



CELL WALL SYMPOSIUM



New Zealand Journal of Forestry Science

39 (2009) 197-205

<http://nzjfs.scionresearch.com>



Probing Cell Wall Structure and Development by the Use of Antibodies: A Personal Perspective[†]

Paul Sutherland,* Ian Hallett and Midori Jones

The New Zealand Institute for Plant and Food Research Ltd, Private Bag 92 169, Auckland, New Zealand.

(Received for publication 11 November 2008; accepted in revised form 1 October 2009)

* Corresponding author: paul.sutherland@plantandfood.co.nz

Abstract

Immunolabelling is a powerful technique that can visualise the spatial and temporal arrangement of polysaccharides within plants, providing detail of localisation within tissues, cell types and individual cell walls not obtainable through chemical extraction methods. An increasing number of highly characterised antibodies to cell wall antigens are now becoming available. When using any of these antibodies there is a need for careful interpretation of the labelling patterns, and adequate controls to ensure specific labelling. This review examines some of the issues involved in obtaining meaningful results based on examples from our own work. We focus in particular on immunolabelling of fixed resin embedded material, providing a basic protocol and illustrating the results that can be obtained using it. We also review precautions that must be taken to verify that the results obtained are meaningful and how to troubleshoot if things go wrong.

Keywords: immunocytochemistry; fluorescence microscopy; fixation; antibody; polysaccharides; pectin

[†] Based on a paper presented at the 3rd Joint New Zealand-German Symposium on Plant Cell Walls, 13-15 February 2008, Auckland, New Zealand

Introduction

The plant cell wall is a structurally complex feature, the precise architecture of which is varied and still not clearly understood (Harris, 2005). The primary wall is composed mainly of polysaccharides with lesser amounts of protein, glycoprotein and phenolics, with the addition of lignin in the secondary wall. Antibodies to specific antigens within the wall have proved to be powerful tools to gain insight into the spatial and developmental aspects of cell wall structure and function, and to complement biochemical analysis (Knox, 1997; Willats et al., 2000; Willats & Knox, 2003). Immunolocalisation of plant cell wall antigens was first reported thirty eight years ago (Knox et al., 1970; Vreeland, 1970). Since then, increasing numbers of antibodies have become available, many of which can be obtained from commercial suppliers such as PlantProbes (University of Leeds,

Leeds, UK; 'JIM' and 'LM' range of monoclonal antibodies), CarboSource (Complex Carbohydrate Research Center, University of Georgia, Athens, USA; 'CCRC' range) and Biosupplies (Parkville, Victoria, Australia). In some cases (e.g. the JIM5 and JIM7 antibodies to epitopes on homogalacturonan pectin or LM5 to (1→4)-β-galactan side chains in rhamnogalacturonan I), antibodies label a wide range of tissue types in many different plants. However, other antibodies may be much more restricted in their range of tissues or species, for example LM8 labels xylogalacturonan in sites of cell detachment and separation whilst LM9 binds to an epitope that is a structural feature of cell wall pectic polysaccharides only of plants belonging to the family Amaranthaceae, such as sugar beet and spinach (Clausen et al., 2004). Examples of some commonly used antibodies and their specificities are shown in Table 1. Although specificity for the polysaccharide or range of polysaccharides

may be clear, the exact epitope or range of epitopes responsible for this specificity is frequently unclear. However, ongoing research will clarify this in greater detail and make interpretation of the labelling more accurate. For example, the specificity of the monoclonal antibodies JIM5 and JIM7 is associated with the degree of methyl esterification of homogalacturonan. However, the exact patterns responsible for antibody attachment have only slowly become apparent (Clausen et al., 2003). Information from antibody labelling should not be treated in isolation, and a range of other techniques can be used to supplement the information. For example, we frequently use conventional stains such as Calcofluor, which is a fluorescent stain for cellulose, chitin and other β -linked polymers (Hughes & McCully, 1975) and autofluorescence under UV excitation which can localise lignin and suberin.

Examples of methods used in immunolabelling

Plant cell wall antibodies can be used to detect epitopes in plants at a range of scales from whole organs, through tissues and cells to the detailed structure within the cell wall. Some labelling techniques use indirect methods. Tissue prints of plant organs can establish the overall distribution of specific polysaccharides over entire organs (Willats et al., 1998). Recently, Moller et al. (2007) developed a methodology that enables the occurrence of cell wall glycans to be systematically mapped throughout the plant in a semi-quantitative high throughput fashion. This technique (comprehensive microarray polymer profiling, or CoMPP) generates

microarrays from extracted glycans of tissue and organs and probes with monoclonal antibodies and carbohydrate-binding modules (CBMs) (McCartney et al., 2004). It allows the relative level of cell wall glycans to be mapped rapidly within and between plants. Even whole organs may be directly labelled. For example, McCartney et al. (2003) labelled intact seedling roots of *Arabidopsis thaliana* (L.) to visualise the variations in epitopes on the root surface during elongation. At the tissue or cellular level, immunolabelling is normally carried out on sectioned tissues which may be fresh, fixed, or fixed and embedded.

