THE DISTRIBUTION OF UN-ESTERIFIED AND METHYL-ESTERIFIED PECTIC POLYSACCHARIDES IN PINUS RADIATA

Tracy L. Putoczki, Juliet A. Gerrard, Brian G. Butterfield and Sandra L. Jackson

School of Biological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8020, New Zealand

SUMMARY

A cationic dye which binds acidic polymers such as pectin and monoclonal antibodies, directed against un-esterified and methyl-esterified (JIM5) and only methyl-esterified (JIM7) pectin epitopes, were used, in conjunction with light microscopy, confocal microscopy and immunogold electron microscopy, to study the spatial distribution of pectin in the xylem tissue of Pinus radiata D. Don. Histochemistry demonstrated that pectin was located in the compound middle lamella (CML) of the maturing tracheid cell wall, in addition to the pit membranes and the CML of the ray cell walls. Immunogold labeling showed differential distribution of the pectin epitopes within the CML of the maturing cell walls. Moreover, in the xylem, the JIM5 and JIM7 epitopes were found to be restricted to distinct tissues. Neither epitope occurred in the secondary walls of the xylem cells. These patterns of epitope expression were not maintained in the mature cell. These results represent the first demonstration of restricted spatial patterns of distribution of these epitopes in the xylem tissue of radiata pine and are consistent with results from other coniferous gymnosperms.

Key words: Cambium, cell wall, pectin, monoclonal antibodies, wood quality, xylem.

INTRODUCTION

Understanding the differentiation of xylem cells is vital to improving wood quality, as their ultra-structure and composition determine softwood properties. The research presented in this paper was performed to establish the distribution of pectic polysaccharides in free standing radiata pine, in order to gain insight into the processes involved in wood formation. These results constitute part of an ongoing investigation directed at understanding wood quality issues, with the long-term goal of enhancement of future radiata pine crops.

Pectic polysaccharides are one of the first cell wall components deposited during growth, are a major constituent of the primary cell walls of conifers, and may play a critical role in determining the physical characteristics of the CML (Thornber & Northcote 1961; Burke et al. 1974; Thomas et al. 1987; Edashige et al. 1995; Edashige & Ishii 1996, 1998; Shimokawa et al. 1999; Willats et al. 2001a). Pectic polysaccharides are believed to be involved in cell adhesion, are linked to the regulation of cell growth,
and are gaining increased recognition for their role in the structure and stability of the cell wall (Knox 1992; Perez et al. 2000; Willats et al. 2001a, 2001b). In addition, pectic polysaccharides are considered to form a gel-like matrix, in which cellulose and other polysaccharides are embedded, allowing pectins to act as a hydrophilic filler that prevents the aggregation and collapse of the cellulose network (Carpita & Gibeaut 1993; O’Neill et al. 1996; Cosgrove 1997; Fleischer et al. 1999; Carpita & McCann 2000).

There are three major pectic polysaccharides which can be further categorized into two groups: those with a homopolymer backbone of galactopyranosyluronic acid residues, such as homogalacturonan (HG) and rhamnogalacturonan-II (RG-II), and those with a heteropolymer backbone of galactopyranosyluronic acid residues interspersed with rhamnopyranosyl residues, such as rhamnogalacturonan-I (RG-I) (Carpita & Gibeaut 1993; Cosgrove 1997). HG is present in the primary cell walls of conifers as seen in Douglas fir (Thomas et al. 1987) and suspension cultured Japanese cedar (Edashige et al. 1995; Edashige & Ishii 1996). It is postulated that HG, RG-I and RG-II are covalently linked to one another, creating a pectin matrix throughout the CML (Willats et al. 2001a; O’Neill et al. 2004).

To aid our understanding of the role of the cell wall in xylem development and wood formation, we have used ruthenium red, a cationic dye that stains pectins dark pink through a reaction with carboxyl groups (Wardrop 1981). In addition, immunocytochemistry coupled to confocal scanning laser microscopy and immunogold transmission electron microscopy studies were undertaken in order to precisely ascertain the distribution of pectic polysaccharides in radiata pine. The monoclonal antibodies that were used in this work are specific to methyl-esterified and un-esterified portions of HG (Knox et al. 1990; Hafren et al. 2000; Willats et al. 2000).

MATERIALS AND METHODS

Plant material

Radiata pine trees from a University of Canterbury field site (planted in 1994) located in Burnham, New Zealand (latitude: -43.6167, longitude: 172.3167), were felled as required during mid-summer of the 2003, 2004 and 2005 growing seasons. Twelve trees from the same clone were chosen to minimize genotypic variation. Stem segments (approximately 1 m) between whorls of branches were removed from a region of the tree approximately 1 to 3 m above the ground and returned to the laboratory.

