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Chemistry of developing bordered-pit rims in balsam-fir trees

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Abstract: Rims of bordered pits form on primary walls of radially enlarged cambial derivatives prior to the onset of general secondary-wall formation. A recent report (Botany, 2014, 92(7): 495-511) raised the possibility that the chemical composition of the rim might be different from that of the secondary wall. To investigate this, early-stage non-fluorescent and late-stage autofluorescent rims were separated from cambial derivatives of Abies balsamea (L.) Mill. and purified to homogeneity by density-gradient centrifugation.

Solid state nuclear magnetic resonance spectroscopy, Raman microspectroscopy, combined gas chromatography - mass spectroscopy, enzyme digestion and chemical resilience data support the interpretation that cellulose alone is the microfibrillar polysaccharide of non-fluorescent early-stage rims. A lignin is additionally present in late-stage rims, and it evidently bonds with cellulose because rims are extraordinarily resistant to hydrolysis by either enzymes or strong acid.

Key words: Abies balsamea, Pinaceae, balsam fir, cambium, cell wall, cellulose, lignin, hemicellulose
INTRODUCTION

During secondary growth in conifers, primary-walled cambial derivatives that are destined to become elements of mature secondary xylem have several possible cellular differentiation fates, viz., tracheids, sclerenchyma, parenchyma or resin-secreting cells. Tracheids by definition are the only cell type having bordered pits.

Investigations into the cell biology underlying bordered-pit development led to the conclusion that at least 16 successive steps are involved, and formation of the bordered-pit rim upon the primary wall of the radially enlarged cambial derivative was identified as the 9th step (Savidge 2014). Rims appear as birefringent rings approximately 10 µm or smaller in diameter (Fig. 1a). The rim is produced around a circular bordered-pit organelle (BPO), and rim growth occurs concomitantly around the circumference of the BPO (Fig. 1a). Remarkably, the growing rim becomes autofluorescent before the general S₁ secondary-wall layer becomes structurally or histochemically evident (Savidge 2014). Accordingly, ‘early-stage’ rims lacking autofluorescence are distinguished from ‘late-stage’ autofluorescent rims.

Microfibrils of the rim have long been thought to be made of cellulose (Savidge 2014). However, this assumption has never been substantiated by direct chemical characterization, and indirect immunohistochemical evidence raised the possibility that one or more hemicelluloses might be in rims of conifer bordered pits (Kim et al. 2010; Kim et al. 2011; Donaldson and Knox 2012). Moreover, in addition to microfibrils of cellulose (β-1,4-glucan), microfibrils of linear β-1,3-xylan, β-1,4 xylan, β-1,4 mannan, β-1,3-glucan, and chitin have been described (Preston 1979; Kopecká 2013), and hemicellulose – cellulose cross-linking is also possible (Renard and Jarvis 1999; Hsieh and Harris 2012). Thus, it seemed possible that rim microfibrils might be either entirely non-cellulosic or a mixture of hemicellulosic polysaccharides with cellulose. Direct chemical investigations to answer this became possible following the discovery that rims can be separated from their adhering primary walls using a pectinase – cellulase combination (Savidge 2014). However, as detailed below, early-stage rims are not
obtained using that pectinase – cellulase method because they are digested. This report describes a non-enzymic method for releasing intact early-stage rims and provides distinct procedures for purifying both early- and late-stage rims.

MATERIALS AND METHODS

General experimental procedures

Equipment and procedures for SEM, epifluorescence, d.i.c and bright-field microscopy were as previously described (Savidge 2014). To gain insight into the organic composition of solid rims, Raman microspectrometry (Raman), solid-state nuclear magnetic resonance (NMR) spectroscopy and combined gas chromatography-mass spectrometry (GC-MS) investigations were done. Raman was done using a Renishaw inVia Reflex Raman microscope. Solid-state NMR data were generated with a Bruker Avance 9.4 T spectrometer using a dual resonance 4 mm HX probe. GC-MS data were acquired using a Varian 3800 GC coupled to a Varian 2000 ion trap mass spectrometer. A Beckman J2-21M centrifuge with JS-13.1 swinging bucket rotor served for high speed centrifugation. Cellulose powder was obtained from Whatman; all other chemicals were from Sigma-Aldrich. Double-distilled water (distilled H₂O) was produced using tandem all-glass units. For additional chemical characterization of rims, high molarity alkaline and acid solutions (NaOH and H₂SO₄) were employed using well established procedures of wood chemistry research (Sjöström and Alén 1999). Microfibrillar cellulose is an exceptional material in its abilities to transform into a swollen state (i.e., to mercerize) in boiling 2 mol-L⁻¹ NaOH and to retain its polymeric constitution in 4 mol-L⁻¹ NaOH, whereas hemicelluloses of wood in general are hydrolyzed into oligo- and mono-saccharides when treated with 2 mol-L⁻¹ or higher concentrations of NaOH, leaving cellulose and some lignin in the solid residue. Lignins in general are resistant whereas all polysaccharides are susceptible to hydrolysis in 72% (sp. gr. 1.6338 at 20 °C) H₂SO₄ (Sjöström and Alén 1999).
Isolation of developing xylem