Immunolabelling is usually reported by some secondary probe that can be visualised: fluorescent molecules, chromophores, or colloidal gold, which can be silver-enhanced to produce large visible particles. Fluorescent probes have the advantage that they tend to produce a 'cleaner', more distinct image than chromophores. Silver enhancement of colloidal gold is also prone to much more non-specific background labelling. Generic fluorophores such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) have to a large extent been replaced by proprietary molecules such as the Alexa Fluor® Dyes (Invitrogen, Carlsbad, USA), which are brighter and more photo-stable. More recently, Quantum Dots or Qdots® (Invitrogen, Carlsbad, USA) have been introduced. These are nanometre-scale atom clusters containing from a few hundred to a few thousand atoms of a semiconductor material (cadmium mixed with selenium or tellurium) that have then been coated with an additional semiconductor

TABLE 1: A selection of antibodies used by the authors for plant cell wall immunolabelling.

Antibody Name	Specificity	Reference
JIM5	anti-homogalacturonan (unesterified, partially esterified)	Clausen et al., (2003)
JIM7	anti-homogalacturonan (methyl-esterified)	Clausen et al., (2003)
LM2	anti-arabinogalactan protein	Smallwood et al., (1996)
LM5	anti-(1→4)- β -galactan	Jones et al., (1997)
LM6	anti-(1→5)- α -arabinan	Willats et al., (1998)
LM8	anti-xylogalacturonan	Willats et al., (2004)
LM9	anti-feruloylatedgalactan	Clausen et al., (2004)
LM10	anti-(1→4)- β -xylan	McCartney et al., (2005)
LM11	anti-(1→4)- β -xylan/arabinoxylans	McCartney et al., (2005)
LM15	anti-xyloglucan (XXXG structural motif)	Marcus et al., (2008)
LM18	anti-homogalacturonan	Verhertbruggen et al., (2009)
LM19	anti-homogalacturonan	Verhertbruggen et al., (2009)
LM20	anti-homogalacturonan	Verhertbruggen et al., (2009)
PAM1	anti-homogalacturonan	Mansfield et al., (2005)
2F4	anti-homogalacturonan (calcium-requiring configuration)	Liners et al., (1989)
CCRC-M1	anti-xyloglucan/fucosylated RG1	Puhlmann et al., (1994)
BGM C6	anti-(1→4)- β -mannan	Pettolino et al., (2001)
	anti-(1→3)- β -glucan	Meikle et al., (1991)

shell of zinc sulphide. The emission range of a particular quantum dot is narrow and dependent on its size, so a single wavelength excitation source is all that is required for viewing Quantum Dots. Because these nanocrystals are particle-based fluorophores, they can be used in correlative light and electron microscopy. However, Quantum Dots are larger than fluorophore dyes and currently much more expensive.

Whilst the distribution of polysaccharide epitopes can be determined over tissues within the plant organ using immunolabelling at the light level, immunolabelling for the electron microscope is required to look in detail at distributions within a cell wall. With this technique, either colloidal gold or Nanogold® (Nanoprobes, Yaphank, N.Y., U.S.A.) is used as a secondary probe. While this technique has greater resolution than fluorescent immunolabelling, the number of cells examined is small and cell type is often difficult to determine (Sutherland et al., 1999). It is thus difficult to determine whether the features being observed are representative of the tissue as a whole. Fluorescein-Nanogold (Nanoprobes) can be used in correlative light and electron microscopy, permitting observation of the same section using both techniques. Ideally, a range of different techniques should be used to build a full understanding of the subject. Initial immunolabelling studies using tissue prints and light microscopy will establish labelling patterns. Electron microscopy can then be used to examine these labelling patterns in greater detail. The last sections of this paper will focus on fluorescent immunolabelling.

Antibody labelling is most successful on fresh, unprocessed material as the antigen and specific epitopes have not been modified. However, depending on the antigen, it can also be carried out on fixed and embedded material, although the extent of processing will tend to reduce the ability to label. Fixation will stabilise the tissue and allow labelling and observation to be carried out at later dates, increasing the number of samples that can be taken and viewed. Embedding allows for sectioning of soft tissues (such as ripe fruit), and for sections to be much thinner than in fresh material (less than 1 µm), improving the image resolution and reducing autofluorescence. Fixation and embedding allows for sectioning and immunolabelling years after the material has been collected. In the sections that follow we describe the techniques we have used for immunolabelling embedded tissue, discuss some of the issues involved in producing meaningful results and illustrate them with examples from a range of plants we have looked at. These examples are illustrated in Figures 1 and 2.

Plant material

Plant materials used as illustrations of immunolabelling in this study are: green and red tomato (*Solanum lycopersicum* L.); *Actinidia arguta* (Sieb. et Zucc.)

Planch. ex Miq. var. *arguta*; green kiwifruit (*Actinidia deliciosa* (A.Chev) C.F.Liang and A.R.Ferguson var. *deliciosa* "Hayward"); bean (*Phaseolus vulgaris* L.); pea (*Pisum sativum* L.); maize (*Zea mays* L.); carrot (*Daucus carota* L.) root; coffee (*Coffea arabica* L.); *Sandersonia aurantiaca* L. (Hook); cherry (*Prunus avium* (L.) L.); and kiwifruit (*Actinidia deliciosa*) leaf infected with fungus (*Cercospora* sp.).

