

MICRODISTRIBUTION OF XYLOGLUCAN IN DIFFERENTIATING POPLAR CELLS

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Recent studies on the ultrastructure and composition of the gelatinous layer (g-layer) in poplar have reported findings of xyloglucan. Using correlated fluorescence, scanning- and transmission electron microscopy, we found evidence for xyloglucan present in and surrounding the g-layer, using the fucosylated xyloglucan specific CCRC-M1 antibody and the carbohydrate binding module FXG-14b. However, labeling of isolated gelatinous layer remained negative.

Keywords: *Populus tremula x tremuloides; Tension Wood; Xyloglucan; CBM; Immunogold labeling*

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INTRODUCTION

Wood is one of the highest valued and most used raw materials in the world. Demand for forest products is rising (Juslin and Hansen 2003), increasing our need to understand wood formation, in an effort to increase wood production and to better utilize our lignocellulose resources.

Tension wood, the reaction wood of hardwoods, forms naturally in the upper part of branches and leaning stems as a response to gravity or mechanical stress (Timell 1969). In softwoods, reaction wood is known as compression wood and in contrast to hardwoods is found in the lower part of branches and leaning stems (Timell, 1969).

Tension wood is not as stiff as normal wood and exerts stronger longitudinal forces, making it more susceptible to drying defects (Hoadley 2000). In combination with lower fiber collapsibility for chemical and mechanical pulps, this makes tension wood less desirable for both solid wood and paper products. Currently, there are no micro-mechanical models that can fully describe the difference between normal wood and tension wood, but is likely linked to the properties of the gelatinous layer (g-layer) that is characteristically found in tension wood fibers (Pilate et al. 2004; Mellerowicz et al. 2008).

Micro-mechanical models for tension wood principally rely on the low microfibril angle found in the g-layer to account for the change in properties compared to normal wood. The g-layer was initially reported as composed of almost pure cellulose formed into macrofibrils (Noberg and Meier 1966). However, more recent analysis suggest that these early chemical findings may have overestimated the cellulose content, and there are now reports of other minor components including pectins (Furuya et al. 1970), hemicelluloses (Arend, 2008; Nishikubo et al. 2007), and lignin (Joseleau et al. 2004).

The diameter of the cellulose crystals found in the g-layer of poplar has been reported as approximately four times greater than that found in the rest of the secondary cell wall, either by large microfibrils or by microfibril aggregation (in to macrofibrils) (Müller et al. 2006; Daniel et al. 2006). The size and angle of the cellulose structures found in the g-layer, including interaction with these newly reported minor components, may all contribute in explaining the g-layer's physical properties.

Of these newly suggested minor components, the hemicellulose xyloglucan is of particular interest. Xyloglucan has an intrinsic affinity for cellulose, and together with the enzyme xyloglucan endotransglucosylase (XET), it could help explain how tension is transmitted from the g-layer to the rest of the cell wall, as discussed by Mellerowicz et al. (2008).

In this study we show experimental evidence for the presence of xyloglucan together with its micro-distribution in developing poplar cells, specifically targeting tension wood fibers. To complement earlier findings, we have combined results from fluorescence microscopy with scanning- and transmission electron microscopy, using the CCRC-M1 antibody together with the novel carbohydrate binding module (CBM) FXG-14b, both specific for fucosylated xyloglucan (Lehtiö et al. 2003; Gunnarsson et al. 2006; von Schantz et al. 2009).

MATERIALS AND METHODS

Plant Material

Ten aspen plants (*Populus tremula x tremuloides*) were grown for 6 months in a climate chamber with a 16-h photoperiod. Seedlings grown *in vitro* in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) were transferred after one month to pots with autoclaved flower soil (Blomjord, Econova Garden AB, Box 90, 616 21 Åby, Sweden). During the light period, the temperature was held at 22 °C, with a relative humidity of at least 70%. During the night cycle the temperature was decreased to 17 °C with 70% relative humidity. Artificial light was provided by Osram Warm White 840 and Osram Fluorlight 77 fluorescent tubes (Osram AB, Box 504, 136 25 Haninge, Sweden). Plants were watered every second day with tap water and treated every second week with a 1:500 solution of liquid Blåkorn (Bayer AB, 245 42 Staffanstorp, Sweden). After four months growth, 5 plants were selected randomly and inclined at a 30° angle for 2 months to induce tension wood formation. After 6 months growth, the 11th internode was collected from all plants and prepared for further analysis.