Saplings approximately 20 years old, 6 m in height and 20-cm basal diameter of Abies balsamea (L.) Mill. growing in wild forest near Fredericton, New Brunswick, Canada, were felled in early and late springtime for preparation of early-stage and late-stage rims, respectively. Bud scales were beginning to separate in the earlier harvest; shoots were actively elongating in the later one. Branches were removed and discarded, and 1 m segments of the trunk were cut and transferred to the lab. The bark surface was brushed and washed free of adhering matter under running cold tap water; then the bark was peeled from the wood and discarded. Using a razor-sharp knife, the exposed wood surface was scraped axially downward to release strips of cambial derivatives and cell sap into a pre-weighed crystallising dish containing 3.4 mol-L\(^{-1}\) NaCl (250 mL, all-glass distilled H\(_2\)O, room temp.). For early-stage rims and late-stage rims, 31 g\(\text{fw}\) and 141 g\(\text{fw}\) of starting tissue, respectively, were processed.

Isolation of early-stage rims

After 1 h incubation at room temp., the tissue was compressed and saline liquid decanted to waste. The tissue contained in a stainless-steel screen was rinsed with an excess of distilled H\(_2\)O, then compressed to remove excess water. The rinsed tissue was shaped into thin plates within Al foil packets, frozen to -80 °C for 24 h, foil removed and frozen tissue fragmented into a fine powder in a pre-frozen mortar (-80 °C). The powder was suspended in distilled H\(_2\)O (100 mL), stirred (room temp, 15 min) and filtered through coarse (~2 mm openings) stainless-steel mesh, discarding the retained solids. The recovered suspension was passed through a stainless-steel strainer (Sigma-Aldrich CD-1, 60 mesh, ~24 wires cm\(^{-1}\) approximately 400 µm openings) and the filtrate centrifuged in 15 mL tapered glass tubes (300 x g, room temp., 5 min). The supernatant was decanted to waste and the pellet resuspended by vortexing in distilled H\(_2\)O, centrifuged again as described, and this washing procedure was repeated 5 times. The pellet resuspended in distilled H\(_2\)O (2 mL) was transferred to a Potter
Elvejhem homogeniser, given three strokes and transferred to a 10 mL glass syringe connected to a stainless steel Swinny assembly (Millipore, 13 mm) housing a stainless-steel support screen with approximately 50 µm x 50 µm square openings. The suspension was forced through the support screen, layered onto the surface of pure glycerol (10 mL in a 15 mL Corex centrifuge tube) and centrifuged in a swinging bucket rotor (Beckman JS13.1) for 30 min (21,500 x g, 20 °C). The tube was overturned and the glycerol supernatant drained (15 min, room temp.) to waste. The pellet recovered in distilled H₂O (3 x 2 mL) was transferred to tapered glass tubes and centrifuged (5 min, 300 x g, room temp); this centrifugal washing of the recovered pellet was repeated 5 further times. The pellet was examined by d.i.c. microscopy and confirmed to consist principally of bordered-pit rims. The resuspended pellet (1 mL distilled H₂O) was transferred to a Potter Elvejhem homogeniser and given three strokes, then loaded onto the top of an aq. KI step gradient (50%, 75%, 90%, 95%, 100%=satd. KI, 2 mL steps, 20 °C) in Corex tube. Following centrifugation (24,500 x g, 1 h, 20 °C), a single prominent band at the interface between 90% and 95% satd. KI was removed by Pasteur pipette, transferred to a tapered glass centrifuge tube and washed by centrifugation (5 min, 300 x g, room temp), repeating the resuspending, washing and supernatant disposal steps 6 times. The washed all-white pellet (~9 mg subscripts{dw}) recovered in absolute ethanol was determined by d.i.c. and epifluorescence microscopy to be an essentially pure preparation of non-fluorescent rims, some of which were broken. Some isodiametric ray cell sclereids from the latewood boundary were also present but represented < 1% of the rim preparation. Other small bits of unrecognizable debris were present in minor quantity.