Standard methodology

All samples were fixed and resin-embedded using our routine method. This is used as a starting point for our samples. Tissue is fixed in a relatively low strength fixative (a minimal fixative of 2% paraformaldehyde (w/v) and 0.1% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) under vacuum for 1 h) to preserve antigenicity (Griffiths, 1993). Tissue is washed in buffer, dehydrated in an ethanol series to 100% dry ethanol and embedded in LR White resin (London Resin, Reading, UK) (for plant material infiltration is usually carried out using several changes of resin over 2-3 days on a rotator with a final polymerisation temperature under 60 °C). Sections are best cut using a diamond knife with a thickness of 200 nm being routinely used to minimise autofluorescence though thicker sections (up to 1 µm) can be successfully used. Sections are mounted on poly-L-lysine coated slides, dried overnight on a hot plate (45-50 °C) and then immunolabelled (Sutherland et al., 2004).

For immunolabelling, sections are blocked with 0.1% bovine serum albumin (BSA-c, Aurion, Wageningen, The Netherlands) in PBS-T (phosphate-buffered saline plus 0.1% Tween 80) for 15 min and then incubated overnight with antibodies diluted in blocking buffer in a humid chamber at 4 °C. The optimal dilution is based on experimentation and may vary considerably from 1 : 5 (e.g. JIM5, JIM7) to 1 : 200 (e.g. LM5, LM6, LM8) for the antibodies used here. After incubation the slides are washed in PBS-T and incubated for 1 h in the appropriate secondary antibody. This will vary depending on the species the primary antibody was raised in, e.g. goat anti-rat for JIM5 and LM5 monoclonals from rat cells. Secondary antibodies we use are conjugated to an Alexa dye (Alexa 488, Alexa 594) (Molecular Probes, Eugene, Oregon, USA.) and are diluted 1 : 600 in PBS. Sections are finally washed in 2-3 ml of PBS-T and mounted in Citifluor AF1 antifadent solution (Citifluor, Leicester, UK).

Immunolabelling is normally carried out in a chamber created by using a PAP pen (Daido Sangyo Co. Ltd, Tokyo, Japan) to draw a hydrophobic barrier on the slide around the sections, although other techniques such as the use of the Shandon coverplate™ technology have been used. This general starting protocol can be modified if specific issues are encountered (different antigens, different materials). Alternative methods can involve microwave

enhanced fixation and processing (Russin & Trivett, 2001) or freezing and freeze substitution (Echlin, 1992) of the samples. Use of osmium tetroxide as a primary or secondary fixative should be avoided, as it severely reduces or eliminates labelling.

Issues to consider when immunolabelling

Care must be taken in interpretation of the results of immunolabelling experiments. Presence of labelling in a specific location will normally be interpreted as indicating presence of the antigen. However, controls must be used to guard against non-specific labelling by either the primary or secondary antibody. Controls are an important part of establishing specific labelling in any immunolabelling study. Even though some cell wall antibodies have been extensively studied (JIM5, JIM7, LM5), it is essential that sufficient controls are performed when studying new material. The most useful controls involve either omitting the primary antibody from the protocol or a pre-incubation of primary antibody with excess antigen before incubation with the sample. The former control detects non-specific binding of the secondary antibody-detection system conjugate to the tissue or section, while the latter determines specificity of the antibody.

Absence of labelling cannot automatically be interpreted as indicating that a constituent itself is absent but may merely reflect the inability of the antibody to bind. Specific epitopes may be structurally altered or masked by other components in the system, and thus inaccessible to the antibody (Knox, 2008). For example, the pre-treatment of sections of plant material with pectate lyase [in *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer] has recently been demonstrated to unmask xyloglucan epitopes masked by pectic homogalacturonan (Marcus et al., 2008). Binding of some cell wall antibodies may be increased by using high pH treatments (Verhertbruggen et al., 2009). Similarly, variations in processing methods used to prepare the specimen may change accessibility. Thus, as in any good experiment, the results of immunolabelling should be able to be verified in different ways.

When labelling any tissue for the first time, it is important to know that the labelling protocol is working properly and that the interaction of antibody and antigen is optimal. By including sections of an appropriate test material on the same slide as the experimental material, it is easy to see whether failure to label is likely to be due to the processing of the sample or the immunolabelling. We have utilised a number of 'test materials' to demonstrate optimal labelling. Tissues assessed for inclusion as test material have been root, stem and leaf of seedlings of maize (Figures 2C and 2D), pea, bean and tomato, fruit tissue from green tomato (Figure 1E), apple, pear, and red tomato

plus carrot root. Some antibodies (e.g. JIM5, JIM7, LM5) labelled regions in a number of the different plants and tissues, one antibody (LM8) only labelled cells in the root cap, shown for maize in Figure 2D.

