Title: SECONDARY CELL WALL DEPOSITION IN DEVELOPING SECONDARY XYLEM OF POPLAR

Running title: Secondary wall deposition in poplar

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Abstract

Although poplar is widely used for genomic and biotechnological manipulations of wood, the cellular basis of wood development in poplar has not been accurately documented at an ultrastructural level. Developing secondary xylem cells from hybrid poplar (*Populus deltoides* x *P. trichocarpa*), which were actively making secondary cell walls, were preserved with high pressure freezing/freeze substitution for light and electron microscopy. The distribution of xylans and mannans in the different cell types of developing secondary xylem were detected with immunofluorescence and immuno-gold labeling. While xylans, detected with the monoclonal antibody LM10, had a general distribution across the secondary xylem, mannans were enriched in the S2 secondary cell wall layer of fibres. To observe the cellular structures associated with secondary wall production, cryofixed fibres were examined with transmission electron microscopy during differentiation. There were abundant cortical microtubules and endomembrane activity in cells during the intense phase of secondary cell wall synthesis. Microtubule-associated small membrane compartments were commonly observed, as well as Golgi and secretory vesicles fusing with the plasma membrane.

Keywords:

Introduction

Poplar has ecological and economical value, and as the first sequenced tree genome, poplar is an important model system for fibre development. During secondary xylem differentiation, tangential and longitudinal cell divisions in the cambial zone produce xylem daughter cells that undergo cell differentiation into diverse cell types (Bailey, 1919; Larsson 1994). The cell types in wood of angiosperms such as poplar are more diverse in function and shape than those of gymnosperms, which consist predominantly of tracheids (Esau 1965). In angiosperms, cells derived from the cambium differentiate into tracheids, vessel elements, fibres, as well as axial, and radial, parenchyma cells.
Tracheids and vessel elements conduct water and ions, while fibres provide physical support for the plant and parenchyma provides metabolic support for the tissue.

Divergence in function of different lignified cell types, i.e. fibres and vessels, might require diverse secondary cell walls but this type of spatial heterogeneity has not been explored. There are common steps in differentiation of lignified secondary xylem cells (reviewed by Larsson, 1994), including cell expansion or elongation (Wenham and Cusack, 1974; Catesson, 1989), secondary cell wall polysaccharide biosynthesis and secretion (Awano et al., 2002; Wardrop and Harada, 1965; Samuels et al., 2002), lignification (Wardrop et al., 1957; Terashima et al., 1979; Takabe et al., 1985), and programmed cell death (O’Brien and Thiman, 1967; Groover et al., 1999; Courtois-Moreau et al., 2008). When developing secondary xylem cells reach their final shape following intrusive growth for fibres and radial expansion for vessels, a thick secondary cell wall, consisting of three major components, is deposited (reviewed by Mellerowicz et al., 2001, Turner et al., 2007). The most abundant component, cellulose, is assembled by the cellulose synthase complex at the plasma membrane, where the angle of deposition of microfibrils is correlated with the array of cortical microtubules. The secondary cell wall is normally made of three layers, called the S1, S2 and S3 layers, defined by different angles of cellulose microfibrils relative to the cell length axis (Bailey and Kerr, 1935; Barnett and Bonham, 2004). The second component is hemicellulose, which helps to aggregate cellulose microfibrils by providing cross-links among cellulose microfibrils (Atalla et al. 1993). During secondary cell wall formation, the cellulose microfibril-hemicellulose matrix provides the environment in which monolignols polymerize into lignin (Awano et al. 2002). Lignification begins at the cell corners and middle lamella, proceeds coincidently with the secondary cell wall polysaccharides biosynthesis and secretion, and finishes after polysaccharide deposition is done (Grünwald et al., 2002).