**Isolation of late-stage rims**

After stirring the saline suspension for 1 h at room temp., the liquid was decanted through a stainless-steel screen to waste. Free liquid was expressed and the tissue washed and decanted thrice (distilled H₂O). The resulting solids (44 g subscripts{fw}) were suspended in 250 mL
distilled H$_2$O containing pectinase (*Aspergillus niger*, Sigma P9932, 6 mL, 10.3 mg-mL$^{-1}$ of protein, 11.6 units-mg$^{-1}$) and cellulase (*Trichoderma reesii*, Serva, 1.0 g, 0.5 units-mg$^{-1}$) and stirred at room temp. for 2.5 h. The digested suspension was passed through a stainless-steel strainer (60 mesh, Sigma-Aldrich CD-1), and solids (2.2 g$_{fw}$) retained by the strainer were discarded. The suspension was centrifuged (12000 x g, 15 min, 22 °C, Beckman JA 14 fixed-angle rotor, 250 mL bottles) and the supernatant discarded. The pellet (6.9 g$_{fw}$) was removed and suspended in 100 mL of 1 mol-L$^{-1}$ NaOH and subjected to 1 h of heating/stirring at 100 °C in 1 mol-L$^{-1}$ NaOH, then allowed to cool to room temp. while continuing the stirring over 24 h. The suspension was centrifuged (12000 x g, 15 min, 22 °C, Beckman JA 14 fixed-angle rotor, 250 mL bottles) and the supernatant discarded. The pellet (< 1 g$_{fw}$, light yellow-brown) was resuspended in distilled H$_2$O (10 mL) and an equal volume of 1.0 mol-L$^{-1}$ NaOH added. The 0.5 mol-L$^{-1}$ NaOH suspension was transferred into Corex tubes and centrifuged (Beckman JS 13.1 swinging bucket, 12000 x g, 20 min, 22 °C). The pellet in distilled H$_2$O after vortexing was transferred into 15 mL tapered glass tubes and centrifuged (300 x g, room temp., 5 min). The supernatant was decanted to waste and the pellet resuspended, repeating this washing procedure 5 times. After Potter Elvejhem homogenisation (3 strokes) of the washed pellet, the suspension was processed through a Swinny assembly (approximately 50 µm x 50 µm square openings). Based on brightfield light microscopy of phloroglucinol-HCl treated cells, particles retained by the Swinny assembly were exclusively secondary-walled lignified fragments of xylem. The filtrate in 2 mL distilled H$_2$O was loaded onto the top of an aq. glycerol step gradient (25%, 50%, 75%, 90%, 100%=satd. glycerol, 2 mL steps, 20 °C) in Corex tube and centrifuged in a swinging bucket rotor (Beckman JS13.1) for 30 min (21,500 x g, 20 °C). The centrifuge tube was overturned and the glycerol supernatant drained (15 min, room temp) to waste. The pellet was resuspended (distilled H$_2$O, 6 mL) by vortexing, transferred to tapered glass tubes and centrifuged (5 min, 300 x g, room temp); the supernatant was decanted to waste and centrifugal washing of the pellet was repeated 5 times. The distilled H$_2$O resuspended pellet was layered
onto a KI gradient and centrifuged as described (see early-stage rims), and a band at the 75% KI – 90% KI interface was transferred by Pasteur pipette to a tapered glass centrifuge tube, washed by centrifugation (5 min, 300 x g, room temp), repeating the resuspending, washing and supernatant disposal steps 6 times. The final washed pellet recovered in absolute ethanol was light yellow in colour and consisted mainly of autofluorescent bordered-pit rims.

**Raman microspectroscopy**

Late-stage rims in ethanol were transferred into distilled H$_2$O and centrifuged (300 x g, 5 min, 20 °C). The pellet after resuspension and vortexing in distilled H$_2$O was processed stepwise (0, 10, 25, 50, 75 and 100 percent acetone) by repeated centrifugation (300 x g, 5 min, 20 °C) and resuspension of the pellet into 100% acetone. The pellet dispersed in acetone was layered onto a glass slide, allowed to air dry and investigated. A slide of dry Whatman cellulose powder was similarly prepared. Coverslipping was not done. Surfaces of sampled sites (2 mm$^2$ field) were focused through a ×50 objective lens (dry, N.A=0.75); backscatter light was detected via the same objective. Excitation was achieved using a 514.5-nm Ar-ion laser, filtered to give ~9 mW total laser energy at the sample surface. Spectra were recorded via a Peltier-cooled silicon-based multichannel array charge-coupled device (RenCam CCD detector). Twenty accumulations were acquired over 1.5 h based on 35 s scans. To enhance intensities of weak signals, baseline subtractions was used. Before-and-after examination of sampled sites confirmed that investigated rims were still intact and uncharred.