Isolation of the G-layer

The g-layer was isolated according to Norberg and Meier (1966). Fresh wood samples with pronounced tension wood were placed in 96% ethanol overnight. The next day, 20 µm sections were cut from the tension wood area and placed in 96% ethanol for 2 h to loosen the g-layer from the outer secondary cell wall. After treating sections with 5 min of ultrasonic vibrations (Branson 220), the g-layer was filtered through a 35-mesh nylon cloth. The purity of the final sample was checked by light microscopy and double staining with Safranin and Astra Blue (Vazquez-Cooz and Meyer 2002).

Production and Purification of CBM FXG-14b

Recombinant FXG-14b was produced, as previously described (Cicortas Gunnarsson et al. 2004). Briefly, CBM carrying a hexa-histidine tag was produced in *Escherichia coli* BL21(DE3) (Novagen, Madison, WI, USA). Harvested bacterial pellets were washed before being resuspended in phosphate buffer (20mM NaH₂PO₄, pH 7.4). Cytoplasmic proteins were released by sonication (3 × 2 min, 50% amplitude (250-D sonifier; Branson Ultrasonics Corp., Danbury, CT, USA)) and separated from cell debris by centrifugation (13000 g, 15 min). Purification of FXG-14b was performed using chromatography on Ni-NTA agarose (Qiagen GmbH, Hilden, Germany). Eluted purified FXG-14b was dialysed against phosphate buffer. The final protein concentration was determined spectrophotometrically using an extinction coefficient calculated based on the sequence of the protein (Gill and von Hippel 1989).

Embedding

Fresh wood samples were cut radially into eight equal sections (10 mm long, 2 mm radius and 2 mm sectors), and fixed with 3% v/v glutaraldehyde containing 2% v/v paraformaldehyde in cacodylate buffer (0.1 M, pH 7.2) for 4 h at room temperature. After fixation, the samples were washed 20 min × 2 with distilled water. The samples were then divided into three equal parts. One part was embedded in Technovit 8100 (Kulzer, Wehrheim, Germany) after having been dried with an ethanol series (see below). Of the remaining two parts, one part was post-fixed with osmium tetroxide (2% w/v cacodylate solution) for 2 h at room temperature. Thereafter both parts were washed 30 min × 3 in distilled water, dried with a ethanol series and embedded in LR White (London Resin, Basingstoke, UK). The ethanol series used for all dehydration series consisted of 20 min steps of 20, 40, 60, 70, 80, 90, 95, and 99.5% ethanol.

Light Microscopy

Fluorescence microscopy labeling was carried out on extracted g-layer, fresh (fixed) and Technovit 8100 embedded semi-thin sections. Labeling was done with the antibody CCRC-M1 (CarbonSource Services, Georgia, US) and the CBM FXG-14b. Fresh material was sectioned (approximately 10 μm) using a Leitz sledge microtome. Technovit embedded material was sectioned at 4 μm on a Microm microtome (HM 350, Microm, Germany). Fluorescence microscopy was conducted using a wide-field Leica DMRE fluorescence microscope fitted with a mercury lamp and I3-513808 filter-cube (Leica, excitation 450-490 nm, emission >515 nm) (Leica Microsystems, Wetzlar, Germany). Extracted g-layer and sections were blocked in 5% w/v bovine serum albumin (BSA)(Sigma) in PBS solution (pH 7.4) for 1 h.

Antibody labeling was performed using a 1:10 solution of CCRC-M1 in PBS (pH 7.4) supplemented with 1% w/v BSA overnight at 4 °C (Puhlmann et al. 1994). After labeling, slides were washed 15 min × 4 with distilled water and the primary antibody detected with 1:500 solution of FITC conjugated (goat) anti-mouse IgG (Sigma) in PBS (pH 7.4) for 1 h at room temperature.

CBM labeling was performed using a 1:200 solution of HIS-tagged FXG-14b in PBS (pH 7.4) overnight at 4 °C. After CBM labeling, sections were washed 15 min × 4 with PBS and labeled with a 1:100 solution of the monoclonal mouse Anti-His antibody

(Amersham Biosciences) in PBS (pH 7.4) for 1 h at room temperature. Thereafter, samples were washed 15 min \times 4 with PBS and the primary antibody detected with FITC-conjugated (goat) anti-mouse IgG (Sigma) as described above.

Finally, all slides were washed 15 min with PBS (pH 7.4) and 15 min with distilled water. Control samples were labeled in parallel with omission of the primary labeling stage.

Scanning Electron Microscopy (SEM)

Small cubes (3-mm sides) of fresh wood, containing cambium, were cut and washed 20 min \times 2 with distilled water. Samples were then blocked with 5% w/v solution of BSA in PBS (pH 7.2) for 1 h at room temperature. After blocking, samples were washed 15 min \times 3 with PBS buffer (pH 7.2), and subsequently labeled with either CCRC-M1 or CBM FXG-14b as described above.