The predominant hemicelluloses in woody angiosperms secondary cell walls are xylans, which can make up 25-35% of the dry biomass of woody tissue, and consist of a β-(1,4)-xylan backbone, decorated with acetyl groups and side chains of α-D-glucuronic acid, 4-O-methyl-α-D glucuronic acid, α-arabinose residues (Ebringerová and Heinze, 2000, McCartney et al. 2006). Monoclonal antibodies, LM10 and LM11, antibodies, raised against a synthetic pentose xylan conjugated to BSA, can recognize glucuronoxylans from beech wood (McCartney et al. 2005). These antibodies were used to localize xylan in metaxylem and protoxylem secondary cell walls in wild-type Arabidopsis inflorescence stems (Persson et al. 2007). The importance of this xylan for secondary cell wall
production in Arabidopsis has been demonstrated in a number of mutants that showed significant reduction of xylan and collapsed xylem with uneven cell wall deposition in stem vascular bundles and interfascicular fibres (Persson et al. 2007; Zhong et al., 2005). Xylan distribution in the secondary cell wall of Fagus crenata was studied with field emission SEM and increasing accumulation of xylan was seen as secondary cell wall deposition progressed (Awano et al. 2002).

Mannans, another class of secondary cell wall hemicelluloses, are abundant in secondary xylem of gymnosperms where they occur as galactoglucomannans. Mannan epitopes were localized in thickened secondary cell walls of primary xylem elements, xylem parenchyma and interfascicular fibres in Arabidopsis inflorescence stem (Handford et al. 2003). Monoclonal antibodies raised against galactomannan (Pettolino et al., 2001) were tested in this study for binding to poplar secondary xylem walls.

The goal of this study was to define the cellular structures involved in secondary cell wall deposition during xylogenesis in poplar vessels and fibres. In the course of autoradiographic studies of developing wood (Kaneda et al. 2008) and published studies of gymnosperm wood prepared with cryofixation (Inomata et al. 1992; Samuels et al., 2002), it became clear that the quality of organelle structure preservation in cryofixed developing wood was very high. In contrast, published reports of cell structure in developing poplar fibres and vessels relied on chemical fixation and demonstrated evidence of osmotic disruption during fixation, such as vesiculated plasma membranes (Arend and Fromm, 2003). In this study, ultrastructure of high pressure frozen/freeze substituted hybrid poplar (Populus deltoides x P. trichocarpa, clone H11-11) was examined with immunofluorescence and TEM with the goal of providing cellular structural context for the process of secondary cell wall deposition.

Results

Secondary xylem and ultrastructure during xylogenesis

An overview of poplar secondary xylem development was obtained using light microscopy sections stained with toluidine blue (Figure 1A), which showed the developmental gradient from cambium to mature xylem. In such transverse sections, it is clear that vessels showed rapid radial expansion during their development. Fibres showed slower, more gradual radial expansion and retained cytoplasm for a longer period prior to programmed cell death. The most striking cellular events during wood development are related to secondary cell wall deposition, which begins immediately after cell expansion.
To define the types of cell wall matrix polysaccharides being deposited during fibre and vessel differentiation, the distribution of hemicelluloses in the poplar secondary xylem was probed with immunofluorescence. Xylan distribution in poplar secondary xylem was tested using the anti-xylan antibody LM10, which recognizes unsubstituted or low-substituted xylan backbone (McCartney et al. 2005). LM10 bound to the tissue in a general distribution on fibres, vessels and ray parenchyma cells, demonstrated by secondary antibody Alexa 594 label on tangential xylem samples (Figure 1B,D). The secondary cell walls, but not the middle lamella, of fibres and vessels (Figure 1B) as well as mature radial tracheids (Figure 1D) were evenly labeled.

Mannan distribution was studied using monoclonal anti-mannan primary antibodies (Pettolino et al. 2001), detected with secondary antibody-Alexa 594. Anti-mannan label was strongly enriched in fibre cell walls, especially the S2 layer (Figure 1C,E,F), suggesting that fibre cells have different relative proportions of hemicelluloses than vessels and rays. Anti-mannan binding to ray tracheids did increase in mature xylem (data not shown), which suggests that ray mannan deposition is delayed compared to axial fibre mannan deposition. To verify the pattern of anti-mannan label at higher resolution, samples were prepared for TEM and the grids incubated with monoclonal β-1-4-mannan antibody and colloidal gold-conjugated secondary antibody. Gold labeled the secondary cell walls of fibres but little label was detected on the walls of rays or vessels (Figure 2), supporting the immunofluorescence results.

