DEVELOPMENTAL LOCALIZATION OF HOMOGALACTURONAN AND XYLOGLUCAN EPITOPE S IN PIT MEMBRANES VARIES BETWEEN PIT TYPES IN TWO POPLAR SPECIES

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ABSTRACT

Localization of homogalacturonan (HG) and xyloglucan epitopes in developing and mature pit membranes from different pit types in xylem of *Populus tremula* L. × *P. tremuloides* Michx. (hybrid aspen) and *Populus tremula* L. (European aspen) was investigated using immunogold labeling. Pit types not mediated by ray parenchyma (intervessel- and fiber pits) showed significant developmental changes in HG epitope localization. Both low- and high methyl-esterified HG epitopes (recognized by LM19 and LM20, respectively) were detected in developing pit membranes of intervessel- and fiber pits until late stages of xylem formation, whereas no HG- and high methyl-esterified HG epitopes were detected in mature intervessel (except for annulus regions of pit membranes)- and mature fiber pit membranes, respectively. In contrast, no notable developmental changes in HG epitope localization were detected in pit types mediated by ray parenchyma (vessel-ray-, ray- and fiber-ray pits) during pit maturation. Vessel-ray- and fiber-ray pits showed abundant low- and high methyl-esterified HG epitopes in pit membranes, while ray pits showed presence of primarily low methyl-esterified HG epitope during all stages of pit development including at maturity. With xyloglucan (recognized by LM15), specific developmental changes in epitope localization were detected in vessel-ray pits. Xyloglucan epitope was detected in developing vessel-ray pit membranes, but was almost absent in mature pit membranes. Instead, xyloglucan was detected in the protective layers of vessel-ray pits showing completely different localization pattern than homogalacturonan, which was only detected in pit membranes. Together, our results suggest that the chemistry of pit membranes varies depending on both the developmental stage and pit type.

**Keywords:** Immunogold labeling, LM15 antibody, LM19 and LM20 antibodies, pit, *Populus*.

INTRODUCTION

Wood (xylem) is composed of various cell types with each type designed for a specific functional role during wood formation. In angiosperms (hardwoods), vessels and fibers
perform mainly the axial conduction of water through the xylem and mechanical support, respectively (Fengel & Wegener 1989). In contrast, water transport and mechanical properties of gymnosperm (softwoods) are predominantly carried out by tracheids (Fengel & Wegener 1989). Parenchyma cells are generally thought to contribute to storage, mobilization and transport of metabolites in the xylem. To maximize the biological function of each cell type, xylem cells are connected through special openings in their walls called pits. Pits are generally thought to facilitate the lateral transport of water and assimilates between xylem cells. In particular, the pit membrane between pits plays an important role in relation to safety and efficiency of the hydraulic system within the xylem (Choat et al. 2008).

In poplar, pits are present in the cell wall of all xylem cells including fibers, vessels and parenchyma cells (ray and axial) with different proportions and anatomy depending on pit types. Bordered pits found predominantly in the cell wall of vessel pairs (intervessel pits) are a major pit type of poplar xylem and allow for most of the water transport in xylem. Unlike the torus-margo structure found in tracheids of gymnosperms, bordered pits in angiosperms including poplar develop relatively homogeneous pit membranes, i.e. have even deposition of microfibrils (Choat et al. 2008). In contrast to the connections between vessels, the cell walls between parenchyma (ray and axial) and vessels develop half-bordered pits, which are thought to play important roles in the transport of solutes (Sauter et al. 1973; DeBoer & Volkov 2003; Nardini et al. 2010) from ray parenchyma to vessels. Pits are also found between fibers, between fibers and parenchyma (ray and axial) and between parenchyma cells (ray and axial), which may also be important in intercellular transport throughout the xylem.