When a negative result occurs in an immunolabelling experiment (that is failure to label a component that has been shown to be present by other techniques), the reason is often not clear. The dot-spot test uses spots of antigen on nitrocellulose strips as a model system for trouble-shooting that makes it possible to pinpoint a problem to a defined part of the immunodetection experiment. It is particularly useful in assessing the effect of fixation and embedding. Ideally, a purified antigen should be used, but any suspension material containing the antigen that can be bound onto nitrocellulose will work. After verifying that the antibody can bind to the antigen in this state, the spots of antigen on nitrocellulose can be used to check steps in an immunolabelling/preparation protocol (Riederer, 1989). For instance, a dilution series of a specific antigen can be spotted on to nitrocellulose strips and either fixed in varying concentrations of glutaraldehyde and paraformaldehyde, or left unfixed. The membranes can then be washed, blocked, incubated with a primary antibody, washed and the primary antibody detected with an appropriate detection system. This test will determine the effect of fixation on the antigen, whether the antigen can be detected or not, and the extent to which detection may be reduced. In the same way, the effect of differences in dehydration or embedding can be tested to find the optimal conditions. In general, any fixation or processing will affect the ability to label. However, although some antigens are highly sensitive others appear relatively unaffected. In some cases, fixation may improve labelling as mobile antigens are attached to other cellular structures.

Another consideration is the host animal from which antibodies are produced. Antibodies from different sources are frequently produced in different hosts. For example, PlantProbes antibodies are generally produced in rats and 2F4 and CCRC-M1 antibodies are produced in mice. Other antibodies may be produced in rabbits or other, more exotic animals. This can be a source of error if the wrong secondary conjugate is used, but it also allows for some informative double labelling experiments, where primary antibodies from different species can be reported with different secondary antibodies on the same sections. In this regard, all labelled material can be counter-stained with dyes (e.g. Calcofluor – fluorescent brightener 28) so long as these do not quench the fluorescence of the label, Figures 1A, 1B, 2D, 2F and 2G illustrate this technique in cherry stigma, maize root tip and green tomato. Visualisation of double labelling is accomplished by combining separate images of label and counter-stain in this case, to relate cellulose and a specific polysaccharide component (see Figures 2E, 2F and 2G).