For both antibodies, and in each replicate experiment, controls that were not incubated with primary antibody, but instead incubated in buffer followed by secondary antibody, did not have significant fluorescent signals in the red spectrum used to detect the Alexa fluorochromes or secondary antibody-gold binding in the TEM (data not shown).

The thickened secondary cell wall layers labeled with immunofluorescence is the product of these cells’ rapid and abundant cellulose deposition at the plasma membrane and secretion of matrix polysaccharides by the Golgi apparatus. To correlate cell structure with this strong biosynthetic activity, the ultrastructure of the developing secondary xylem of poplar from their birth in the cambium, to death as mature empty xylem elements, was examined in TEM preparations optimized for morphology by freeze substitution in osmium and embedding Spurr’s epoxy resin.

As cells exit the cambial zone, the cell structure was still dominated by the large central vacuole with the cytoplasm restricted to the cell periphery (Figure 3A). As the developing poplar xylem cells differentiate, vessels mature and undergo programmed cell death more rapidly than fibres (Courtois-
Moreau et al., 2009). In thin sections for TEM, few vessels were observed that retained their cytoplasm. Fibres, in contrast, mature more slowly and the development of their thickened secondary cell walls was correlated with cortical cytoplasm rich in cytoskeleton and endomembranes (Figure 3B-D; Figure 4A). In tangential sections, cortical microtubules lined the plasma membranes in these cells and aligned with cellulose microfibril orientation (Figure 4A; Figure 5), as described by Chaffey et al. (1998, 1999) for Aesculus hippocastanum.

During secondary cell wall biosynthesis, the cortical cytoplasm contains a variety of endomembrane structures as well as the prominent microtubule array. Tubular membrane structures with similar staining characteristics as the plasma membrane were often seen in the cortex (Figure 3B). These often had clathrin coated vesicles associated with them so could be considered ‘partially coated reticulum’ but it is not possible to determine if they represent anterograde or retrograde membrane traffic. In addition, a set of vesicles of consistent diameters in the range of 150 nm were often observed in tangential sections (Figure 3D) and often co-localized with microtubules (Figure 3B, C). In addition, secretory vesicles could be observed fusing with plasma membrane, forming “slit-like or horseshoe shaped structures” (Figure 4A, arrows), which have been reported in cryofixed pine developing tracheids (Samuels et al. 2002) and carrot and sycamore-maple suspension cultured cells (Staehelin and Chapman 1987). Staehelin and Chapman (1987) proposed that these are intermediary structures resulting from fusing secretory vesicles, which have been collapsed by turgor pressure and are in the process of undergoing membrane recycling.

The Golgi in developing fibres often showed signs of active polysaccharide production (Figure 4B), such as vesicular structures at the trans-most cisterna. However they lacked the elaborate TGN and the grape-like clusters of Golgi vesicles that were found in developing pine tracheids (Samuels et al. 2002) and, in many cases (see Figure 4B) lack a TGN within the plane of section.

The plasma membrane of cryo-fixed developing poplar fibres was generally smooth and pressed against the cell wall by turgor pressure (Figures 3 and 4). However, in some domains, the membrane appeared to form regular ridges or tubules (Figure 5A, 5B). Where the plane of section was parallel, or nearly parallel, to the plane of the plasma membrane bilayer, the plasma membrane appears as a darkly electron-dense region with the cortical cytoplasm on one side and the secondary cell wall on the other. The membrane appears to form ridges or tubules between microtubules (Figure 5B, arrow) and these were co-aligned with both the cortical microtubules (Figure 5B) and the cellulose microfibrils (Figure 5A, double-headed arrow). High pressure
freezing can create some membrane-related artifacts, such as bulges in the nuclear envelope, so these data should be treated as preliminary.