In gymnosperms, many studies have reported clear evidence for the presence of non-cellulosic compounds such as pectins and hemicelluloses in pit membranes (Bauch et al. 1968; Daniel et al. 1996; Hafrén et al. 2000; Putoczki et al. 2008). However, the chemistry of pit membranes in angiosperms varies greatly depending on species and methods of analysis (reviewed by Choat et al. 2008; Gortan et al. 2011; Plavcová & Hacke 2011). For example, a recent cytochemical study of Lauraceae species has suggested that pectins are present in intervessel pit membranes (Gortan et al. 2011), while an immunogold labeling study of four angiosperm species (Betula papyrifera, Populus balsamifera, Prunus virginiana, Amelanchier alnifolia) indicated that mature intervessel pit membranes contain very little/or no homogalacturonans (HG) and rhamnogalacturonan-I (RG-I) (Plavcová & Hacke 2011). Earlier studies have also proposed that non-cellulosic polysaccharides may be hydrolyzed from the pit membranes of intervessel and vessel-ray (between vessel elements and ray parenchyma) pits at late stages of xylem formation in plants, such as willow, lilac, blackberry and ferns (O’Brien & Thimann 1967; O’Brien 1970; Morrow & Dute 1998), indicating a different chemical composition of pit membranes depending on developmental stage. However, the change in chemistry of membranes during pit development and the variation between different pit types is still poorly understood.

To extend our understanding of developmental dynamics and chemistry of pit membranes, we investigated the microdistribution of HG and xyloglucan, major polysaccharides of the primary cell wall, in pit membranes of two poplar species using...
immunogold labeling. Temporal and spatial changes of HG and xyloglucan distribution in pit membranes were systematically examined in both developing and mature xylem. Variation of chemical properties and distributional correlation between HG and xyloglucan in pit membranes between different pit types were also studied.

MATERIALS AND METHODS

Plant materials

Three month old hybrid aspen (Populus tremula L. × P. tremuloides Michx., clone T89) grown in the greenhouse (Kim et al. 2012) and mature European aspen (Populus tremula L.) grown in wild conditions (Kim & Daniel 2012) were used for experiments. Sample preparation was conducted according to procedures described previously (Kim & Daniel 2012; Kim et al. 2012). In brief, small sectors were taken from each sapling or tree and fixed using a mixture of paraformaldehyde and glutaraldehyde. After dehydration through a graded ethanol series, sectors were embedded in LR White resin (London Resin Co., UK). Early stages of secondary xylem formation (i.e. S₁ formation stages of xylem cells) were not observed in the developing xylem of European aspen (Kim & Daniel 2012).

Immunogold labeling

Labeling was conducted according to procedures described previously (Kim & Daniel 2012). Transverse ultrathin sections (ca 90 nm) mounted on nickel grids were incubated in blocking buffer (pH 8.2, Tris-buffered saline (TBS) containing 1% w/v BSA and 0.1% w/v NaN₃) for 30 min at room temperature. Grids were then incubated in LM15, LM19 or LM20 antibodies (PlantProbes, UK; 1:20 dilution in blocking buffer) for 2 days at 4°C. LM15 is specific for XXXG motif of xyloglucans (heptasaccharide with 3 xylosyl and 4 glucosyl residues, Marcus et al. 2008). LM19 binds to partially methyl-esterified and un-esterified HG (low methyl-esterified HG), whereas LM20 binds only to methyl-esterified HG (high methyl-esterified HG) (Verhertbruggen et al. 2009). Subsequently, grids were incubated with goat anti-rat secondary antibody conjugated with 10-nm colloidal gold (BBInternational, UK, 1: 50 dilution in blocking buffer) for 4 h at 35°C and stained with 4% w/v uranyl acetate for 10 mins. As control, sections were incubated with only secondary antibody and processed as above. Grids were examined using a Philips CM12 Transmission Electron Microscope (TEM, USA) operated at 80 kV. Negative TEM films were scanned using a film scanner (Epson Perfection Pro 750, USA). Results reflect observations on two hybrid aspen saplings and three different sections prepared from three different embedded blocks of European aspen.

