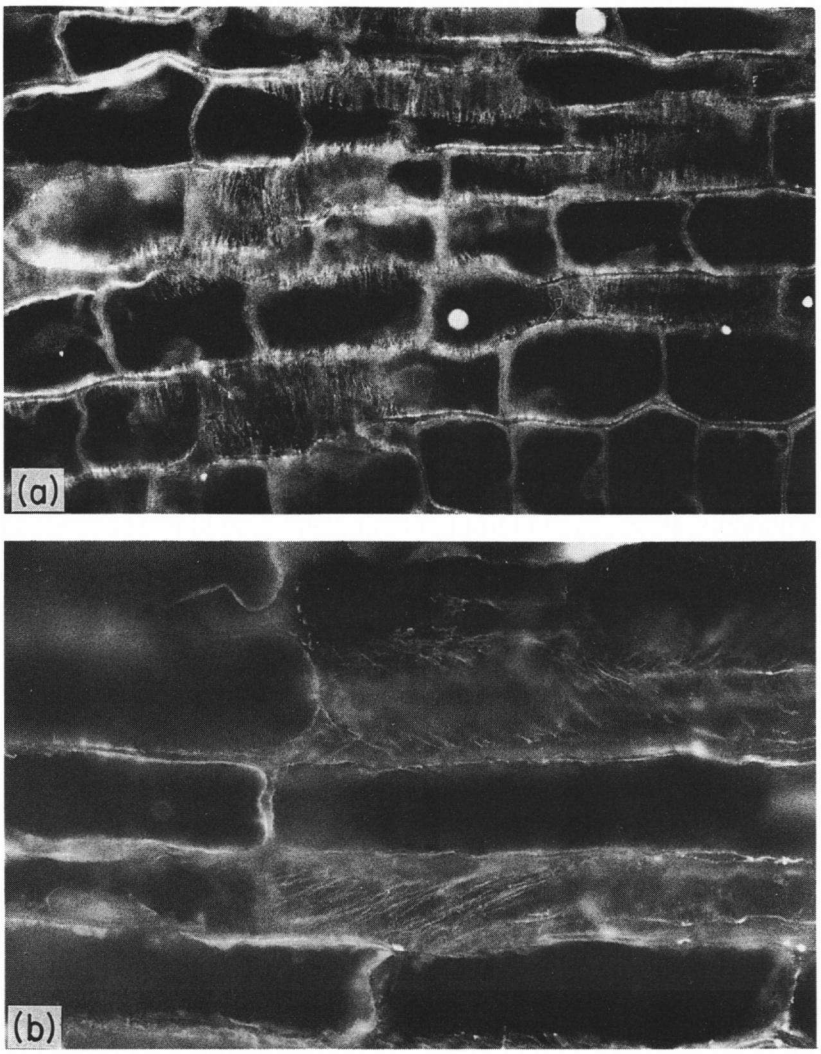

Fig. 3. Immunofluorescent staining of cortical microtubules in thick (8–10 μm) cryosections of pea roots. Behind the meristem (A) cortical microtubules are predominantly transverse in orientation, whilst in larger vacuolate cells of the root (B) the microtubules are oblique. Roots were fixed in 3% paraformaldehyde, infiltrated with 1·5 M sucrose and 10% (w/v) gelatine. Cryosections were stuck onto heat activated poly-L-lysine coated multi-well slides. All washes after primary antibody staining contained 1% fish gelatine to reduce background fluorescence (see Hush *et al.* 1990). Micrographs courtesy of Julia Hush, Biology Department, University of Sidney. A bar = 30 μm, B bar = 60 μm.

cut dry on a glass knife with an ultramicrotome and transferred to a slide on a drop of sucrose solution (Hawes 1988).

Resin sections: The advantage of using resin-embedded material for immunochemistry lies in the fact that it is possible to cut sections of tissue that are difficult to cryo-section and that the same block can be used for both thick and thin sectioning for the ultrastructural localization of antigens. In most cases the resin must be chemically removed or etched from the surface of the section to expose the antigenic sites prior to staining

(Perrot-Rechenmann & Gadal 1986). With epoxy resins this can be carried out with sodium ethoxide or methoxide (Hawes 1988). In many cases, however, the use of this family of resins is not suitable as satisfactory levels of antigenicity in the tissues are not retained. Gubler (1989) reported on the successful immunofluorescent localization of microtubules in sections of buty-methyl methacrylate-embedded root tips. This resin was polymerized by long wave ultraviolet at room temperature and dissolved out of the section with acetone. Resin removal may not be necessary with low-temperature Lowicryl-embedded sections (Gubler *et al.* 1987). A summary of these various approaches to tissue preparation is given in Table 5.