Discussion

In poplar secondary xylem, changes in cell structure were correlated with the production of the secondary cell walls during development. As the secondary cell wall thickened, the cells contained abundant microtubules, fusing vesicles and Golgi stacks. The cryofixation and freeze substitution method for fixation of developing poplar wood allowed high-resolution ultrastructure when contrasted with previously published TEM work in this tissue. Cell structure did not show signs of plasma membrane forming large internal spheres, several micrometres in diameter, as seen in developing poplar xylem prepared by conventional chemical fixation (Arend and Fromm, 2003). In that study, extensive infoldings of plasma membrane, including vesicle-filled large (micrometer-size range) membrane structures, were observed penetrating through the cortical cytoplasm and into the vacuole. The authors interpreted these large protrusions of plasma membrane as representing a vesicular flow of material into the vacuole. Here, there was no evidence of that vesicular flow: cells showed intact organelles, smooth membranes on the Golgi and tonoplasts and generally smooth plasma membranes.

On the plasma membrane, areas with ridged or tubular membrane structures were observed, in the size range of tens of nanometers, which were closely associated with microtubules. These may correspond to the ‘plasmatubules’ described in cryofixed material by Chaffey and Harris (1985). To determine the three-dimensional morphology of these structures, with respect to the plane of the plasma membrane bilayer, will require electron tomography. We speculate that these plasma membrane structures may represent a specialized domain of the plasma membrane bilayer found in cells actively depositing cellulose microfibrils. The ridges themselves are well below the limit of resolution of the light microscope so would not be detected in live cell imaging. The collection of ridges, however, might be large enough to be visualized if a marker of the microdomain is found.

The microtubule-associated compartments that were abundant in the cortical cytoplasm are interesting when considered in the light of recent advances in live cell imaging of cells expressing cellulose synthase (CesA)-fluorescent protein constructs. In both primary cell wall (Crowell et al. 2009; Gutierrez et al. 2009) and secondary cell wall (Wightman and Turner 2007) producing cells, small microtubule-associated compartments were visualized. These compartments are postulated to be involved in delivery and removal of CesA complexes from the
plasma membrane. Correlative confocal and transmission electron microscopy localized the CesA fusion proteins to the plasma membrane and Golgi but the nature of these small microtubule-associated compartments was elusive in cryo-sections labeled with anti-GFP (Crowell et al. 2009). In this study, samples were optimized for morphology, rather than immuno-labeling so it is not possible to assess whether the striking 150 nm vesicles or tubular structures in the cortex contain cellulose synthase complexes but they are consistent with the compartments described in terms of size and location.

Golgi morphology during xylogenesis was observed in poplar, in particular, a large number of small vesicles at the periphery of the Golgi stacks and less elaborate trans-Golgi networks. Golgi structure observed was unlike other secretory polysaccharide systems such as pine where TGN were made up of grape-like clusters (Samuels et al. 2002) or seed coat cells where the TGN consists of interconnected vesicular clusters (Young et al. 2008). Possible explanations for different Golgi morphologies might be that the arrangement of glycosyltransferases producing the hemicelluloses might vary or the way that the polysaccharide product is packaged at the trans-most cisterna could be different in poplar.

The basic structure of secondary cell walls in vessel elements and fibres are similar but distinct. This histo- and cytochemical study established that mannans and xylans occur in different patterns in poplar wood. Xylan showed a general distribution in developing xylem using monoclonal anti-xylan LM10. That data agreed with studies of secondary xylem in tobacco, showing that LM10 bound to all cell types in the secondary cell walls in secondary xylem (McCartney et al. 2005). Over all, the localization of xylan in poplar supports the view that xylan is a major material as a general hemicellulose in secondary cell walls of diverse cell types. In contrast, mannann epitopes were detected in a cell type specific localization pattern where the fibre secondary cell wall, especially the S2 layer was strongly labeled while vessel and rays were not. In Arabidopsis, mannans are found in secondary cell walls (Handford et al. 2003). However, the authors mentioned that xylem vessels consistently showed lower labeling of mannann epitopes than fibres and xylem parenchyma cells (Handford et al. 2003).