RESULTS

Detection of HG epitopes in pits membranes of hybrid aspen

Both LM19 and LM20 antibodies bound in similar manner to intervessel pit membranes. LM19 (Fig. 1a–c) and LM20 (not shown) epitopes were detected in the pit membranes of developing intervessel pits until late stages of secondary cell wall
formation in vessels, but were not detected in the main part of mature intervessel pit membranes (Fig. 1d, e). LM19 (arrow in Fig. 1d) and LM20 (arrows in Fig. 1e) epitopes were detected in only the annulus regions (or membrane-rim; Schmid & Machado 1968;
In developing fiber pits (pits between fibers), LM19 (Fig. 2a, b) and LM20 (Fig. 2d, e) epitopes were detected in pit membranes. However, with mature fiber pits, only the LM19 epitope was detected in the actual pit membrane (Fig. 2c), whereas the LM20 epitope was not detected (Fig. 2f).

Figure 2. Immunogold labeling of low (LM 19, a–c) and high (LM 20, d–f) methyl-esterified homogalacturonan epitopes in fiber pit membranes (arrows) of hybrid aspen. The LM 19 epitope was detected in pit membranes, regardless of pit developmental stage (a–c). In contrast, the LM 20 epitope was detected in pit membranes of developing fiber pits until late stages of xylem formation (a, b), but was not detected in mature fiber pits (f). F = fiber. — Scale bars = 500 µm.

Plavcová et al. 2010; Plavcová & Hacke 2011) of mature intervessel pit membranes. In developing fiber pits (pits between fibers), LM19 (Fig. 2a, b) and LM20 (Fig. 2d, e) epitopes were detected in pit membranes. However, with mature fiber pits, only the LM19 epitope was detected in the actual pit membrane (Fig. 2c), whereas the LM20 epitope was not detected (Fig. 2f).
Figure 3. Immunogold localization of low methyl-esterified homogalacturonan (HG) epitope by LM19 in vessel-ray pit membranes (m) of hybrid aspen. The epitope was detected in pit membranes of developing (a−c) and mature (d) pits. No epitope was detected in the protective layer (pl in b−d) during pit developmental stages. Note gradual decrease of HG epitopes in middle lamella regions (asterisks) during pit development. V = vessel; R = ray parenchyma. — Scale bars = 500 µm.
Figure 4. Immunogold localization of low (LM 19, a–c) and high (LM 20, d) methyl-esterified homogalacturonan (HG) epitopes in ray pit membranes (m) of hybrid aspen. The HG epitope was detected in pit membranes during both developmental and mature stages, regardless of antibody type (a–d). The LM 19 epitope (c) was much more abundant than LM 20 (d), regardless of developmental stage. Asterisks indicate middle lamella regions. PW, primary cell wall in ray parenchyma (R); SW = secondary cell wall in ray parenchyma. — Scale bars = 500 µm.
Figure 5. Immunogold localization of low (LM 19, a−c) and high (LM 20, d) methyl-esterified homogalacturonan (HG) epitopes in fiber-ray pit membranes (m) of hybrid aspen. The HG epitope was detected in pit membranes during both developmental and mature stages (a−d). The LM 19 epitope (c) was slightly more abundant than the LM 20 epitope (d) during all pit formation stages. F = fiber; R = ray parenchyma. — Scale bars = 500 µm.

Unlike intervessel- and fiber pits (pit types that are not mediated by ray parenchyma), developmental changes in the distribution of LM19 and LM20 epitopes were not significant in pit membranes of vessel-ray, ray- and fiber-ray pits (pit types that are mediated by ray parenchyma). LM19 (Fig. 3) and LM20 (not shown) epitopes were abundant in vessel-ray pit membranes during all pit developmental stages including mature pits. No epitopes were detected in the protective layer (Chafe 1974) of vessel-ray pits by the two antibodies (Fig. 3b−d). In ray pits (pits between ray parenchyma), LM19 epitope (Fig. 4a−c) was abundant in pit membranes, whereas LM20 (Fig. 4d) gave very weak labeling during all pit developmental stages including mature pits. Fiber-ray pits also showed the presence of the two epitopes in pit membranes during all pit developmental stages including mature pits with slightly more abundance of LM19 (Fig. 5a−c) than LM20 (Fig. 5d).