Visualizing the antigen. The two-layer technique, using a second antibody conjugated to a fluorochrome, is still the method of choice for most LM immunochemical localizations. The exact protocols used, including buffer types, blocking agents and antibody concentrations, vary greatly between tissues and with different antibodies. Therefore, no set procedure should be taken as standard and staining schedules have to be developed for each particular experiment if the immunostaining is to be optimized (Wick & Duniec 1986). An example of how the successful localization of an antigen can be dependent on the preparative techniques is elegantly demonstrated in a recent paper by Goodbody *et al.* (1989). Staining of fibrillar bundles in carrots with a monoclonal antibody was achieved in air-dried protoplasts which had been treated with Triton X-100 and dipped in methanol. However, a microtubule-like distribution of the same antigen could only be observed in preparations without methanol treatment.

Suitable controls have to be constructed for each experiment. Commonly substitution of the primary antibody with pre-immune serum is used as the control with polyclonal antibodies. With monoclonals an antibody, which should not cross-react with the tissue being stained can be used as the control as well as staining of tissues which should not contain the antigen.

One major problem with immunofluorescence is non-specific staining and general background fluorescence, the latter due to a wide variety of naturally occurring auto-fluorescent compounds found in many plant cells, such as various phenols and pigments. In our laboratories, in addition to the use of blocking agents such as BSA, the addition of 1% fish gelatine to all solutions after incubation in the primary antibody has been found to reduce greatly the level of background fluorescence (B. Satiat-Jeunemaitre personnal communication).

It should be remembered that other markers as well as fluorescent probes are available to visualize the second layer antibody. Peroxidase labelling techniques can be used on sectioned material (Van Driessche *et al.* 1981; Bullock & Petrusz 1982) although problems can arise in tissues with naturally high levels of peroxidase activity and other enzymes linked to the second antibody may have to be used.

The use of colloidal gold as a marker at the LM level is increasing in popularity. It may be used in its own right, whereby the gold is seen as a dark red stain over sites of antibody binding and as such has been used to great effect in the study of microtubules in the liquid endosperm cells of *Scadoxus (Haemanthus)* sp. (De Mey *et al.* 1982; Morejohn *et al.* 1987). A more satisfactory technique, however, is to amplify the signal from the gold by silver enhancement (Danscher 1981; Holgate *et al.* 1983; Van den Bosch 1986). With this procedure a shell of silver is grown, under controlled conditions, around the gold particles. With recently developed reagents this technique can be carried out in the light and the reaction monitored microscopically and stopped when the required density of staining is

Table 5. Examples of various tissue preparative techniques for immunofluorescence localization of antigens

Technique	Specimen	Antigen	Reference
Permeable protoplasts	Carrot protoplasts	Tubulin	Lloyd *et al.* 1980
Protoplast ghosts	Carrot cells	Clathrin	Hawes *et al.* 1989
	Tobacco cell cultures	Tubulin	Van der Valk *et al.* 1980
	Carrot cells	Intermediate filaments	Goodbody *et al.* 1989
Permeable suspension culture cells	Soy bean cell cultures	Connexin	Meiners & Schindler 1989
	Carrot cell cultures	Tubulin	Lloyd *et al.* 1980
Square protoplasts	Onion root tips	Tubulin	Wick *et al.* 1981
	Pea & Onion root tips	Calmodulin/tubulin	Wick & Duniec 1986
Thin cryosections (0·5–1 μm)	Carrot roots	Arabinogalactan-proteins	Knox *et al.* 1989
Thick cryosections	Carrot embryos	Arabinogalactan-protein	Stacy *et al.* 1990
	Pea roots	Tubulin	Hush *et al.* 1990
	Vinca shoot apices	Tubulin	Sakaguchi *et al.* 1988
PEG embedding	*Gasteria* pollen	Tubulin	Van Lammeren *et al.* 1985
Resin embedding	Sorghum leaves	PEP carboxylase	Perrot-Rechenmann & Gadal 1986
	Barley aleurone cells	α-amylase	Gubler *et al.* 1987
	Onion root tips	Tubulin	Gubler 1989
Tissue strips	Epidermal strips	Tubulin	Roberts *et al.* 1985
Thick tissues—confocal	Leaves (various spp.)	Tubulin	Wick *et al.* 1989