Ruthenium red staining

Stem segment explants (50 mm axial × 50 mm tangential × 50 mm radial) that had been fixed in 4% formaldehyde in ¼ × PBS were dehydrated in a series of tert-butyl-alcohol solutions, and embedded in paraffin wax. Sections (10 μm) thick were cut with a Reichert-Jung 2040 rotary microtome and mounted on glass slides. The slides were de-waxed, immersed in ammonium hydroxide to remove surface lignin (Micheli et al. 2002), stained in 0.25% (w/v) ruthenium red for 45 minutes, washed thoroughly with distilled water.
Fluorescence microscopy

A small block (3 mm tangential × 2 mm radial × 2 mm longitudinal) was removed from the outer growth ring of the collected stem explants and fixed in 4% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde in 0.1 M cacodylate buffer. The samples were rinsed with 0.1 M cacodylate buffer and dehydrated in an ethanol series, after which they were embedded in LR White resin. Transverse sections (3 μm thick) were cut using a glass knife mounted on a LHB Bromma 11800 pyramitome (Stockholm, Sweden) and placed on poly-L-lysine-coated glass slides (Micheli et al. 2002). Immunocytochemistry was performed on the sections with JIM5 (1:30) or JIM7 (1:30) rat monoclonal antibodies kindly provided by Dr. Paul Knox (Centre for Plant Sciences, University of Leeds, UK). The sections were rinsed in PBS containing 0.1% (w/v) BSA (PBS/BSA), blocked in goat serum for 1⁄2 hour (1:30 in PBS-T/0.1% (w/v) BSA) followed by multiple rinses in PBS/BSA. The samples were incubated in the appropriate dilution of primary antibody (in PBS/0.01% (w/v) BSA) overnight at 4 °C in a humidified chamber. Control sections were incubated in PBS/BSA in place of the primary antibody. The sections were rinsed in PBS/BSA followed by incubation in goat anti-rat IgG FITC secondary antibody (1:100 in PBS/0.01% BSA; Sigma) for one hour in a dark humidified chamber. The sections were then washed in PBS/BSA followed by distilled water and mounted in a 90% glycerol (containing 0.01 M PB, 0.0027 M KCl, 0.137 M NaCl pH 7.4 (Zhang et al. 2003)) and 1% p-phenylenediamine dihydrochloride anti-fade solution. Confocal microscopy was performed on an Olympus IX70 inverted microscope with a BioRad μRadiance confocal scanning system. A Kalman filter was used to collect Z-series images with a 0.5 μm focus step.

Immunogold transmission electron microscopy

Samples were fixed and embedded in LR White resin as described for the fluorescence microscopy. Ultra-thin sections (100 nm) were cut using a LKB 2128 Ultrotome and mounted on formvar-coated nickel grids. Labeling was performed using a Leica EM IGL as per AURION™ Immunogold Reagent Methods, using AURION™ reagents (Wageningen, The Netherlands). Briefly, residual aldehyde groups, present after aldehyde fixation (J. Leuisson, University of Otago, pers. comm.), were inactivated by incubation in a glycine/PBS solution (0.05 M glycine, 0.1 M phosphate). Grids were blocked using AURION™ blocking solution, washed with incubation buffer and incubated in the appropriate dilution of primary antibody made in PBS/0.1% (w/v) BSA for two hours (JIM5 and JIM7, 1:20). Following incubation, the grids were washed in incubation buffer and then incubated in AURION™ Ultra Small Gold Conjugate Reagent (goat anti-rat US (GARa); 1:100) diluted in incubation buffer for 1 hour and 30 minutes. The grids were then washed in incubation buffer, 0.1 M PBS, post-fixed in glutaraldehyde (2% made in 0.1 M phosphate) and washed further with phosphate buffer and distilled water. The grids were silver enhanced using the AURION™ R-GENT Silver Enhancing Kit, washed with distilled water and stained with 1% uranyl acetate and lead citrate in an LKB Bromma 2168 ultrastainer (Bromma, Sweden), prior to viewing with a Phillips TEM at 80 kV.
RESULTS