**Cross Polarisation – Magic Angle Spinning Nuclear Magnetic Resonance (CP/MAS NMR)**

Standard cellulose (Whatman) and rim spectra were acquired using 7 kHz MAS, using the $^{13}$C carbonyl resonance of glycine to set the magic angle and achieving cross polarization with TPPM decoupling. Larmor frequencies were 400.23 MHz for $^1$H and 100.65 MHz for $^{13}$C. A contact time of 2.6 ms was used for both samples; 2048 scans were acquired for the rims and
1064 scans for the cellulose standard. Due to the small mass of rims available for analysis (17 mg\textsubscript{dw}) dead space in the 4 mm rotor was filled with Teflon tape and Teflon spacers, and a pulse delay of 3 s was used. The cellulose standard relaxed more slowly, allowing a 7 s pulse delay.

Combined Gas Chromatography – Mass Spectrometry (GC-MS)

Alditol acetate standards were prepared from selected mono- and di-saccharides. Sulfuric acid hydrolysis and BaCO\textsubscript{3} neutralisation of the hydrolysate were as previously described (Savidge and Colvin 1985). The \textit{in vacuo} dried (thin-film rotary evaporation, 50 °C) samples were reduced with sodium borohydride in dimethyl sulphoxide and the resulting alditols acetylated using L-methylimidazole as the catalyst (Blakeney et al. 1983). Blanks and standards were processed through the identical procedures.

Splitless injections (1 µl, 220 °C injection port, He at 10 psi) in chloroform onto a 5% phenylmethylsilicone cross-linked capillary column (0.25 mm i.d., 30 m) with He flowing at 1.2 mL-min\textsuperscript{−1} yielded baseline resolution of alditol acetates. The column oven was held at 80 °C for 1 min, then ramped to 180 °C over 10 min, 240 °C over 15 min, 270 °C over 3 min, and 320 °C over 6 min followed by holding at 320 °C for 1.67 min; total run time was 40 min. Transfer line was constant at 240 °C. These conditions allowed both mono- and di-saccharides to be investigated in a single run. EI spectra were gathered in full scan mode (\textit{m/z} 60 – \textit{m/z} 450) from 7 – 40 min., emission current 10 µAmp, maximum ionization time 25000 µs. Alditol acetates obtained from rims were positively identified by mass spectra and co-chromatography with standards.

RESULTS

Early-stage rims with negligible autofluorescence began appearing on radial walls of primary-walled radially enlarged cambial derivatives soon after commencement of cambial growth in early springtime (Fig. 1a). Broken and intact early-stage rims sedimented through a
KI step gradient to a band at density ~1.6 g·cm⁻³. By d.i.c. microscopy, >95% of the KI band consisted of rims, and some unidentifiable debris and ray-cell sclereids were also noted as minor components. From 31 g₉w of living cambial derivatives, a purified preparation of ~9 mg₉w of early-stage rims was obtained from a 6-m tall tree trunk.

Light microscopy observations of purified early-stage rims revealed that each rim comprises several discrete rings of fibrils (Figs. 1b – 1f). In the light microscope it was apparent that the rim is a composite of fibrillar rings formed by deposition of one upon the next (Figs. 1d, 1e, 1p, 1r). In the SEM, the fibrillar rings appeared as ridges separated by valleys having long axes paralleling the orientation of the rim circumference (Fig. 1f). The rim surface in contact with the primary wall appeared smooth and evidently homogeneous (Fig. 1g). As the developing rim increased in thickness, its interior edge arched upward and propagated inward (Figs. 1b, 1c). Purified early-stage rims after being treated 24 h at 100 °C in 4 mol·L⁻¹ NaOH were swollen (Figs. 1h-1j) and displayed surfaces less corrugated than untreated ones, but the fibrils showed little evidence for end peeling, unraveling or dispersing. When early-stage rims were left setting in 2 mol·L⁻¹ NaOH at room temp. for 3 months, then washed and examined, rims broken or weakened during mortar and pestle crushing or Potter Elvejhem homogenization had separated into linear bunches of aggregated fibrils (Fig. 1k) but some rims remained structurally intact (Fig. 1l). Both the fibrillar bunches and intact rims were quickly digested into a clear solution when incubated with cellulase. Calcofluor white (Fig. 1m) and Congo red (Fig. 1n), both cellulose-binding reagents (Herth and Schnepf 1980; Anderson et al. 2010; Woodcock et al. 1995), displayed strong affinity for early-stage rims. As early-stage rims increased in size they became increasingly autofluorescent (Figs. 1o, 1p) and lost their affinity for Calcofluor white and Congo red adsorption.