Antibody labeling was performed using a 1:100 solution of CCRC-M1 in PBS (pH 7.2) supplemented with 1% w/v BSA overnight at 4 °C. After washing 20 min \times 2 with distilled water, the primary antibody was detected with gold-conjugated anti-mouse IgG (10 nm Au, Sigma-Aldrich) in Tris buffer (pH 8.2) for 1 h at room temperature. After detection, samples were washed 20 min \times 2 with Tris buffer and 20 min \times 2 with distilled water.

CBM labeling was performed with a 1:10 solution of HIS-tagged FXG-14b in PBS (pH 7.2) supplemented with 1% w/v BSA overnight at 4 °C. Samples were washed 15 min \times 4 with PBS and labeled with a 1:100 solution of monoclonal mouse Anti-His antibody (Amersham Biosciences) in PBS (pH 7.2) for 1 h at room temperature. Thereafter, samples were washed 15 min \times 4 with PBS and the primary antibody detected with a 1:200 solution of gold-conjugated (goat) anti-mouse IgG (10 nm Au, Sigma-Aldrich) in Tris buffer (pH 8.2) for 1 h at room temperature.

After washing 10 min \times 2 with PBS and 10 min \times 2 with distilled water, both antibody- and CBM gold labeled samples were enhanced with silver (Amersham Biosciences, AuroProbe LM and IntenseSEM), applied for 6 min according to the manufacturer's instructions. After enhancement, samples were critical-point dried using a Polaron E5000 CPD apparatus with acetone as the substitution fluid. Samples were then mounted on SEM stubs and coated with platinum and gold using an Agar High Resolution Coater. Observations were made using a Hitachi FE-SEM operated at 20 kV. Control samples were labeled in parallel, with the exclusion of the primary labeling protein.

Transmission Electron Microscopy (TEM)

For transmission electron microscopy (TEM), silver and gold ultrathin sections were cut from LR White embedded samples using a Reichert-Jung Ultracut E ultramicrotome and collected on 100 and 200 mesh nickel grids. Prior to labeling, sections were etched for 1 min by floating on drops of 10% H₂O₂ placed on parafilm, followed by washing 20 min \times 2 with PBS (pH 7.2).

Antibody labeling was performed with a 1:10 solution of CCRC-M1 in PBS (pH 7.2) supplemented by 1% w/v BSA overnight at 4 °C. Samples were subsequently washed 15 min \times 4 with PBS (pH 7.2) and the primary antibody detected with a 1:200

solution of gold-conjugated anti-mouse IgG (10 nm Au, Sigma-Aldrich) in Tris buffer (pH 8.2) for 1 h at room temperature.

CBM labeling was performed with a 1:10 solution of HIS-tagged FXG-14b in PBS (pH 7.2) for 4 h at room temperature. Samples were then washed 15 min \times 4 with PBS and labeled with a 1:200 solution of the monoclonal mouse Anti-His antibody (Amersham Biosciences) in PBS (pH 7.2) for 1 h at room temperature. Thereafter, the samples were washed 15 min \times 4 with PBS and the primary antibody detected with a 1:100 solution of gold-conjugated (goat) anti-mouse IgG (10 nm Au, Sigma-Aldrich) in Tris buffer (pH 8.2) for 1 h at room temperature.

After labeling, all grids were washed 15 min \times 4 with Tris buffer (pH 8.2) and 5 min \times 2 with distilled water and air dried. Sections were observed using a Philips CM/12 transmission electron microscope (TEM) operated at 80 kV. Control samples were labeled in parallel, with the exclusion of the primary labeling protein.

RESULTS AND DISCUSSION

By combining labeling results from both the CCRC-M1 antibody with the novel CBM FXG-14b, we were able to show xyloglucan labeling throughout the secondary cell wall, including the g-layer. This is in agreement with recent chemical analysis that has supported the possibility of xyloglucan being a major component in the g-layer (Nishikubo et al. 2007) challenging the long standing theory that the g-layer is almost exclusively composed of pure crystalline cellulose (Norberg and Meier 1966).

The presence of xyloglucan has previously been shown in the primary cell wall and compound middle lamellae of dicotyledons using immunogold labeling (Baba et al. 1994; Bourquin et al. 2002; Nishikubo et al. 2007; Bowling et al. 2008). In addition, Nishikubo et al. (2007) also presented some indications of xyloglucan's presence in developing g-layers in poplar using immunofluorescence microscopy.

Nishikubo et al. (2007) postulated from chemical sugar analysis that xyloglucan is a major component in the g-layer, up to 10-15% (based on extracted g-layer). If this is indeed the case and unless it is masked, altered or removed, the expectation is that the g-layer should show distinct labeling of xyloglucan both in-situ and extracted.