Distribution of pectic polysaccharides in xylem tissue

As seen in Figure 1a, ruthenium red pectin staining was observed in the CML of cells throughout the xylem. This included cambial and newly divided cells (Fig. 1a, C/ND), differentiating xylem cells (Fig. 1a, D), mature xylem cells (Fig. 1a, M) and latewood (Fig. 1a, LW). This staining was particularly evident in the cambial and newly divided xylem cells, suggesting that its deposition and/or presence may be developmentally regulated (Fig. 1a). As the differentiation of a xylem cell progressed towards maturity,
Figure 2. Transverse sections of xylem tissue showing the distribution of JIM5 and JIM7 epitopes as observed by confocal laser scanning microscopy. JIM5 (a, c, e) and JIM7 (b, d, f) labelled tissue. Cambial cells (a, b) showed the greatest pectin labelling, followed by the radially expanding cells (c, d). Ray cell walls also showed labelling (c, d, arrows). Mature cells (e, f) showed labelling mainly in the ray cell walls and pit membranes (arrows). — Scale bar (a–f) = 20 μm.
observed by tracing the cells in a radial cell file back to their ‘mother cell’, pectin localization remained in the CML and was not observed to spread into the secondary cell walls (Fig. 1a, b, c). Pectin staining was also strong in other regions of the xylem cells, specifically, the CML of the ray cell walls (Fig. 1b) and the bordered pit membranes (Fig. 1c) of differentiating cells.

**Localization of JIM5 and JIM7 epitopes in xylem tissue**

JIM5 and JIM7 labeling was observed for each stage of xylogenesis in radiata pine. JIM5 labeling was strongest in the CML of the cambial/newly divided cell junctions and radial cell walls, and was slightly weaker in the CML of the tangential cell walls (Fig. 2a). As differentiation progressed, JIM5 epitopes were present in the CML of the radially expanding cell walls (Fig. 2c), and were absent in the CML of lignified mature wood (Fig. 2e). JIM5 labeling was, however, present in the ray cell walls (Fig. 2c, arrow) and the pit membranes (Fig. 2e, arrow) of both radially expanding and mature cells. JIM7 labeling was more intense than JIM5 labeling (Fig. 2b, d, f). JIM7 labeling was strong and homogeneous in the CML of both the radial and the tangential cell walls of the cambial/newly divided cells (Fig. 2b), was present in the CML of radially expanding xylem (Fig. 2d), and was drastically reduced in fully lignified cells (Fig. 2f). Similar to the JIM5 results, JIM7 labeling was evident in the ray cell walls (Fig. 2d) and in the pit membranes (Fig. 2f) throughout the xylem. Overall, a gradient distribution of both

![Image](https://via.placeholder.com/150)

**Figure 3.** Transverse sections of xylem tissue showing the distribution of JIM5 and JIM7 epitopes in the cell wall as observed by immunogold electron microscopy. JIM5 (a, c), JIM7 (b, d). Newly divided cells (a, b) and cells that have undergone the initial stages of secondary wall deposition (c, d) show differential pectin distribution. — Scale bars (a, b) = 0.5 μm, (c, d) = 2 μm.
the JIM5 and JIM7 epitopes was observed in the xylem tissue, with this distribution being greatest in the cambial and newly formed cells, and lowest in the mature wood (Table 1).

Table 1. Summary of the distribution of JIM5 and JIM7 epitopes in radiata pine.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cambium</th>
<th>Radial expansion region</th>
<th>New secondary wall region</th>
<th>Mature wood</th>
</tr>
</thead>
<tbody>
<tr>
<td>JIM5</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JIM7</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

++++ = strong intensity; +++ = moderate intensity; ++ = weak intensity; + = weak/no intensity

Figure 4. Transverse sections of xylem tissue showing the distribution of JIM5 and JIM7 epitopes in the pit membranes and ray cell walls as observed by immunogold electron microscopy. JIM5 (a, c), JIM7 (b, d). Pit membranes (a, b, arrows) and ray cell walls (c, d, arrows). — Scale bars (a, b) and (c, d) = 5 μm.
Distribution of JIM5 and JIM7 epitopes in the CML of developing xylem cells

Immunogold micrographs, shown in Figure 3, indicated that the JIM5 and JIM7 epitopes were present in different regions of the CML as differentiation progressed. Consistent with the confocal images, JIM5 and JIM7 labeling were greatest in the cambial and newly divided cells (Fig. 3a, b). However, in the cells undergoing later stages of differentiation, evidenced by secondary wall deposition, JIM5 labeling was predominantly present in the CML of the radial and tangential cell walls, and was not homogeneous in the CML of the cell junctions (Fig. 3c). On the other hand, JIM7 labeling remained homogeneous in the CML of the cell junctions, in addition to the CML of the radial and tangential cell walls (Fig. 3d). Also consistent with the confocal images, JIM5 and JIM7 labeled pit membranes (Fig. 4a, b, respectively) and ray cell walls of maturing xylem cells (Fig. 4c, d, respectively). JIM5 labeling was slightly stronger (Fig. 4a) in the pit membranes than the JIM7 labeling (Fig. 4b).