Late-stage rims after KI density-gradient centrifugation were 99% pure. From 141 g₉w of cambial derivatives, the purified rim preparation yielded 24 mg₉w. All late-stage rims were autofluorescent; however, autofluorescence intensity varied among rims from weak to strong...
(Fig. 1q). Rims remained intact and autofluorescent after 4 mol-L\(^{-1}\) NaOH (Figs. 1q) and 72% H\(_2\)SO\(_4\) (Fig. 1s) treatments. Those treatments were initially done at room temp., and at room temp. the identical 72% H\(_2\)SO\(_4\) solution when tested on cotton fibres yielded a clear solution within 10 min. A suspension of late-stage rims in 72% H\(_2\)SO\(_4\) was held for 4 h at 120 °C and, again, rims remained intact; however, when viewed by bright-field microscopy, they had a rust-orange coloration (Fig. 1t). Late-stage rims were also resistant to prolonged boiling in 0.5 mol-L\(^{-1}\) HCl.

Relatedly, it was found that mature conifer (spruce/pine/fir) bordered-pit rims could be recovered from kraft pulp newsprint by suspending, beating and sieving an aqueous suspension, and rims were also noted to be weakly present in suspensions of raw black liquor fines (i.e., white liquor containing ~2.5 mol-L\(^{-1}\) NaOH and ~0.5 mol-L\(^{-1}\) Na\(_2\)S, digesting at ~175 °C for 2 h). Bordered pit structure for the most part remained intact and autofluorescent on kraft pulp tracheids (Fig. 1u). However, after bleaching (ClO\(_2\)) of oxygenated kraft pulp, the overarching bordered was entirely devoid of autofluorescence and scarcely resolvable by d.i.c. microscopy.

Purified early-stage rims not pretreated with NaOH solution were rapidly converted into a clear solution when suspended in 72% H\(_2\)SO\(_4\). After complete hydrolysis, reduction and acetylation, GC-MS of early-stage rims indicated the presence of glucitol acetate alone (Figs. 2a, 2b), whereas additional monosaccharides were present in late-stage rims (Fig. 2c). After a shortened period of rim hydrolysis, peaks with retention times of both glucitol acetate and cellobiotol acetate were obtained from early-stage rims, and when co-injected with glucitol and cellobiotol acetate standards, they co-chromatographed, thus confirming \(\beta\)-1,4-glucan structure.

Raman microspectroscopy shifts produced by late-stage rims (Figs. 3b, 3c) were in agreement with those of standard cellulose (Fig. 3a), and a 1603 cm\(^{-1}\) shift confirmed the presence of lignin in the rims (compare Gierlinger and Schwanninger 2006; Hänninen et al. 2011). The intensity of the 1603 cm\(^{-1}\) lignin shift varied between different sampling positions on
the slide (compare Fig. 3b with Fig. 3c), in agreement with the observation that autofluorescence intensity varied among rims (Fig. 1q).

Solid-state $^{13}$C and $^1$H CP-MAS resonances of standard cellulose (Figs. 4a and 4b, respectively) corresponded well with those produced by purified late-stage rims (Figs. 4c and 4d). A 105 ppm resonance, diagnostic of the C-1 of cellulose glucose, and two characteristic C-4 shifts, one for crystalline cellulose at 89 ppm and one for amorphous cellulose at 84 ppm, constituted conclusive evidence for cellulose. Weak signals characteristic of lignin were generated at 151 and 147 ppm and generally over the range 160 – 110 ppm (Fig. 4c). A relatively intense $^{13}$C shift near 25 ppm signal was also present (Fig. 4c).

Following H$_2$SO$_4$ hydrolysis of insoluble late-stage rims and neutralization of the hydrolysate with BaCO$_3$, the residual dry wt of non-hydrolysable rims was ~98% of that of the starting material. In other words, late-stage rims were almost entirely resistant to hydrolysis using a common method for plant cell wall investigations (Sjöström and Alén 1999); when applied to particles of whole balsam-fir wood that method yielded abundant hemicellulosic monosaccharides (data not shown). The small yield of hydrolysable compounds obtained from late-stage rims was in accord with the fact that the structural integrity of rims remained intact after several hours of treatment in both 4 mol-L$^{-1}$ NaOH and 72% H$_2$SO$_4$. GC-MS of alditol acetates produced from the minor fraction of hydrolysate revealed mannose, galactose and xylose as the more abundant components, but glucose, arabinose and rhamnose were also present (Fig. 2c). Each component yielded the carbohydrate-diagnostic m/z 43 fragment as base peak. No evidence for diagnostic fragments (e.g., m/z 87, m/z 99, m/z 101, m/z 117, m/z 189) of methylated hexoses was found in the total ion current mass spectra of any of the GC alditol acetate peaks. The m/z 210 fragment of inositol hexaacetate was not present, nor were ion pairs characteristic of acetylated amino hexoses present.
DISCUSSION