Detection of xyloglucan epitopes in pit membranes of hybrid aspen

During intervessel pit development, the LM15 epitope for xyloglucan was detected in pit membranes even though it was very weak (Fig. 6a, b). At mature stages, no LM15 epitope was detected in mature intervessel pit membranes (Fig. 6c). LM15 epitope was also not detected in fiber pit membranes during all developmental stages including mature stage pits (not shown).
Figure 6. Immunogold localization of xyloglucan epitopes by LM 15 in intervessel pit membranes (m) of hybrid aspen. The epitope was detected in pit membranes during pit developing stages (arrowheads in a, b), but was not detected in mature pits (c). V = vessel. — Scale bars = 500 µm.

In vessel-ray pits, the LM15 epitope was detected in pit membranes until early stages of protective layer formation (Fig. 7a, b). However, labeling of the LM15 epitope decreased gradually in pit membranes during protective layer formation and subsequently increased in the protective layer (Fig. 7c, d). At the mature stage of vessel-ray pits, the LM15 epitope was mostly detected in the protective layer (Fig. 7d). The epitope was also detected in overarching regions of the pit border near ray parenchyma (arrows in Fig. 7c, d). In ray pits, only a weak heterogeneous distribution of the LM15 epitope was detected in pit membranes during the entire phase of xylem development (Fig. 8a–c). No LM15 epitope was detected in pit membranes of fiber-ray pits during pit development or mature stages (not shown).

**Distribution of HG and xyloglucan epitopes in pits membranes of European aspen**

Localization patterns of HG and xyloglucan epitopes in pit membranes of intervessel-, vessel ray- and ray pits were further advanced and confirmed in European aspen. Although European aspen (Fig. 9) showed overall more abundant LM19 and LM20 epitopes in mature intervessel-, vessel ray- and ray pit membranes than hybrid aspen (Fig. 1d, e; 3d; 4c, d), localization patterns of the two epitopes were almost identical between the two taxa. With LM15 localization, the epitope was detected across vessel-
Figure 7. Immunogold localization of xyloglucan epitopes by LM 15 in vessel-ray pit membranes (m) of hybrid aspen. The epitope was detected in pit membranes during early stages of pit formation (a) with movement from the pit membranes to protective layers (pl) during pl formation (b, c). In mature pits, the epitope was mostly detected in pl regions (d). Note strong labeling in middle lamella regions (asterisks). V = vessel; R = ray parenchyma. — Scale bars = 500 µm.
Figure 8. Immunogold localization of xyloglucan epitopes by LM 15 in ray pit membranes (m) of hybrid aspen. Only weak detection of xyloglucan was detected in pit membranes during developmental and mature stages (a–c). Asterisks indicate middle lamella regions. PW = primary cell wall in ray parenchyma (R); SW = secondary cell wall in ray parenchyma. — Scale bars = 500 µm.

Ray pit membranes at late stages of xylem formation (Fig. 10a), but was only observed in outermost pit membranes adjacent to ray parenchyma in mature xylem (Fig. 10b). The epitope was also abundant in overarching regions of the pit border near ray parenchyma (arrows in Fig. 10a, b). These labeling patterns were similar with LM15 epitope localization in vessel-ray pit membranes of hybrid aspen (Fig. 7). This indicates that European aspen may also have a thin protective layer in vessel-ray pits as is seen in hybrid aspen and that similar developmental change in LM15 epitope localization may occur in vessel-ray pit membranes between the two taxa. In case of mature ray pits, LM15 epitope localization in pit membranes was almost identical to hybrid aspen (not shown). Therefore, we conclude that localization patterns of HG and xyloglucan epitopes in mature intervessel-, vessel ray- and ray pit membranes may be overall similar between European aspen and hybrid aspen. The only exception was found with LM15 epitope
Figure 9. Immunogold localization of low (LM 19) and high (LM 20) methyl-esterified homogalacturonan (HG) in pit membranes (m) of European aspen. All micrographs show the mature stage of pits. As in hybrid aspen (Fig. 1d, e), LM 19 and LM 20 epitopes were only detected in the annulus regions of intervessel pit membranes (arrows in a, b). In vessel-ray pits, LM 19 and LM 20 epitopes were detected in pit membranes (c, d), except for the innermost membranes adjacent to ray parenchyma (arrowheads in c, d). In ray pits, the LM 19 epitope (e) was abundant in pit...
Figure 10. Immunogold localization of xyloglucan epitopes by LM 15 in vessel-ray pit membranes (m) of European aspen. The epitope was detected across pit membranes in late stages of xylem formation (a), except for innermost membranes adjacent to the vessel wall (V = arrowheads in a). Note epitope localization in overarching regions of the pit border (arrows in a, developing protective layer). In mature pits, the epitope was only detected in the region of the protective layer (arrows and inset in b, see text). Note epitope localization in middle lamella regions (asterisks). R = ray parenchyma. — Scale bars = 500 µm.