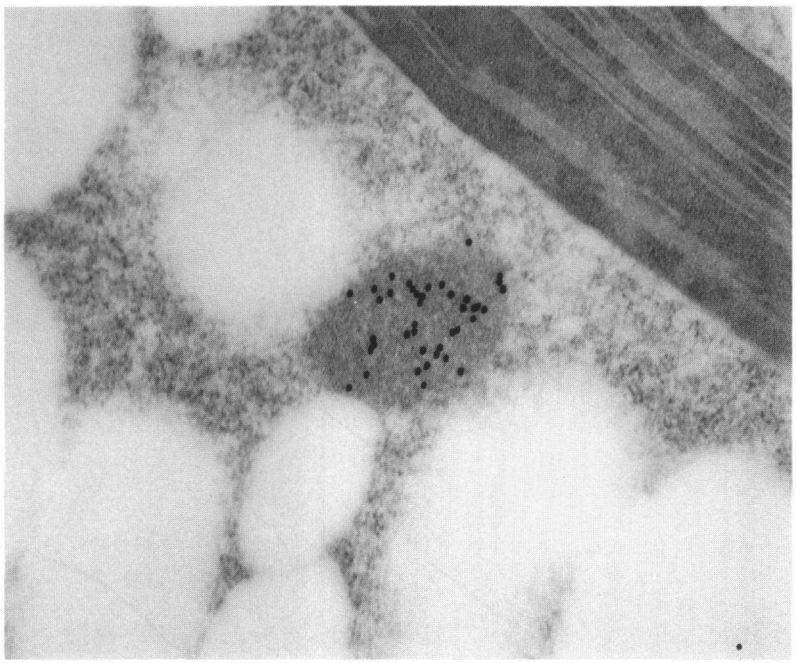

Fig. 4. Immunogold localization of serine glyoxylate amino transferase (SGAT) on a peroxisome in a section of pumpkin cotyledon embedded in LR White resin and polymerized at 50°C for 24 h. Specimens were fixed with 1% paraformaldehyde and 1% glutaraldehyde in 0·1 M PIPES buffer, pH 6·9. Staining was with a rabbit anti-SGAT polyclonal serum followed by 15 nm gold labelled anti-rabbit IgG. Staining buffer was 20 mM Tris-HCl (pH 8·2) with 0·5 M NaCl, 0·1% (w/v) Tween-20 and 1% globulin-free BSA. Bar = 200 nm. (Micrograph courtesy of Sue Montgomery, Plant Sciences, Oxford.)

achieved. To give the best signal, a small particle size colloidal gold sol (5 μm) should be conjugated with the second antibody thus giving the optimum number of nucleation sites for growth of the silver deposits. This technique is not necessarily restricted to sectioned material or naked cells. Recently, B. Satiat-Jeunemaitre (1989) successfully immunolocalized microtubules in epidermal strips of mung bean hypocotyls by making the cell walls permeable with enzymes (or methylamine personal communication) prior to labelling with 5 nm gold and silver enhancement. The recent introduction of 1-nm colloidal gold probes and gold-conjugated Fab fragments (papain cleaved 45 kD immunoglobulin fragments with a single antigen binding site), should make this staining procedure available to a wide range of plant cell types.

The main advantage of the use of silver-enhanced gold staining at the LM level is that the distribution of antigen within cells can be seen with transmitted light and further staining of the tissue is possible. In combination with epipolarized light, which will impart a silver–blue fluorescence to the deposited silver, this becomes a very impressive technique and should prove applicable to the staining of plant cells as well as animal tissues (Ellis *et al.* 1989).

Future developments. In addition to the introduction of ultra-small gold probes in combination with silver enhancement and refinements to the various embedding and staining protocols, it is likely that the most significant developments in LM immunochemistry