The quantities of purified early-stage and late-stage rims produced by the described methods were limited and, considering that every cambial derivative in the process of becoming a tracheid produces multiple bordered pits, there undoubtedly were major losses of rims associated with the processing procedures. Nevertheless, the use of glycerol and KI gradients was effective in achieving homogeneous rim preparations in their native state and free of extraneous matter and enabled direct chemical characterizations.

It was previously suggested that rim microfibrils might be composed of xylan (Savidge 2014); however, the 105 ppm resonance in the $^{13}$C CP-MAS spectrum of late-stage rims (Fig. 4c) is highly diagnostic of the C-1 of glucose in cellulose (Zawadzki and Wisniewski 2002; Virtanen et al. 2008; Fernandes et al. 2011; Foston 2014), whereas the C-1 in xylan resonates near 102 ppm (Renard and Jarvis 1999). The C-4 shifts at 89 ppm and 84 ppm constituted additional confirmatory evidence for cellulose, as also did the $^1$H data. Thus, based on alditol acetate data (glucose and cellobiose in early-stage rims), rapid digestion by cellulase of early-stage rims, and late-stage Raman and NMR data, it can be concluded that rim microfibrils consist solely of cellulose. The possibility that one or more hemicelluloses may have been present in early-stage rims but removed by KI during density-gradient purification cannot be entirely excluded, because some ionic solutions have that capability (e.g., Cai and Zhang 2005). Because no alternative method to purify rims was available, the possibility that hemicelluloses were extracted from early-stage rims into KI solution was not investigated.

The finding of mannose, galactose, xylose, glucose and other sugars in hydrolysate from late-stage rims appears to support several antibody-based reports suggesting hemicellulose structures associated with bordered-pit rims (Kim et al. 2010; Kim et al. 2011; Donaldson and Knox 2012). However, the present findings indicate that the hemicellulose content is minor (~2%) and that the acid-, alkali- and cellulase-resistant late-stage rim consists dominantly of cellulose and lignin. Evidence for hemicelluloses such as reported by Kim et al. (2010; 2011)
and Donaldson and Knox (2012) was based on indirect immunolabeling methods and, in general, results from immunohistochemical investigations although of high anatomical resolution can be equivocal, not only in relation to inferring molecular abundance based on labeling frequency but also because uncertainty attends immunobinding specificity. For example, when proteins (i.e., primary and secondary antibodies) are incubated with a material that is known to be chemically complex and variable but nevertheless incompletely characterized – certainly the case for xylem (Hon and Shiraishi 2001) – it is possible that antibodies might be aberrantly adsorbed. Cross-reactivity of antibodies with unintended or undiscovered epitopes is another common problem, and numerous others concerns affect interpretations of chemical composition based on immunolabeling (Gaster et al. 2011).

This study is probably the first characterization by direct chemistry methods of the composition of the bordered-pit rim and, possibly, it is more generally the first to characterize a particular cell-wall substructure in actively differentiating cells. Earlier work using direct methods of chemical analysis provided insight into the overall chemical composition of cell walls of actively differentiating cambial derivatives (Kataoka and Kondo 1996; Kakegawa et al. 1998). However, developing bordered pits and other minor structures within individual cell types were not accounted for in those investigations, and there evidently have been no past attempts to characterize the chemical composition of mature bordered pits using direct methods despite many thousands of publications having addressed wood chemistry (e.g., see Hon and Shiraishi 2001).

The research has revealed that cellulose microfibrils in early-stage rims occur as composite rings aggregated into larger rings of fibrils (Figs. 1d-1f, 1k, 1p, 1r). The continuing existence of structural integrity of each fibrillar ring following treatment of early-stage rims with 4 mol-L⁻¹ NaOH appears to be an indication that each polymeric β-1,4-glucan chain forms as a cohesive loop rather than as a continuous circumvoluted coil having two ends, as might be expected if a terminal rosette complex were traveling around and around the BPO to generate
microfibrils (e.g., see Taylor 2008; Fujii et al. 2010; Morgan et al. 2013; Thomas et al. 2013; McFarlane et al. 2014).