membranes, while LM 20 epitope (f) was very weak as in hybrid aspen (Fig. 3c, 4c). Some epitopes were also detected in plasmodesmata regions of ray pit membranes (arrowheads and inset in e). Note different localization patterns in middle lamella regions (asterisks) between LM 19 and LM 20 epitopes. V = vessel; R = ray parenchyma. — Scale bars = 500 µm.
localization. Unlike hybrid aspen, some epitope distribution was detected in mature intervessel pit membranes with variations in amounts between pits (Fig. 11). We expect that this minor difference between the two taxa is probably due to the difference in age of samples (i.e. young hybrid aspen and mature European aspen).

**DISCUSSION**

*Distribution patterns of HG and xyloglucan epitopes in pit membranes varies between pit types*

Table 1 shows distributional characteristics of HG and xyloglucan epitopes in pit membranes of different pit types in hybrid aspen. During developing stages of xylem, differences in HG epitope labeling patterns were not significant between pit types except for ray pits, which showed a weak high methyl-esterified HG (LM20 epitope) presence in pit membranes. In mature xylem, homogalacturonans were absent in mature intervessel pit membranes except for annulus regions in agreement with previous studies (Schmid & Machado 1968; Plavcová & Hacke 2011), while HG epitope was abundant across the pit membranes of other pit types (i.e. vessel-ray-, ray-, fiber- and fiber-ray pits) (Table 1). Our results also indicate that vessel-ray- and fiber-ray pit membranes are composed of a mixture of low (LM19 epitope)- and high methyl-esterified HG at the mature stage of xylem development, while ray- and fiber pit membranes are composed predominantly of low methyl-esterified HG (Table 1). With xyloglucan localization (LM15 epitope) and in contrast with other pit types, vessel-ray pits showed strong epitope distribution in developing pit membranes and dynamic changes in distribu-
Table 1. Distribution of homogalacturonan (HG) and xyloglucan epitopes in developing and mature pit membranes reflected by intensity of immunogold labeling of hybrid aspen. Annulus regions and protective layers (pl) are not included in pit membranes of intervessel- and vessel-ray pits, respectively. Number of ‘+’ indicates intensity of labeling, ‘nd’ not detected, ‘tr’ trace amounts of labeling.

<table>
<thead>
<tr>
<th>Pit type</th>
<th>Low methyl-esterified HG (LM19 epitope)</th>
<th>High methyl-esterified HG (LM20 epitope)</th>
<th>Xyloglucan (LM15 epitope)</th>
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<td>Developing</td>
<td>Mature</td>
<td>Developing</td>
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<tr>
<td>Intervessel</td>
<td>++</td>
<td>nd (+ in annulus)</td>
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<tr>
<td>Fiber</td>
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<td>Vessel-ray</td>
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Localization patterns during xylem maturation (i.e. from the membrane to protective layers) (Table 1). Our results suggest therefore that the chemical composition of pit membranes may be highly variable not only between wood species (Choat et al. 2008; Gortan et al. 2011; Plavcová & Hacke 2011), but also dependent on the developmental stage of pits and between pit types in hybrid aspen.