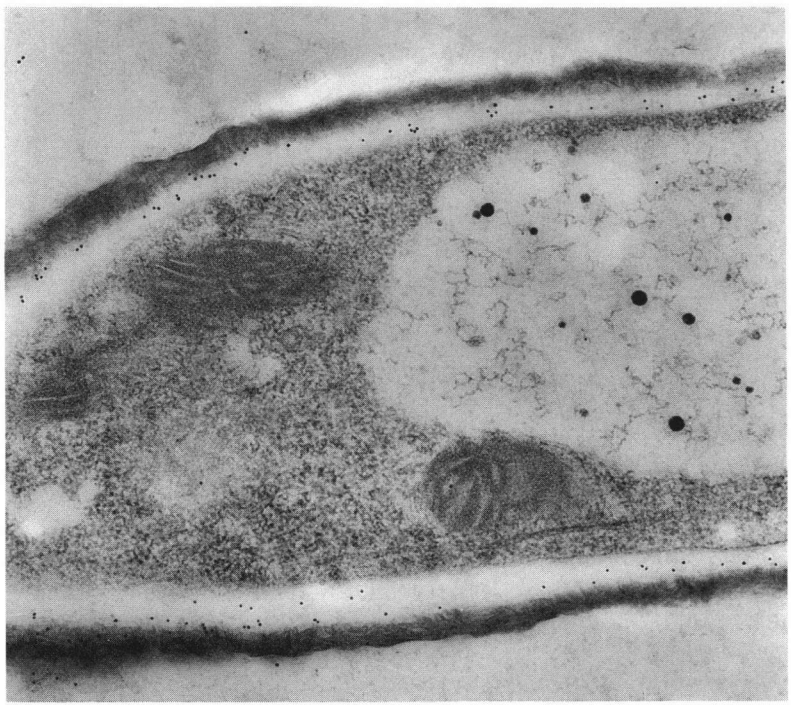

Fig. 5. Immunogold localization of a species specific antigen in the hyphal wall of *Penicillium islandicum* with a 10 nm gold-conjugated mouse monoclonal antibody. Material was fixed in 1% glutaraldehyde and 1% paraformaldehyde in 0·1 M PIPES buffer (pH 7·2) and low temperature embedded in LR White resin. Bar = 400 nm.

will be in the imaging apparatus. The introduction of the laser scanning confocal microscope, which can be used in the epifluorescence mode, and associated image processing will make a significant contribution to our understanding of the three-dimensional organization of cells (Shotton 1989). With the removal of out-of-focus blur, resolution in immunofluorescence images will be improved and three-dimensional reconstructions from serial optical sections can easily be made. Another potentially important use of this microscope is that relatively large strips of tissue, more than one cell thick, can be stained and the distribution of antigens below the surface cell layer probed (Wick *et al.* 1989).

Immuno-electron microscopy

Post embedding labelling. At the electron microscope level the options available in terms of the variety of preparative techniques appear to be limited. The pre-embedding labelling procedures, used with such great effect in animal cell biology to locate structural antigens such as cytoskeletal proteins in detergent-treated cells (Lawson 1986), cannot be used in plant cells due to the presence of the cell wall. Likewise plasma membrane-bound surface antigens can only be located after preparation of protoplasts, which undoubtedly causes rearrangement of molecules on the cell surface. The labelling of thick cryosections, prior to re-embedding in resin and thin sectioning, has been used (Raikhel *et al.* 1984), but this technique has not proved very popular due to the lack of penetration of gold probes into the thick cryosection and the technical difficulties of resectioning.

Due to these constraints by far the overwhelming majority of studies have employed post-embedding labelling techniques on ultra-thin sections of resin-embedded material. These techniques are now routine in many laboratories. General protocols will not be discussed here but can be found in the many books and review articles on immunocytochemistry (Bullock & Petrusz, 1982, 1983, 1985; Polak & Varndell 1984; Wang 1986; Hawes 1988). For a review on the application of these techniques to plant cells the reader is referred to Herman (1988). Here we will limit the discussion to the choice of fixatives and embedding resins which are perhaps the two most crucial decisions to be made prior to undertaking an immunoelectron microscopy study.

Satisfactory retention of antigenicity in the tissue is the major goal to be achieved when preparing the specimen for immunoelectron microscopy. Many antigens are denatured both by fixatives and the curing reaction of resins particularly if heat is involved. In most cases the use of osmium tetroxide is precluded although it has been used in protocols to localize storage proteins in developing seeds (Craig & Goodchild 1984) and microbody enzymes in leguminous nodules (Van den Bosch & Newcomb 1986). However, even in these cases osmication undoubtedly had a major effect on the antigenicity in the sections. Thus, in the majority of investigations, fixation will be restricted to paraformaldehyde, glutaraldehyde or mixtures of both (e.g. Shaw & Henwood 1985; Gubler *et al.* 1987; Herman 1988). This will inevitably lead to a reduction in the quality of structural preservation and reduction in contrast and post-sectioning staining procedures have to be modified accordingly.