Watanabe et al. (2015) provided immunological evidence that spiralling bands of microtubules and a putative cellulose synthase (CESA7) are both constrained to secondary-wall domains during protoxylem development in Arabidopsis thaliana. There is anatomical evidence that spiraled and reticulated secondary-wall thickenings can merge seamlessly into circular secondary-wall thickenings characteristic of bordered-pit rims (Savidge 1983, 1996), and circular bands of microtubules have been observed to be associated with the circumferential margins of developing bordered-pit rims in conifers (Uehara and Hogetsu 1993; Funada et al. 1997). There can be little doubt that microtubules contribute to the formative process, to either microfibril orientation or deposition, or both. However, based on their findings Watanabe et al. (2015) inferred that cellulose synthases delivered in both Golgi-associated and non-associated cellulose synthase “compartments” to plasma membrane at sites of secondary-wall formation do not depend upon microtubules for insertion into the membrane. Thus, interesting questions remain about how β-1,4-glucan chains, microfibrils and fibrillar rings are actually formed.

A bordered-pit organelle (BPO, Fig. 1a) is spatiotemporally associated with and undoubtedly the source of the β-1,4-glucan chains that aggregate to form the fibrillar rings of the rim upon the primary wall. Based upon current understanding, torus and margo of the bordered pit also are dominantly made of cellulose microfibrils, and they evidently have their origin within the BPO (Savidge 2014). In other words, torus and margo microfibrils appear to arise within an organelle independently of the plasma membrane (Savidge 2014). Earlier work made it apparent that BPOs probably begin as Golgi vesicles (Savidge 2014), and higher plants are known to synthesize β-1,4-glucan chains within Golgi (Buckeridge et al. 2001). Moreover, vesicular secretion of β-1,4-glucan followed by polymerisation into cellulose microfibrils is well established in bacteria and single-celled eukaryotes (Choi and O’Day 1984; Fritz and Triemer 1985). In other words, the cohesively looped cellulose fibrils generated during early-stage rim
formation appear to contradict the rosette hypothesis of cellulose microfibril biogenesis.
Investigation of BPOs could provide an entirely novel research avenue for resolving in vitro how the most abundant biological substance on earth is produced.

Raman spectroscopy confirmed the earlier interpretation that autofluorescence of late-stage rims was due to lignin (Savidge 2014). However, the expected 53.5 ppm $^{13}$C shift for methoxy resonance was absent in CP-MAS NMR data (Fig. 4c). Lignin resonance in the vicinity of 170 ppm is also usual, and its absence in late-stage rims further indicates an unusual lignin. Moreover, the $^{13}$C shift near 25 ppm is anomalous in relation to $^{13}$C CP-MAS spectra generally obtained from woods (Fig. 4c). Resonance at 25 ppm is produced by methylated aromatic compounds. Methyl and acetyl groups of methylated hexoses and uronic acid resonate in the region 20-23 ppm, but no evidence for methylated hexose was detected by GC-MS and those groups would not be expected to survive strong alkali treatment. Thus, it can be suggested that the lignin in rims must be novel.

Bordered-pit rims may be chemically as well as anatomically unique structures in wood in having lignin and cellulose but almost no hemicellulose. Rim lignification occurs well before onset of general S$_2$ secondary-wall formation on otherwise non-lignifying primary walls. This precocious lignification probably has its explanation in the activity of coniferyl alcohol oxidase, a cell-wall bound enzyme localized to developing bordered pits at this early stage of cellular differentiation (Savidge and Udagama-Randeniya 1993).

Late-stage rims are exceptionally resistant to chemical hydrolysis, and thus it can be suggested that lignin must be bonded to cellulose in rims and that rim lignin probably contributes significantly to the small amount of residual lignin remaining in Kraft pulp (Froass et al. 1996; Namane et al. 2015).

The need to acquire high resolution 2D NMR data to acquire additional insight into the chemistry of rims was apparent. Preparations of late-stage dry rims were investigated using the solution state NMR methods of Lu and Ralph (2003), Cai and Zhang (2005) and Kim and Ralph.
However, despite rims being ~10 µm in diameter and their walls being only about 1 µm in thickness, rims remained particulate and yielded unacceptably weak poorly resolved $^{13}$C and $^1$H spectra (data not shown). Cross-linking between the rim’s microfibrils evidently is exceptionally strong and distinct from that of the lignin-carbohydrate bonds existing in the bulk of wood material.

Late-stage rims did not bind Calcofluor white whereas early-stage rims did. Molecular shielding of Calcofluor adsorption was earlier noted (Fritz and Triemer 1985) and, in the case of late-stage rims, a hypothetical explanation is that rim microfibrils are unrecognizable due to lignin cross-linking.