Both fibres and vessels have abundant cellulose and lignin in thick secondary cell walls. There are differences in lignin composition between the two cell types with vessel cell walls showing high guaiacyl (G) lignin content, while fibre cell walls contain more syringyl (S) lignin (Fergus and Goring, 1970; Grünwald et al., 2002). The differences of hemicellulose matrix in secondary cell walls might be
linked to the differences in lignin content, e.g. hemicellulose could help to organize selective monolignol binding. Atalla discussed the influence of the hemicellulose–cellulose matrix, which provides a link between lignin and the polysaccharide matrix, with hemicelluloses in particular playing a central role in selecting lignin precursors (reviewed in Atalla, 2005).

This study provides evidence that the endomembrane system and cortical cytoskeleton are closely associated during secondary cell wall formation. The lack of large vesicular bulges in the cytoplasm of developing poplar secondary xylem and close association of the plasma membrane with secondary cell wall during biosynthesis indicate that cryo-fixation is the most appropriate technique for studies of cell structure during wood biosynthesis.

**Materials and methods**

**Plant materials and growth conditions**

Hybrid poplar; *Populus deltoides* x *P. trichocarpa* (H11-11) were grown in pots (15x15x25 cm) in the greenhouse after whips were propagated.

**High pressure freezing.**

The actively growing poplar stems were dissected for cryofixation in 0.2 M sucrose as an extracellular cryoprotectant and high pressure frozen using either a Bal-Tec HPM 010 (Bal-Tec AG, Balzers, Liechtenstein) using Ted Pella ‘B’ sample carriers or Leica EM HPM 100 (Leica Microsystems GmbH, Wetzlar, Germany) using 6 mm sample carriers. For morphological observations, samples were freeze-substituted with 2% osmium tetroxide (Electron Microscopy Sciences) and 8% dimethoxypropane (Aldrich) in anhydrous acetone for 120 hours, using a dry ice-acetone bath that equilibrated at -80°C. The samples were then warmed to –20°C in a freezer for 4 hours, and to 4°C in a refrigerator for 4 hours, after which time they were brought to room temperature. The stem segments were transferred to fresh acetone and then Spurr’s resin was gradually added over 2 hours to bring the concentration to approximately 25%. The slices were then transferred to 50% resin for 2 hours, to 75% resin in open vials for 12 hours, and finally to 100% resin with changes two times per day, for 3 days. The infiltrated samples were polymerized in fresh Spurr’s resin at 60°C overnight.

Light microscope sections (0.5 um) were cut on glass knives using a Leica UCT ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany), dried onto glass slides and stained with Toluidine
Blue. Seventy nm sections were cut with a diamond knife on the Leica UCT Ultramicrotome, picked up on formvar-coated copper grids and stained for 10 min in 2% (w/v) uranyl acetate and 5 min in Reynold’s lead citrate. Sections were examined using a Hitachi H-7600 Transmission Electron Microscope (Hitachi High-Technologies Canada, Toronto, ON).

**Immunofluorescent labeling using anti-xylan (LM10) and anti-mannan antibodies.**

For immunofluorescent labeling, cryofixed samples were fixed and substituted with 0.25% glutaraldehyde and 8% DMP in anhydrous acetone, then infiltrated and embedded in LR-white resin. Teflon coated multiwell slides (EMS cat#63424-06) were coated with poly-L-lysine and left to dry. Sections (300, 500 nm) were placed on a drop of water in each slide well and air-dried over night in a 37°C incubator. Slides were placed in a coplin jar filled with 5% non-fat milk blocking buffer with TBST (10 mM Tris-buffer, 0.25 M NaCl, pH 7, with 0.1% Tween) for 20 min. After washing for 10 min with TBST, sections were incubated with 1/100 concentration anti-β-(1-4)-D-mannan (catalogue #400-4) monoclonal antibody (Biosupplies Australia Pty Ltd) or anti-xylan LM10 antibody (kind gift of Dr. J. Paul Knox, www.plantprobes.co.uk) for 1 hour, followed by several washes and 1/100 secondary antibody-Alexa 543 for 1 hour. Fluorescent localization was observed by Leica DRM light microscope using a Texas Red filter.

