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Immunolocalization of (1,4)-β-galactan in tension wood fibers of poplar

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Summary The occurrence and distribution of (1,4)-β-galactan in wood cells of poplar (*Populus trichocarpa* Torr. & A. Gray × *P. koreana* Rehder) were studied by immunolabeling with a monoclonal antibody (LM5) specific to this carbohydrate epitope. Immunofluorescence microscopy revealed exclusive binding of the LM5 antibody to tension wood fibers, indicating the specific presence of (1,4)-β-galactan in cell walls of this wood cell type. Higher magnifications achieved with the fluorescence microscope and additional immunogold electron microscopy showed that the binding of the LM5 antibody was mainly restricted to a narrow cell wall area between the gelatinous *G*-layer and the secondary cell wall. This labeling pattern strongly suggests a role of (1,4)-β-galactan in cross-linking the *G*-layer and secondary cell wall. Furthermore, the exclusive localization of (1,4)-β-galactan in tension wood strengthens the view that this carbohydrate epitope can be considered a highly specific marker of reaction wood formation in mechanically stressed trees.

Keywords: cell wall, galactan, G-layer, xylem fibers.

Introduction

The wood cell wall is a multi-layered composite formed by cellulose, lignin and different fractions of matrix polymers. Three major cell wall layers—middle lamella, primary and secondary cell wall—can be identified easily in microscopic sections because of differences in molecular structure and chemical composition. In fibers, the cell builds a thick secondary cell wall that gives stems and branches of trees their mechanical strength.

In certain cases, however, the general structure and chemistry of the fiber cell wall change and reaction wood develops in response to mechanical stress such as wind and the action of gravity on branches and leaning stems. The reaction wood of conifers, known as compression wood, has increased amounts of lignin on the lower sides of branches and leaning stems, whereas the reaction wood of angiosperms, known as tension wood, has increased amounts of cellulose on the upper sides of branches and inclined stems (Timell 1986, Bamber 2001, Pilate et al. 2004). In tension wood, an additional gelatinous layer, the *G*-layer, is deposited on the lumen side of the fiber cell wall, partly replacing the secondary cell wall. The *G*-layer consists mainly of highly crystalline cellulose (Côté and Day 1965, Norberg and Meier 1966, Daniel et al. 2006), although other components such as hemicelluloses (Furuya et al. 1970, Nishikubo et al. 2007), arabinogalactan proteins (Lafarguette et al. 2004) or lignin (Joseleau et al. 2004) may also be present.

Because of the biological and economic significance of tension wood, it has been extensively studied in recent years. Substantial progress has been made in its physical analysis leading to a better understanding of how the *G*-layer contributes to the development of tensile stress in tension wood (Clair and Thibaut 2001, Clair et al. 2003, 2006, Coutand et al. 2004, Yamamato 2004, Yamamato et al. 2005). Nevertheless, uncertainty remains about the active role of the *G*-layer in tension wood properties, especially how the stress-generating *G*-layer adheres to the secondary cell wall (Clair et al. 2005). Ultrastructural studies have revealed a significant change in the cellulose microfibril angle between the *G*-layer and adjacent secondary cell wall leading to a lower density of cellulose microfibrils in the contact zone (Araki et al. 1982, Prodhan et al. 1995). This, in turn, creates a mechanically weakened area, an effect that must be counteracted by other cross-linking cell wall polymers such as pectins or hemicelluloses.

In the present study, the occurrence of such cell wall polymers in tension wood fibers was proven by an immunohistochemical approach. Three monoclonal antibodies (LM5, LM6 and JIM5) were tested as specific probes for pectic and hemicellulosic carbohydrate epitopes in combination with fluorescence and transmission electron microscopy (TEM). A (1,4)-β-galactan-containing polymer was exclusively labeled in tension wood fibers using the LM5 antibody. Labeling was abundant in the contact zone between the *G*-layer and secondary cell wall suggesting that (1,4)-β-galactan has a role in cross-linking these cell wall layers.