In summary, these investigations have revealed that bordered-pit rims in balsam-fir trees when first produced consist of fibrillar rings of evidently pure cellulose microfibrils. Several discrete fibrillar rings are produced one after the other to form the rim, and the fibrillar rings are bonded together with lignin prior to the onset of general secondary-wall formation. The rim is resistant to hydrolysis by enzymes, strong acid and strong alkali; as such, the rim is a novel component of wood cell walls and its formation and chemistry are deserving of further investigation.

**Conflict of interest**

The author declares that there are no conflicts of interest.

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FIGURES AND LEGENDS

Figure 1. D.i.c. (a, b, e, h, l, k, l), brightfield (d) and SEM (c, f, g, j) images of rims. a) Hand-cut radial section of live tissue mounted in 50% glycerol showing successively later stages, from left to right, of growth of rims (black arrows) upon the radial primary walls of 3 fusiform cambial cell derivatives. Bordered-pit organelles (white arrows) are associated with all three stages. b) An isolated developing rim with the surface that was against the primary wall foremost (black arrow). Inward accretion of loops of rim fibrils has produced an overarching structure resulting in the aperture side having decreased diameter (white arrow). c) A portion of a developing rim separated from the primary wall. The edge that was in contact with the primary wall is indicated by the black arrow and the aperture side by the white arrow. The star indicates the residue of a bordered-pit organelle. d) A rim with at least 4 distinct fibrillar loops (black arrows) with separations evident between the loops (white arrow). e) Another example of how the rim comprises a number of discrete highly birefringent fibrillar loops that, prior to lignification, readily separate from one another (white arrow). f) Multiple ridges (white arrows) and valleys of cellulose fibrils in a developing rim are reminiscent of collenchyma lamellae. g) High magnification image of the rim surface in contact with the primary wall; note the fine microfibrillar lineation (arrow). h) Swollen rims (one arrowed) after treatment with hot 1 mol-L⁻¹ NaOH. i) Higher magnification of one of the rims shown in Fig. 1h. j) A greatly swollen rim after NaOH treatment and critical point drying; lines of weakening (one arrowed) in the cohesive structure have evidently developed. k) Bundles of broken dispersed rim fibrils having lengths similar to the rim circumference were common after early-stage rims were incubated at room temp. for 3 months in 2 mol-L⁻¹ NaOH. l) Still-intact rims such as that arrowed were also present in the preparation described in Figure 1k. m) Epifluorescence (B1 filter) of an early-stage rim after incubation for 15 min in Calcofluor White M2R solution (Fluorescent Brightener
28, Sigma-Aldrich product no. F3543, 100 µL of a 10⁻³ mol-L⁻¹ aq. stock solution adjusted to pH 10 with NaOH). n) Epifluorescence (B1 filter) of two early-stage rims after incubation for 15 min in Congo Red solution (Sigma-Aldrich product no. C6277, 100 µL of a 0.1 mg-mL⁻¹ aq. stock solution). o) Autofluorescence (B1 filter) and p) d.i.c. of the same rim, transitional between early and late stages. q) Autofluorescence (B1 filter) of late-stage rims after they were incubated in 4 mol-L⁻¹ NaOH and then wash; some produced only weak (arrow) fluorescence. r) D.i.c. and s) autofluorescence of the same two late-stage rims following incubation in 72% H₂SO₄. t) Autofluorescence (B1 filter) of late-stage rims after incubation in 72% H₂SO₄ for 4 h at 120 °C followed by washing. u) Autofluorescence (B1 filter) of a conifer tracheid after kraft pulping showing intense rim fluorescence in four bordered pits. Scale bars for m) to u) = 5 µm.

**Figure 2.** Monosaccharide (alditol acetate) analysis of rims by GC-MS; the three chromatograms share the same baseline. a) Standard monosaccharides as alditol acetates. b) Early-stage rims; co-injection of this with glucitol acetate yielded a singular peak. c) Late-stage rims yielded trace amounts of several monosaccharides.

**Figure 3.** Raman shifts acquired from dry, non cover-slipped Whatman cellulose and dry, non cover-slipped late-stage rims. a) Cellulose; b) Late-stage rim spectrum; c) A second location on the slide of late-stage rims.

**Figure 4.** NMR data. a) ¹³C CP/MAS spectrum of standard Whatman dry cellulose powder; b) ¹H CP/MAS of standard Whatman dry cellulose powder; c) ¹³C CP/MAS spectrum of dry late-stage rims; d) ¹H CP/MAS spectrum of dry late-stage rims.