**Immun-o-gold labeling for xylem tissue by monoclonal mannan antibody.**

LR-White or Spurr’s resin blocks were cut into 60 to 80 nm sections and mounted on formvar coated nickel grids. Before immuno-gold labeling, Spurr sections were treated in 10% H2O2 solution for 20 min to etch the resin, and then rinsed with ddH2O. For blocking non-specific protein binding, grids were incubated with 5% (w/v) bovine serum albumin (BSA) (Sigma) in TBST for 20 min. Grids were floated on a drop of primary antibody of anti-mannan with antibody solution (1% BSA in TBST) for 1 hour at room temperature. After washing in TBST, grids were transferred to secondary antibodies (1:100 dilution in antibody solution). Secondary antibodies were conjugated to 15 nm colloidal gold (Ted Pella) with goat anti-mouse IgG + IgM for anti-mannan. Following washing in TBST and ddH2O, sections were poststained with 2% (w/v) uranyl acetate for 15 min and Reynold’s lead citrate for 5 min and examined as above.
Figure legends

Figure 1: Overview of Poplar secondary xylem development during secondary cell wall deposition.  A) Light micrograph of radial sections of inner bark of hybrid poplar illustrates developmental gradient from cambium (Cam) (Left) to developing xylem (Dev.Xy) and mature xylem (Mat.Xy). V: vessel, F: fibre. Bar is 50 µm. B) and D) Immunofluorescent label of secondary cell walls of poplar sections, embedded in LRWhite labeled with LM10 antibody and anti-rat secondary antibody conjugated to Alexa 594.  C, E and F) Strong mannan immunolabeling in fibre secondary cell walls and relatively lower label in vessel and ray secondary cell walls. Monoclonal rat anti-mannan antibody was used and anti-rat conjugated to Alexa 594 was used as secondary antibody. V: vessel, F: fibre. Bars = 50 µm.

Figure 2: Anti-mannan immunogold labeling in poplar developing xylem. A) Profuse labeling in fibre secondary cell wall, especially S2 layer (arrows) but less labeling in vessel secondary cell wall. C) Abundant labeling in fibre cell wall but less in ray parenchyma secondary cell wall. Bars = 500 nm.

Figure 3: Developing fibres of poplar secondary xylem. A) Fibre cells in oblique longitudinal section contained large central vacuole and peripheral cytoplasm. Bar = 2 um. B) Cortical cytoplasm of developing fibre with microtubules (mt) and both tubular and vesicular endomembrane structures (arrow). Bar = 500 nm. C) 150 nm vesicle associated with cortical microtubule. Bar = 100 nm. D) Tangential section through the cortical cytoplasm, plasma membrane and cell wall with abundant microtubules and darkly staining vesicles (arrow). Bar = 500 nm.

Figure 4: Secretory apparatus of developing poplar fibres. A) Tangential section through the cell surface showing regular arrays of microtubules and, in the cell wall, cellulose microfibrils (double-headed arrow) as well as fusing vesicle profiles (arrow).  A Golgi body (G) is cut parallel to the plane of one cisterna. B) Multiple Golgi with typical anatomy of cis/trans polarity. Some Golgi stacks lacking apparent TGN associated with their trans-face and with many small associated vesicles/fenestrae around their peripheries. Bars = 500 nm.
Figure 5: Domains of plasma membrane ridges or tubules (arrows) associated with microtubules (mt) and aligned with cellulose microfibrils (mf). A) An en face view where the section passes through the plane of the darkly stained plasma membrane (PM), with the cortical cytoplasm (cyt) and newly deposited secondary cell wall (CW). Bar = 500 nm. B) A tangential section through the plasma membrane where the membrane forms a group of ridges intertwined with microtubules. Bar=100 nm.

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References