Materials and methods

Wood sample preparation

Stem tissue was collected in late summer from three, 2-year-old poplar trees (*Populus trichocarpa* Torr. & A. Gray × *P. koreana* Rehder) grown under field conditions at the Munich University of Technology. Small pieces of stem tissue, including the current- and the previous-year ring, were fixed in

2% (w/v) formaldehyde in PBS (137 mM sodium chloride, 10 mM phosphate, 2.7 mM potassium chloride, pH 7.2) for 2 h and then washed several times in PBS. Fixed wood tissue was either transversely sectioned (30 µm) with a sliding microtome for immunofluorescence microscopy or further processed for immunogold electron microscopy. The occurrence of tension wood in the stem tissue was checked by light microscopic observations of tissue sections double-stained with anilin blue and safranin.

Immunofluorescence microscopy

Transverse sections containing tension wood were chosen for immunofluorescence microscopy. To reduce unspecific labeling, sections were blocked for 30 min with 100 mM glycine in PBS containing 0.2% (v/v) Tween 20 and for a further 60 min with 1% (w/v) bovine serum albumin (BSA) and 1% goat normal serum in PBS. The sections were then incubated overnight at 4 °C with monoclonal rat antibodies specific to different pectic epitopes (Table 1), diluted 1:10 in PBS containing 0.5% BSA. Following washing in PBS, bound rat antibodies were labeled with Cy3-coupled goat anti-rat IgG + IgM (Dianova, Germany) for 1 h at 37 °C. The sections were washed again in PBS containing 0.2% (v/v) Tween 20 and then examined with a Zeiss axiophot microscope (Zeiss, Germany) fitted with the filter combination of a 546 nm exciter and a 590 nm emitter. Controls were incubated either without primary antibody or with primary antibody saturated with a mixture of citrus fruit pectins (Sigma Chemicals, catalog numbers P9135 and P9561).

Immunogold electron microscopy

Fixed wood tissue was dehydrated in a graded series of ethanol. After embedding in LR white acrylic resin, ultra-thin sections of 100 nm were cut with a diamond knife on an LKB Ultramicrotome and transferred to formvar-coated nickel grids. Sections were blocked for 30 min with 100 mM glycine in PBS containing 0.2% (v/v) Tween 20 and for a further 60 min with 1% (w/v) BSA and 1% goat normal serum in PBS. The sections were then incubated for 1 h at 37 °C with LM5 monoclonal rat antibody, diluted 1:10 in PBS containing 0.5% BSA. Following washing in PBS, bound rat antibodies were labeled with 12 nm gold goat anti-rat IgG (Dianova, Germany) for 1 h at 37 °C. The sections were washed again in PBS containing 0.2% (v/v) Tween 20 and distilled water, post stained with $KMnO_4$ and then examined with a Zeiss EM 10c transmission electron microscope operated at 80 kV. Controls were incubated with primary antibody saturated with a mixture of citrus fruit pectins (Sigma P9135 and P9561). A semi-quantitative determination of the labeling intensity was carried out by counting the particle density in 11 randomly selected cell wall areas in sections from two trees.

Table 1. Monoclonal antibodies tested with poplar tension wood fibers.

Antibody species	Specificity to epitopes in cell wall polymers	Immunoreactivity with tension wood
LM5	(1,4)-β-D-Galactan in pectins and hemicelluloses; Jones et al. (1997)	Strong
LM6	(1,5)-α-L-Arabinan in pectins; Willats et al. (1998)	Faint
JIM5	Homogalacturonan in pectins; Knox et al. (1990)	Not detectable

Results

The occurrence of tension wood in stem tissue was checked by light microscopic observations of sections double-stained with anilin blue and safranin. Tension wood was clearly distinguishable from normal wood because the *G*-layers in tension wood fibers stained intensively blue (Figure 1A). In most tension wood fibers, *G*-layers were partly detached from secondary cell walls because of the mechanical forces applied during sectioning.