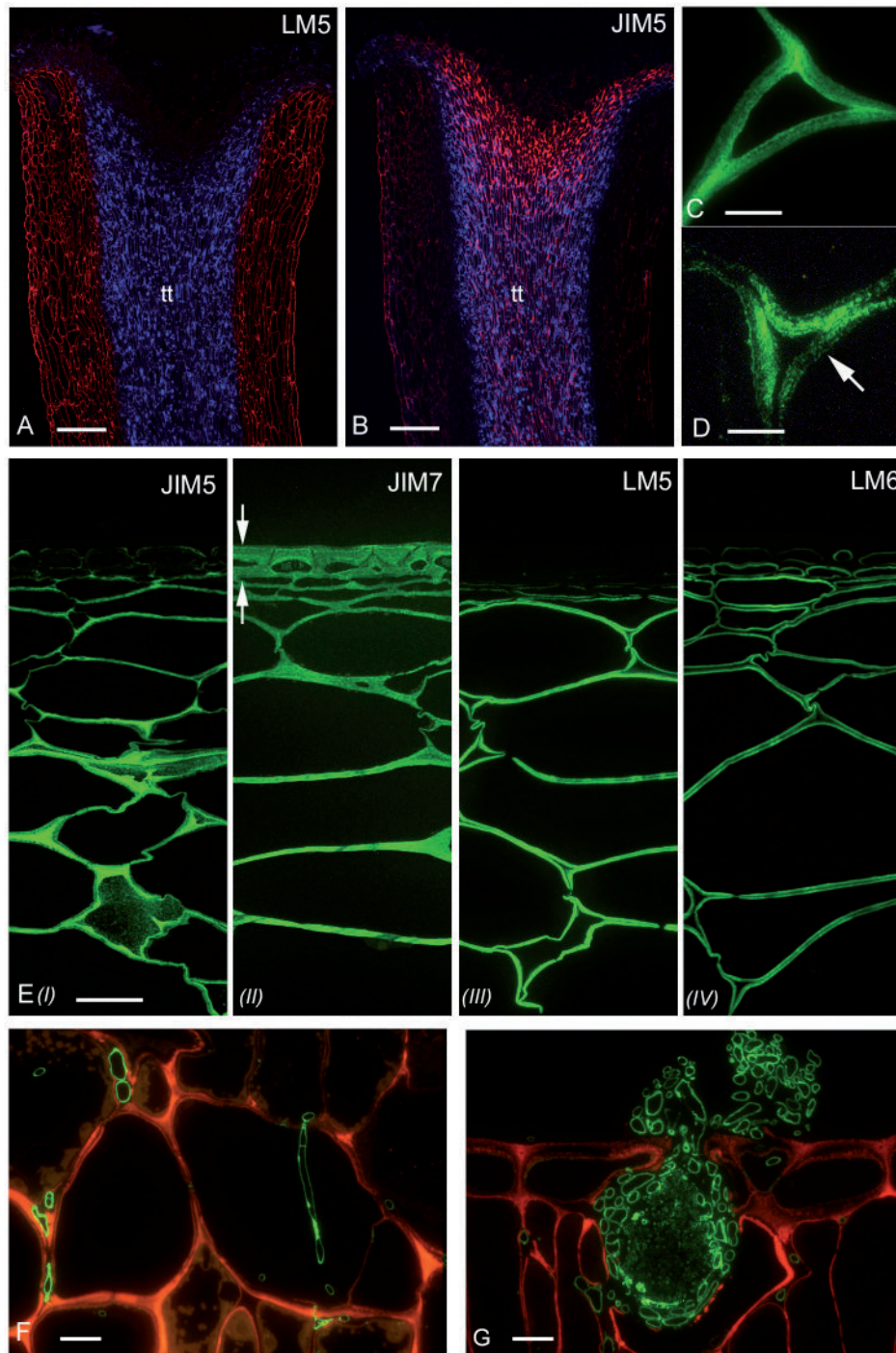


FIGURE 1: A- Longitudinal resin section through a cherry stigma immunolabelled with LM5 and Alexa 594 (red) and counterstained with Calcofluor (blue), tt-transmission tissue;
 B- Section immunolabelled with JIM5 and Alexa 594 (red) and stained with Calcofluor (blue);
 C- Cell walls at the junction of 3 cells of firm *Actinidia deliciosa* (green kiwifruit) labelled with JIM5;
 D- Cell walls at the junction of 3 cells of ripe *Actinidia deliciosa* (green kiwifruit) labelled with JIM5 (arrow indicates loss of labelling);
 E- Green tomato tissue labelled with JIM5 E(i) , JIM7 E(ii), LM5 E(iii), LM6 E(iv) (epidermis is arrowed);
 F- Kiwifruit leaf infected with a *Cercospora* sp. fungus. The leaf cell walls are labelled with JIM5 (red) and the fungal cell walls with anti-(1→3)-β-glucan antibody (green).
 G- *Cercospora* sp. fruiting body on kiwifruit leaf, labelling as in F.

Bar in 1A, 1B, 1E =100 μm; Bar in 1C, 1D, 1F, 1G = 10 μm.

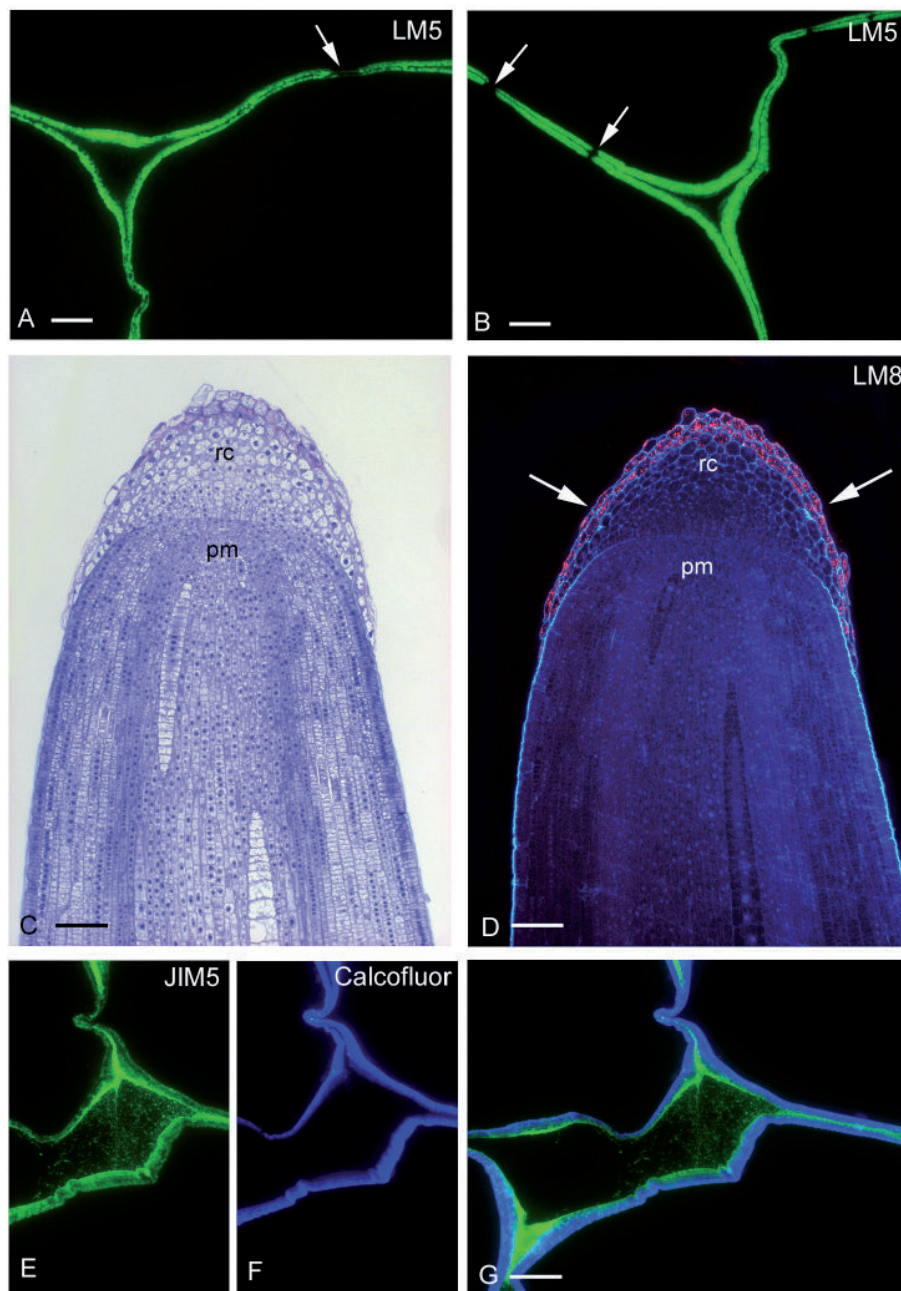


FIGURE 2: A- LM5 labelling of cell walls at the junction of 3 cells in the outer pericarp of *Actinidia deliciosa* (green kiwifruit) (plasmodesmatal pitfields arrowed).