The use of cryofixation techniques and freeze substitution to aid in the retention of antigenicity will undoubtedly increase in popularity during the current decade (Elder 1989). Antigens which have extreme sensitivity to fixation, such as higher plant actin, have now been immunostained at the EM level in freeze-substituted cells (Lancelle & Hepler 1989). Recent developments in freezing technology, such as the high-pressure freezing apparatus (Moor 1987), which will permit good freezing of plant tissues to a hitherto unobtainable depth within tissues (Craig & Staehelin 1988), will allow the localization of fixation-sensitive antigens deep within freeze-substituted tissues.

The choice of embedding procedure can be critical in the preservation of antigenicity. Although the epoxy resins generally give superb structural preservation and have good sectioning characteristics, they are generally unsuitable for immunocytochemical applications due to loss of antigenicity which is also exaggerated by the heat curing process. The hydrophilic Lowicryl resins were specifically designed for the low-temperature polymerization required of many immunocytochemical procedures (Carlemalm *et al.* 1982) and have been used in various applications (Shaw & Henwood 1985; Gubler *et al.* 1987) but have been reported to be difficult to use with many plant tissues (Herman 1988).

However, the London resin LR White has proved to be an extremely reliable resin for plant immunocytochemical applications (Craig & Miller 1984; Van den Bosch *et al.* 1989). This acrylic resin has the hydrophilic properties of Lowicryl and many other advantages. The resin has an extremely low viscosity, needs no mixing and is not toxic. It can also be polymerized by a variety of methods as follows: (1) Heat curing at 60 or 50°C (Fig. 4),(2) Catalytic curing at room temperature (the reaction is extremely exothermic), (3) low temperature ultraviolet polymerization using the Lowicryl catalyst benzoin methyl ether (Fig. 5) (Van den Bosch *et al.* 1989). Thus, the resin can be used with osmicated material for conventional electron microscopy or with unosmicated specimens and polymerized by the most convenient technique for retaining antigenicity. In the authors' laboratories low-temperature polymerization is carried out at −20°C in a cryostat fitted with a suitable

ultraviolet source. Figure 5 shows the localization of a species-specific antigen in the cell walls of *Penicillium islandicum* after aldehyde fixation and low-temperature embedding in LR White.

Other techniques. Ultrathin cryosectioning is frequently used with great success on animal tissues (Tokuyasu 1986). However, this is another technique that has largely been ignored by plant scientists. It can be difficult to cut ultra-thin cryosections of plant cells, but with suitable sucrose infiltration it is by no means impossible. Greenwood & Chrispeels (1985) used this technique with great success to immunolocalize phaseolin and phytohemagglutinin in developing bean cotyledons. One modification of the standard Tokyasu technique that should prove useful is the final embedding of the labelled cryosection in a thin layer of LR White resin rather than in methyl cellulose or polyvinyl alcohol. Unlike the low temperature resin-embedding techniques, post labelling osmication of the cryosections can be carried out. This will result in an acceptable level of preservation and contrast in the cellular membranes, thus overcoming one of the major shortcomings of aldehyde-only fixation.

Labelling of unembedded cells can be achieved on broken open cells and protoplasts. In a modification of the dry cleave technique, whole mounts of paraformaldehyde-fixed cortical root cells were broken open under buffer and the microtubule cytoskeleton immunolabelled prior to fixing with glutaraldehyde and osmium tetroxide followed by dehydration and critical-point drying (Traas & Kengen 1986). Similar labelling of intermediate filament antigens on cleaved protoplast 'footprints' has recently been described by Goodbody *et al.* (1989). This technique should prove useful for the localization of antigens associated with the cytoplasmic face of the plasma membrane and the cortical cytoskeleton of cells.

One area of immunochemical technology that has been used extensively on animal cells but which has yet to be applied to plant tissues is that of freeze-fracture cytochemistry. This is the combination of freeze-fracture techniques with various immunogold labelling protocols. Various methodologies have been developed for labelling and freeze-fracturing or freeze-fracturing and labelling cells (Severs 1989). These may prove useful for studying the distribution of antigens on the surface of membranes and even within the lipid bilayer if cells are fractured before labelling. As techniques improve for the cryopreservation of plant tissues it is likely that these techniques will be developed and modified for the study of plant antigens.

CONCLUSIONS

Antibodies are now essential tools in plant science and, unless research is to be severely limited, the skills and resources involved should be available to laboratories engaging in such areas. The authors' experiences indicate that establishing such facilities over a number of years need not be a daunting task, and useful results can quickly be obtained, for instance by using antibodies purchased or given by other laboratories. The key to success in using antibodies in plant science is in obtaining preparations of high specificity and titre; this involves paying attention to every step, including careful and thorough screening of antibodies by a variety of techniques.

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