DISCUSSION

Localization of pectin to the CML

Using ruthenium red staining and antibody labeling, we were able to establish the spatial distribution of pectin deposition throughout the various stages of xylogenesis in radiata pine. Pectin staining was observed in the region of cambial cell division, the initial stage of xylogenesis (Fig. 1a, 2a, b, 3a, b; Table 1). This was not surprising, as previous reports suggest that it is during the early stages of development that pectin is deposited into the cell walls (Northcote 1986; Follet-Gueye et al. 2000). As development progressed, histochemistry allowed for the visualization of pectin in the CML of radially expanding cells, as well as cells undergoing secondary wall deposition (Fig. 1a), suggesting that pectin is present in the CML at all stages of development. In contrast, antibody labeling did not imply that pectin was present in mature cells (Fig. 2e, f). The intensity of pectin histochemical staining and antibody labeling was observed to show a gradient decrease throughout maturation (Fig. 1a, 2a, b; Table 1) suggesting that pectin deposition, esterification and/or degradation could be developmentally regulated and that pectin, and its various constituents, may possess specific functions at the level of cell wall development (Vincken et al. 2003).

Differential distribution of JIM5 and JIM7 epitopes

JIM5 recognizes un-esterified pectins, as well as those with a low degree (up to 50 %) of methyl-esterification (Knox et al. 1990; Hafren et al. 2000; Willats et al. 2000), while JIM7 recognizes only those with a high degree (35–90 %) of methyl-esterification (Knox et al. 1990; Hafren et al. 2000; Willats et al. 2000), neither antibody recognizes RG-I (Knox et al. 1990). These antibodies may also recognize portions of RG-II, believed to be covalently linked to HG, which may form a backbone for RG-II synthesis, contributing to a macromolecular pectin complex (Ishii & Matsunaga 2001; Ridley et al. 2001). It must be noted that the specific epitope for these antibodies is not fully defined, and the range of esterification they recognize could lead to an overlap in their labeling (Knox et al. 1990; Micheli et al. 2000; Willats et al. 2001a; Clausen et al. 2003).
Pectin is in a methyl-esterified form when it is transported to the cell wall in Golgi vesicles, to prevent premature ionic interactions with its carboxyl groups (Zhang & Staehelin 1992; Goldberg et al. 1996). Once in the cell wall, the methyl-esters are removed by pectin methylesterases (PME), which regulate the ratio between methyl-esterified and acidic, or unesterified, pectins (Goldberg et al. 1996; Guglielmino et al. 1997; Willats et al. 2001b), explaining the high methyl-esterified and un-esterified pectin epitope expression levels observed at early stages of xylogenesis in radiata pine (Fig. 2a, b, 3a, b). Young elongating cells characteristically have a high amount of methyl-esterified pectin, which allows for cell expansion by preventing calcium cross-links (Micheli et al. 2000). This was seen in the cambial and radially expanding cells of radiata pine which labeled strongly with JIM7 (Fig. 2b, 3b). In other reports, un-esterified pectin has been determined to be a critical factor for growth of tobacco plants (Capodicasa et al. 2004) and is present in poplar cambium during active growth and cell elongation (Baier et al. 1994). The role of un-esterified pectins in growth is likely to involve alteration of the pH of the cell wall, which would, in turn, promote cell wall loosening or the preferential interaction of polygalacturonases on HG without methyl-esterification (Willats et al. 2001b; Capodicasa et al. 2004). To a certain extent, un-esterified pectin was also prevalent in radiata pine cells, observed as strong JIM5 labeling in the cambial cells (Fig. 2a, 3a), which may encourage cell expansion. A decrease in methyl-esterified pectin has also been linked to the reduction of growth and cell wall stiffening, as a result of the formation of calcium cross-links with HG (Catesson 1994; Willats et al. 2001a; Vincken et al. 2003; Capodicasa et al. 2004). As such, the literature shows that PMEs can produce complex, often antagonizing, effects in the cell wall, in that they can both stimulate and inhibit cell expansion (Guglielmino et al. 1997). As a result of the potential for overlap in JIM5 and JIM7 labeling, these results do not demonstrate unambiguous esterification changes (Micheli et al. 2000). However, the distribution of JIM5 and JIM7 labeling does agree with the results reported previously for hybrid aspen (Follet-Gueye et al. 2000), poplar cambium (Guglielmino et al. 1997), and Pinus sylvestris (Hafren et al. 2000). These results suggest that pectin, and its state of esterification, is important to the processes involved in xylem differentiation, particularly cell expansion.