B- LM5 labelling in similar junction in the cortex of green tomato fruit. Note the similar labelling pattern.

C- longitudinal resin section through a maize root tip stained with toluidine blue, pm-root primordium, rc-root cap.

D- Adjacent section immunolabelled with LM8 (red - arrowed) and Calcofluor.

E-G- Section of green tomato cortex (E- Immunolabelled with JIM5, F- Stained with calcofluor and G- Overlay of F and G).

Bar in 2A, 2B, 2G = 10 μ m; Bar in 2C, 2D =100 μ m..

Application examples from our work

Our use of antibodies to understand plant cell wall structure and its changes falls into three main areas: development and senescence of plant structures, ripening and softening of fruit, and effects of fungal infection. The following examples illustrate these areas. All have been prepared using the methods outlined above.

Development and senescence of plant structures

A typical application is shown in Figure 1, where within the developing cherry flower stigma, two cell wall polymers have distinct locations (Figures 1A and 1B). In this case, LM5 labels the stigma wall and not the transmission tissue, whereas JIM5 labels the transmission tissue intensely. Staining with Calcofluor determines the location of cellulose and hemicellulose.

The chemical composition and structure of the cell wall polysaccharides of the coffee bean has been the subject of extensive study (Redgwell et al., 2002). However, just how the individual polymers were arranged or distributed throughout the cell wall was largely unknown. Using immunolabelling in conjunction with staining, a "structural map" was obtained for the location of the major groups of non-cellulosic polysaccharides (arabinogalactan-proteins (AGPs), galactomannans and pectins) (Sutherland et al., 2004). There was strong labelling of the whole cell wall by LM2, specific for AGPs, and BGM C6, specific for (1→4)- β -mannan. Using LM6 produced a completely different labelling pattern, which was located in a single layer of epidermal cells and in a layer adjacent to the cell lumen in the remaining cells of the bean. LM5 labelled a narrower layer than LM6. JIM7 labelled the middle lamella only.

In cell walls of *Sandersonia aurantiaca* petals, distinct differences in labelling patterns of outer/inner epidermal walls occurred compared with the internal parenchyma cells. While the intensity of some labels [galactan (LM5), arabinan (LM6)] decreased as flower petals became wilted and senesced, partially esterified pectin antibody (JIM5) labelling changed little, either in intensity or distribution, and for xyloglucan (CCRC-M1) appeared to increase (O'Donoghue & Sutherland, 2007).

Ripening and softening in fruit

To understand changes occurring as fruit ripen and soften the immunolabelling patterns of unripe fruit must first be established as a comparison for a range of antibodies. In green tomato, JIM5 labels the middle lamella and cell junctions strongly, JIM7 labels the entire wall evenly, while LM5 and LM6 label the edges of the cell wall, but not the middle lamella and plasmodesmatal pitfields (Figure 1E).

Changes occurring during fruit ripening and softening can be very marked. In green kiwifruit, ripening is characterised by a significant decrease or loss of JIM5 labelling over the cell wall (Figures 1C and 1D) (Sutherland et al., 1999). This contrasts with changes that occur in another member of the *Actinidia* species. *Actinidia arguta* is a small, ovoid fruit with an edible skin (also known as "baby kiwifruit", "baby kiwi" or "hardy kiwifruit"). As this fruit softens JIM5 labelling is retained throughout the cell wall and there are large amounts of labelled material in the cytoplasm of the cell (unpublished results). This indicates that the pectic polymer recognised by JIM5 has migrated from the cell wall to the cell lumen as polymers long enough to be able to be recognised by the JIM5 antibody. This could explain the gelatinous texture of ripe *A. arguta* compared with the soft juicy texture of *A. deliciosa*, which shows no pectin labelling within the cell when ripe.

Fungal infection

Cell wall antibodies can also be used to locate fungi during infection of plant tissue and to determine position and dissolution of the cell wall (Figures 1F and 1G). Figure 1F shows fungal hyphae ramifying through kiwifruit leaf tissue while Figure 1G shows a fungal fruiting body at the surface of the leaf. The fungal cell wall is labelled with an antibody to (1→3)- β -glucan reported with a green fluorochrome, while the plant cell wall is visualised with JIM5 and a red fluorochrome. The anti-(1→3)- β -glucan also labels callose (not shown in these figures), which is deposited in fungal induced cell wall papillae and adjacent to plasmodesmatal pit fields. Double labelling of fungal and plant cell walls is useful in illustrating the ways in which the hyphae penetrate into cells. Figure 1F shows that penetration occurs with very little loss of pectin. In other examples (not shown), walls associated with fungi lose labelling with JIM5 (and JIM7 and LM5) as the wall is macerated by fungal enzymes.