**Localization patterns of HG epitope differ between pit types in association with ray parenchyma**

Interestingly, our results indicate that developmental changes in HG epitope distribution occur only in pit types that are not mediated by ray parenchyma (i.e. intervessel- and fiber pits, Table 1). In particular, the chemistry of intervessel pit membranes was significantly changed during late stages of secondary xylem formation, i.e. HG epitope was absent in mature pit membranes (except at the annulus) even though the HG epitope was abundant in developing pit membranes until late secondary xylem formation. These temporal changes of cell wall components in intervessel pit membranes were also observed in our previous study of xylan localization in hybrid aspen (Kim et al. 2012). The results are also consistent with earlier cytochemical studies (O’Brien & Thimann 1967; O’Brien 1970), which showed loss of cell wall materials (i.e. polysaccharides including HG) from intervessel pit membranes at the final stage of pit formation. This was hypothesized as caused by the activity of hydrolases on intervessel pit membranes that are un lignified. However, several questions remain in order to explain the enzymatic degradation of HG, such as why the enzymes do not attack HG in vessel-ray pit membranes and why hydrolysis of HG is only limited to highly methyl-esterified HG in fiber pit membranes. A similar change in cell wall components has also been reported in softwoods. For example, galactoglucomannan distribution in Japanese cedar was gradually reduced from the bordered pit membranes of...
tracheids and cross-field pit membranes with the polysaccharide not detected in mature pit membranes (Kim et al. 2011). Although the enzymatic removal of non-cellulosic polysaccharides including HG from the pit membranes needs further investigation, our present and previous results indicate that if enzymatic degradation is truly involved in the removal of non-cellulosic polysaccharides from pit membranes, the mechanism is likely to vary among both pit types and wood species and that several different types of enzymes may be selectively involved in the process. Our results also suggest that variation in HG epitope distribution patterns between pit types may be related to the different biological function between pit types, particularly those in association with ray parenchyma.

Localization patterns of HG and xyloglucan epitopes differ significantly in vessel-ray pits
The HG epitope was consistently detected in pit membranes during all developmental stages of vessel-ray pits, while the distribution of the xyloglucan epitope gradually changed from the pit membranes to protective layers during vessel-ray pit maturation. In fully developed xylem, the vessel-ray pits showed almost opposite distribution patterns for HG and xyloglucan epitopes (i.e. HG epitope was in pit membranes and xyloglucan epitope was in protective layers). Based on previous studies showing the masking effect of HG during xyloglucan epitope localization (Marcus et al. 2008), it can be expected that xyloglucan labeling may be masked by HG in mature vessel-ray pit membranes. However, strong xyloglucan epitope labeling was detected in developing pit membranes and compound middle lamella (CML) regions even though the HG epitope was abundant in developing pit membranes and CML regions, indicating that the masking effect of xyloglucan epitopes by HG is not significant in vessel-ray pit membranes. Although several processes, such as enzymatic degradation of xyloglucan from pit membranes and production of new xyloglucan from living ray parenchyma during protective layer formation may be involved in developmental changes of xyloglucan epitope distribution in vessel-ray pits, exactly how the distribution is changed in vessel-ray pits is unknown. With xylan epitope localization in vessel-ray pit membranes of hybrid aspen, our previous study (Kim et al. 2012) showed similar distribution patterns to xyloglucan epitopes as this study, indicating that xyloglucan and xylan are closely related to protective layer formation in vessel-ray pits. This suggests that a similar mechanism may be involved in developmental changes of xyloglucan and xylan distribution in vessel-ray pits.

In conclusion, this study extends our understanding of variation in pit membrane chemistry. Variation in the chemical composition of pit membranes between species is frequently defined in previous studies, whereas variation depending on the developmental stages of pits and between pit types is rarely considered. Our results show clearly that distribution patterns of HG and xyloglucan epitopes in pit membranes are highly variable depending on the developmental stage of pits and between pit types in poplar. The study also provides new insights into developmental changes of HG epitope distribution in pit membranes. A developmental change of HG epitope distribution occurs only in intervessel- and fiber pit membranes and does not occur in pits mediated
by ray parenchyma (i.e. vessel-ray-, ray-, fiber-ray pits). Our observations also show that HG and xyloglucan epitopes are situated in completely different regions in mature vessel-ray pits (i.e. HG in pit membranes, xyloglucan in protective layers). However, the physiological understanding of our findings in relation to the function of pits is still poor. Further biochemical and immunocytochemical studies will provide more comprehensive understanding of the relationship between biological function of pits and chemical variation of pit membranes.

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