*Pectin is predominantly present in the methyl-esterified form in developing wood*

The role of pectin esterification in the walls of growing and developing plant cells is not well understood. In the cells undergoing later stages of secondary wall deposition, JIM5 was predominantly present in the CML of the radial and tangential cell walls, and its presence was only weakly observed at the junction between cells (Fig. 3c). JIM7, on the other hand, displayed a relatively homogeneous distribution throughout the CML, including the cell junctions (Fig. 3d). This suggests that pectin is predominantly present in the methyl-esterified form at later stages of tracheid development. An explanation for the decrease in un-esterified pectin at this point in development remains elusive (Hafren et al. 2000). Some researchers believe that calcium ions may be involved, as they play a dual role in pectin cross-linking and as stabilizers of superoxide radicals during

---

**Putoczki et al. — Pectin distribution in radiata pine**

122
lignification (Westermark 1982; Hafren et al. 2000). The release of calcium ions from pectin cross-links, as a result of pectin degradation, may be required for lignification to occur (Westermark et al. 1986; Hafren et al. 2000). Calcium ions have been localized to the radial walls and cell junctions in poplar (Guglielmino et al. 1997), placing them in close proximity to the pectin distribution observed in this work, suggesting that calcium ions may indeed be liberated from HG present in this region.

**Distribution of pectin in mature wood cells**

It is not known if pectin remains in the cell wall at maturity, partially masked by cellulose and lignin, or if its presence is degraded because its primary role is during early development (Westermark et al. 1986; Follet-Gueye et al. 2000; Hafren et al. 2000). In the lignified walls observed in this work, pectin was difficult to stain and label with pectin-specific antibodies, similar to research described in previous reports (Guglielmino et al. 1997; Hafren et al. 2000). However, the notion of pectin masking is corroborated by the ruthenium red stained xylem tissue (Fig. 1a, c), in view of the fact that with the chemical removal of lignin, pectin staining was evident in the CML of the cell walls of maturing xylem cells. The histochemistry, confocal scanning laser microscopy, and immunogold TEM results all suggest that pectin is not present in the secondary cell wall, which corroborates previous suggestions that pectin is the only major class of polysaccharide restricted to the primary cell wall (Willats et al. 2001a). This is further supported by the presence of pectin in the pit membranes, and ray cell walls (Fig. 1, 2 & 4) which contain primary cell wall.

**Role of pectin in wood quality**

Mapping the distribution of pectin in xylem is important for understanding the biology of the cell wall in radiata pine. The esterification patterns within the walls of radiata pine xylem cells is of interest to the biochemical function of pectins, and indicate that this function may be developmentally regulated. In particular, the esterification patterns may encourage and/or inhibit cell expansion through regulation of calcium cross links (Micheli et al. 2000). As a result, failure of pectin deposition to proceed properly, or modifications in esterification patterns, may contribute to the incidence of wood quality flaws in radiata pine. For example, turgor pressure is known to produce tensile forces on cell walls that create an opportunity for the three-way junctions to separate (Jarvis 1992), which could create cracks or ‘checks’ in the wood. Pectin may contribute to the maintenance of cell-cell wall contacts at these points through calcium mediated cross-links (Willats et al. 2001b). Recent works also suggest that borate cross-linking of pectin is also important in the maintenance of cell wall structure (O’Neill et al. 2004). As such, understanding the distribution of pectin, and the incidence of esterification, may be critical to our understanding and prevention of wood quality flaws.

In this study, the use of anti-pectin monoclonal antibodies allowed for the observation of changes in pectin esterification in the context of cell wall architecture and development for the first time in radiata pine. Future studies will aim to establish direct links between pectin, esterification and wood quality flaws.
ACKNOWLEDGEMENTS

This project and T. Putoczki were funded by the Wood Quality Initiative Ltd., New Zealand. The authors would like to thank Dr. Paul Knox for the kind gift of the JIM5 and JIM7 antibodies and for assistance with the manuscript preparation. The authors would like to dedicate this manuscript to the memory of Dr. Sandra Jackson, who passed away during the course of this study.

REFERENCES