Microscopic sections containing tension wood and normal wood were chosen for immunofluorescence microscopy and probed with the monoclonal antibodies LM5, LM6 and JIM5. Among these antibodies, only LM5 showed clear and specific immunoreactivity with tension wood, whereas LM6 and JIM5 gave no significant reaction (Table 1). Both LM6 and JIM5 labeled other wood cell structures such as pits, middle lamellae and ray parenchyma, which were not labeled by LM5 (data not shown). Examination of several sets of different microscopic sections confirmed that LM5 reacted specifically with tension wood fibers of current-year and previous-year rings (Figures 1C–F), whereas no other wood cell structures were labeled by the antibody (Figures 1G and 1H). In all tension wood fibers, the labeling was mainly restricted to a narrow band between the *G*-layer and secondary cell wall even when the *G*-layer itself exhibited a weak, diffuse labeling (Figures 1E and 1F). In fibers where the *G*-layer was detached from the secondary cell wall, the intensity of labeling seemed greater, but this was the result of deeper penetration of the primary and secondary antibodies. Control sections, in which the primary antibody was co-incubated with pectin, showed a strongly reduced immunoreactivity with tension wood (Figure 1B) or no immunoreactivity when the primary antibody was omitted (data not shown).

To verify the exclusive binding of the LM5 antibody to an epitope in the contact zone between the *G*-layer and secondary cell wall, further immunogold labeling experiments were carried out with ultra-thin sections and TEM. In good agreement with the results obtained by immunofluorescence microscopy, TEM demonstrated specific labeling of tension wood fiber cell walls but no such labeling was present in normal wood fiber cell walls. The labeling of tension wood fibers was mainly restricted to a narrow area between the *G*-layer and secondary cell wall, including small parts of both cell wall layers (Figures 2A and 2B, Table 2). Labeling of most other parts of the *G*-layer was less intense, and no specific labeling was observed in other cell wall structures. Control sections, in which the primary antibody was co-incubated with pectin, showed no immunolabeling (Figures 2C and 2D).