Characteristic labelling patterns

Antibodies to cell wall components often have a characteristic labelling pattern across different plants and plant organs which reflects the underlying structure of plant cell walls. For example, in a range of diverse tissues from different plants such as tomato, potato and pea cotyledons, LM5 labels the edge of the cell wall adjacent to the cell lumen, but does not label the middle lamella or plasmodesmatal pit field region (Jones et al., 1997; Bush & McCann, 1999; McCartney et al., 2000). Similarly, the labelling pattern of LM5 on the cell walls of the outer pericarp of green kiwifruit (Figure 2A) is virtually identical to that of the cortex of green tomato (Figure 2B). The absence of (1→4)- β -galactan (LM5) from cell walls throughout the region of pit fields of both species illustrated indicates the existence of a distinct cell wall architecture in these regions (Orfila & Knox, 2000; Willats et al., 2000).

Conclusion

The use of immunolabelling techniques to study plant cell walls can clearly provide much valuable information on the underlying structure of the walls, their synthesis and degradation, and the role they play in plant structures. The value of such information will only increase as greater numbers of antibodies are produced and knowledge of the specificity of their labelling increases. However, interpretation of results depends on a good knowledge of the limits placed on the technique by the processes involved in obtaining the sample and by the chemistry of the wall itself.

References

- Bush, M. S., & McCann, M. C. (1999). Pectic epitopes are differentially distributed in the cell walls of potato (*Solanum tuberosum*) tubers. *Physiologia Plantarum*, 107(2), 201-213.
- Clausen, M. H., Willats, W. G. T., & Knox, J. P. (2003). Synthetic methyl hexagalacturonate hapten inhibitors of anti-homogalacturonan monoclonal antibodies LM7, JIM5 and JIM7. *Carbohydrate Research*, 338(17), 1791-1800.
- Clausen, M. H., Ralet M-C., Willats, W. G. T., McCartney, L., Marcus, S. E., Thibault, J-F., & Knox, J. P. (2004). A monoclonal antibody to feruloylated-(1→4)-β-D-galactan. *Planta*, 219(6), 1036-1041.
- Echlin, P. (1992). *Low-temperature microscopy and analysis*. New York, USA and London UK: Plenum Press.
- Griffiths, G. (1993). Fixation for fine structure preservation and immunocytochemistry. In, *Fine structure immunocytochemistry* (pp. 26-89). Berlin and Heidelberg, DE: Springer-Verlag.
- Harris, P. J. (2005). Diversity in plant cell walls. In R. J. Henry (Ed.), *Plant Diversity and Evolution: Genotypic and Phenotypic Variation in Higher Plants* (pp. 201-227). Wallingford, UK: CAB International Publishing.
- Hughes, J., & McCully, M. E. (1975). The use of an optical brightener in the study of plant structure. *Stain Technology*, 50, 319-329.
- Jones, L., Seymour, G. B., & Knox, J. P. (1997). Localization of pectic galactan in the tomato cell walls using a monoclonal antibody specific to (1→4)-β-D-galactan. *Plant Physiology*, 113(4), 1405-1412.
- Knox, R. B., Heslop-Harrison, J., & Read, C. (1970). Localization of antigens associated with the pollen grain wall by immunofluorescence. *Nature*, 225(5237), 1067-1068.
- Knox, J. P. (2008). Revealing the structural and functional diversity of plant cell walls. *Current Opinion in Plant Biology*, 11, 1-6.
- Knox, J. P. (1997). The use of antibodies to study the architecture and developmental regulation of plant cell walls. *International Review of Cytology*, 171, 79-120.
- Liners, F., Letesson, J-J., Didembourg, C., & Van Cutsem, P. (1989). Monoclonal antibodies against pectin. *Plant Physiology*, 91(4), 1419-1424.
- Manfield, I. W., Bernal, A. J., Moller, I., McCartney, L., Reiss, N. P., Knox, J. P., & Willats, W. G. T. 2005: Re-engineering of the PAM1 phage display monoclonal antibody to produce a soluble, versatile anti-homogalacturonan scFv. *Plant Science*, 169(6), 1090-1095.
- Marcus, S. E., Verhertbruggen, Y., Hervé, C., Ordaz-Ortiz, J. J., Farkas, V., Pedersen, H. L., Willats, W. G. T., & Knox, J. P. (2008). Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BMC Plant Biology*, 8, 60 doi: 10.1186/1471-2229-8-60
- McCartney, L., Ormerod, A. P., Gidley, M. J., & Knox, J. P. (2000). Temporal and spatial regulation of pectic (1→4)-β-D-galactan in cell walls of developing pea cotyledons: implications for mechanical properties. *The Plant Journal*, 22(2), 105-113.
- McCartney, L., Steele-King, C. G., Jordan, E., and Knox, J. P. (2003). Cell wall pectic (1→4)-β-D-galactan marks the acceleration of cell elongation in the Arabidopsis seedling root meristem. *The Plant Journal*, 33(3), 447-454.
- McCartney, L., Gilbert, H. J., Bolam, D. N., Boraston, A. B., and Knox, J. P. (2004). Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. *Analytical Biochemistry*, 326(1), 49-54.
- McCartney, L., Marcus, S. E., & Knox, J. P. (2005). Monoclonal antibodies to plant cell wall xylans and arabinoxylans. *Journal of Histochemistry and Cytochemistry*, 53(4), 543-546.
- Meikle, P. J., Bonig, I., Hoogenraad, N. J., Clarke, A. E., & Stone, B. A. (1991). The location of (1→3)-β-glucans in the walls of pollen tubes using a (1→3)-β-glucan-specific monoclonal antibody. *Planta*, 185(1), 1-8.

- Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J. D., Knox, J. P., Bacic, A., & Willetts, G. T. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *The Plant Journal*, 50(6), 1118-1128.
- O'Donoghue, E. M., & Sutherland, P. (2007). Maps of the cell wall: using molecular tools to determine polysaccharide arrangement in plant tissues. In U. Schmitt, A. P. Singh, & P. J. Harris (Eds.). *The Plant Cell Wall - Recent Advances and New Perspectives*: No. 223: 61-68. Proceedings of the 2nd New Zealand-German Workshop on Plant Cell Walls, Hamburg, 4-6 October 2006. Hamburg, Germany: Mitteilungen der Bundesforschungsanstalt für Forst- und Holzwirtschaft.
- Orfila, C., & Knox, J. P. (2000). Spatial regulation of pectic polysaccharides in relation to pit fields in cell walls of tomato fruit pericarp. *Plant Physiology*, 122(3), 775-781.
- Pettolino, F. A., Hoogenraad, N. J., Ferguson, C., Bacic, A., Johnson, E., & Stone, B. A. (2001). A (1→4)-β-mannan-specific monoclonal antibody and its use in the immunocytochemical location of galactomannans. *Planta*, 214(2), 235-242.
- Puhlmann, J., Bucheli, E., Swan, M. J., Dunning, N., Albersheim, P., Darvill, A. G., & Hahn, M. G. (1994). Generation of monoclonal antibodies against plant cell wall polysaccharides. I. Characterization of a monoclonal antibody to a terminal alpha-(1,2)-linked fucosyl-containing epitope. *Plant Physiology*, 104(2), 699-710.
- Redgwell, R. J., Curti, D., Fischer, M., Nicolas, P., & Fay, L. B. (2002). Coffee bean arabinogalactans: acidic polymers covalently linked to protein. *Carbohydrate Research*, 337(3), 239-253.
- Riederer, B. (1989). Antigen preservation tests for immunocytochemical detection of cytoskeletal proteins: Influences of aldehyde fixatives. *The Journal of Histochemistry and Cytochemistry*, 37(5), 675-681.
- Russin, W. A., & Trivett, C. L. (2001). Vacuum-microwave combination for processing plant tissues for electron microscopy. In R. T. Giberson & R. S. Demaree (Eds.), *Microwave: Techniques and Protocols*. Totowa, NJ, USA: Humana Press.
- Smallwood, M., Yates, E. A., Willats, W. G. T., Martin, H., & Knox, J. P. (1996). Immunochemical comparison of membrane-associated and secreted arabinogalactan-proteins in rice and carrot. *Planta*, 198(3), 452-459.
- Sutherland, P., Hallett, I., Redgwell, R., Benhamou, N., & MacRae, E. (1999) Localization of cell wall polysaccharides during kiwifruit (*Actinidia deliciosa*). *International Journal of Plant Science*, 160(6), 1099-1109.
- Sutherland, P. W., Hallett, I. C., MacRae, E., Fischer, M., & Redgwell, R. J. (2004). Cytochemistry and immunolocalisation of polysaccharides and proteoglycans in the endosperm of green Arabica coffee beans. *Protoplasma*, 223(2/4), 203-211.
- Verhertbruggen, Y., Marcus, S. E., Haeger, A., Ordaz-Ortiz, J. J., & Knox, J. P. (2009). An extended set of monoclonal antibodies to pectic homogalacturonan. *Carbohydrate Research*, 344(18), 1858-1862.
- Vreeland, V. (1970). Localization of a cell wall polysaccharide in brown alga with labelled antibody. *Journal of Histochemistry and Cytochemistry*, 18, 371-373.
- Willats, W. G. T., Marcus, S. E., & Knox, J. P. (1998). Generation of a monoclonal antibody specific to (1→5)-α-L-arabinan. *Carbohydrate Research*, 308(1/2), 149-142.
- Willats, W. G. T., Steele-King, C. G., McCartney, L., Orfila, C., Marcus, S. E., & Knox, J. P. (2000). Making and using antibody probes to study plant cell walls. *Plant Physiology and Biochemistry*, 38(1/2), 27-36.
- Willats, W. G. T., & Knox, J. P. (2003). Molecules in context: probes for cell wall analysis. In J. K. C. Rose (Ed.) *The plant cell wall* (pp. 92-110). Oxford, UK: Blackwell Publishing Ltd.
- Willats, W. G. T., McCartney, L., Steele-King, C.G., Marcus, S. E., Mort, A., Huisman, M., Van Alebeek, G-J., Schols, H. A., Voragen, A. G. J., Le Goff, A., Bonnin, E., Thibault, J-F. & Knox, J. P. (2004). A xylogalacturonan epitope is specifically associated with plant cell detachment. *Planta*, 218(4), 673-681.