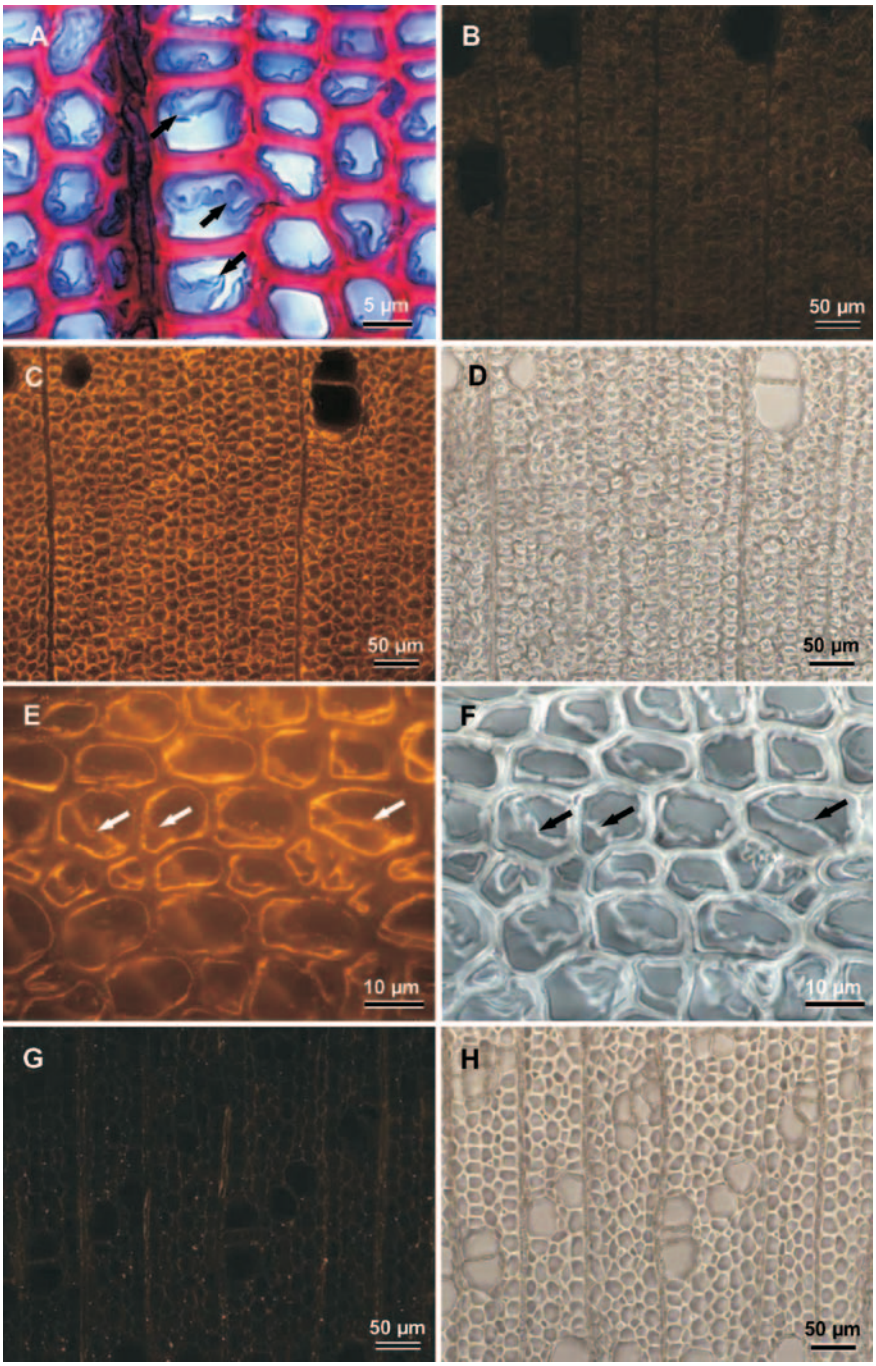

Figure 1. Light microscopic detection of tension wood and immunofluorescence labeling of (1,4)-β-galactan. (A) Light micrograph of tension wood with blue-stained *G*-layers in tension wood fibers (arrows). (B) Fluorescence micrograph of tension wood incubated with pectin saturated LM5 antibody (control). (C and D) Tension wood in a previous year ring incubated with LM5 antibody showing yellow labeling for (1,4)-β-galactan ((C) fluorescence micrograph; (D) light micrograph). (E and F) Higher magnification of tension wood fibers in a current-year ring with yellow labeling between *G*-layer and secondary cell wall ((E) fluorescence micrograph; (F) light micrograph). The *G*-layers themselves show only a weak, diffuse labeling (arrows). (G and H) Normal wood incubated with LM5 antibody lacking yellow labeling for (1,4)-β-galactan ((G) fluorescence micrograph; (H) light micrograph).

Discussion

Reaction wood in trees develops in response to mechanical stress in branches and leaning stems. Tension wood formed in angiosperm trees is characterized by fiber cells that form an additional gelatinous *G*-layer on the lumen side of the fiber cell wall. This *G*-layer is believed to consist mainly of pure cellulose, although small amounts of other cell wall polymers such as hemicelluloses (Furuya et al. 1970), AGPs (Lafarguette et al. 2004) or lignin (Joseleau et al. 2004) may also be present.

In this immunohistochemical study, a (1,4)-β-galactan epitope was exclusively localized in poplar tension wood by the monoclonal antibody LM5. This finding is consistent with former chemical analyses showing increased amounts of galactan and galactose in tension wood of angiosperm trees (Timell 1969, Ruel and Barnoud 1978). These previous studies, however, provided no further information about the cellular localization of galactan in tension wood. In this study, it was shown that, in poplar, (1,4)-β-galactan is localized in cell walls of tension wood fibers and that its occurrence in these cell walls is mainly restricted to the interface between the *G*-layer and the adjacent secondary cell wall. This finding might explain why former chemical analysis of isolated *G*-layers failed to detect

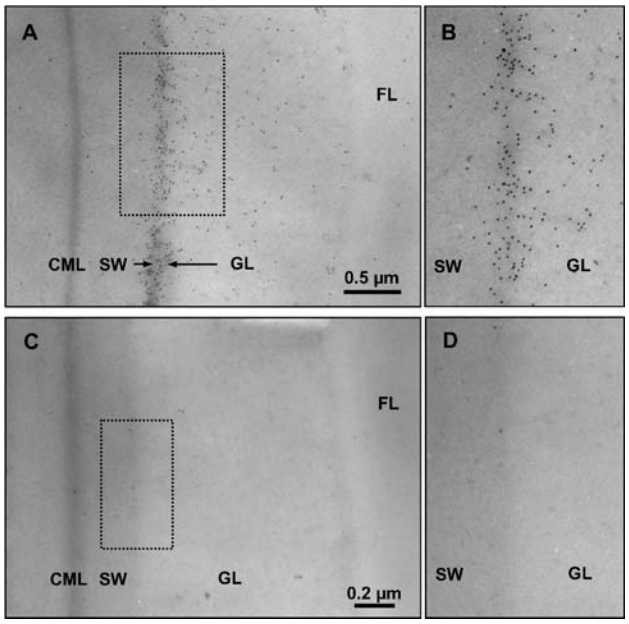

Figure 2. Immunogold labeling of (1,4)-β-galactan in tension wood fibers of a previous-year ring. (A) Part of a cell wall in a tension wood fiber composed of compound middle lamellae, secondary cell wall and *G*-layer. Intense labeling occurs in the contact zone between the *G*-layer and the adjacent secondary cell wall. The *G*-layer itself shows only weak, diffuse labelling. (B) Higher magnification of A. (C) Cell wall of a tension wood fiber incubated with pectin saturated LM5 antibody. The cell wall is unlabeled. (D) Higher magnification of C. Abbreviations: (CML) compound middle lamellae; (SW) secondary cell wall; (GL) *G*-layer; and (FL) fiber lumen.

the presence of (1,4)-β-galactan (Nishikubo et al. 2007). The (1,4)-β-galactan has been also detected in gelatinous fibers of coiling red vine tendrils (Meloche et al. 2007) and in compression wood fibers of gymnosperms (Altaner et al. 2007). These results support the view that (1,4)-β-galactan can be considered a specific marker of reaction wood formation in angiosperm and gymnosperm species.

The striking occurrence of (1,4)-β-galactan in reaction wood raises the question of its function in mechanically stressed wood tissue. A specific role in tension wood might be related to the ability of galactan-rich cell wall polymers to interact with cellulose microfibrils. Galactan-rich pectins have been shown to bind to cellulose microfibrils through their galactan (and arabinan) side chains, thus cross-linking single cellulose microfibrils (Zykwinska et al. 2005, 2007). In tension wood fibers, such cross-linking of cellulose microfibrils might strengthen the attachment of the cellulose-rich *G*-layer to the secondary cell wall, given that (1,4)-β-galactan is exclusively localized in the contact zone between these cell wall layers. It is noteworthy that other epitopes of cross-linking cell wall polymers, which are recognized by LM6 and JIM5 antibodies, were not detected in the *G*-layer or the adjacent secondary cell wall even though they were abundant in other wood cell structures such as pits, middle lamellae and ray parenchyma. This finding emphasizes the exclusive role that (1, 4)-β-galactan appears to play in cross-linking the *G*-layer and secondary cell wall in tension wood fibers.

Taken together, the present findings strengthen the view that (1,4)-β-galactan is a specific marker of reaction wood formation in mechanically stressed trees. Its distribution in the cell walls of tension wood fibers strongly suggests a role in cross-linking the tensile-stressed *G*-layer to the adjacent secondary cell wall. In this role, (1,4)-β-galactan-containing cell wall polymers would presumably act in parallel with hemicellulosic xyloglucan, which has been recently proposed to fulfill a similar function (Nishikubo et al. 2007).

Table 2. Labelling intensity achieved with the LM5 antibody in different cell wall areas of tension wood fibers (values are means ± SE; $n = 11$). Abbreviations: CML, compound middle lamellae; SW, secondary cell wall; CZ, contact zone between secondary cell wall and *G*-layer; and GL, *G*-layer.

Cell wall area	Labelling intensity (particles μm^{-2})
CML	12.2 ± 2.6
SW	13.4 ± 3.4
CZ	429.5 ± 65.1
GL	71.1 ± 13.2

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