Using fluorescence microscopy with both antibody CCRC-M1 and CBM FXG-14b on fresh sections with fibers showing g-layer formation, we were able to record labeling of the inner developing g-layer and primary cell wall of fibers, vessels and rays, as shown in Fig. 1. While we did not observe as pronounced labeling of the g-layer as reported by Nishikubo et al. (2007), there was good agreement between the two probes, with similar staining patterns.

However, labeling of extracted g-layers was completely negative using either the antibody or CBM (data not shown). This raises questions about how xyloglucan is incorporated into the g-layer and whether it is lost or masked during isolation. As reported by Fry (1988, 1989), xyloglucan is chemically stable, but can be extracted using strong base or precipitated using ethanol above 50%. This may suggest that xyloglucan found in the g-layer is either loosely incorporated or that only free xyloglucan is available for labeling.

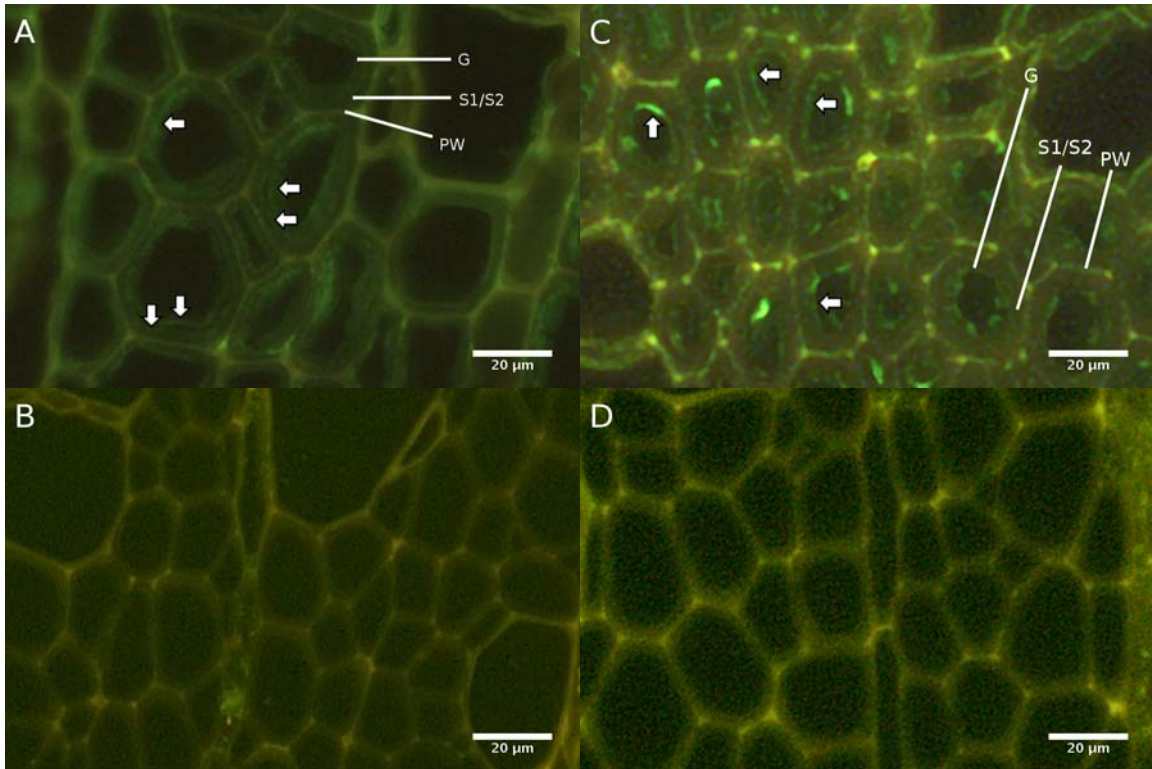


Fig. 1. Immunofluorescence labeling with CCRC-M1 (A and B (control)) and CBM FXG-14b (C and D (control)). Arrows show sites of signal. Fluorescence was mainly found in the primary wall, coinciding with the main autofluorescence signal from lignin, or in the g-layer, particularly at the interface with the S2 layer and on the lumen wall. The g-layer is not apparent in the controls because of lack of fluorescence. Abbreviations: PW (primary cell wall), S1, S2 (secondary cell wall layers), G (g-layer).

As previously reported for CCRC-M1, immunogold labeling of xyloglucan is most abundant in the primary cell wall, with labeling increasing as the fibers reach maturity. To the extent we have been able to replicate these findings, this also holds true for FXG-14b. In fusiform fiber cells, labeling was mainly found in vesicles and occasionally incorporated into the forming cell wall, as shown in Figs. 2A-2B.

In mature cells CCRC-M1 labeling was mainly found in the primary cell wall, consistent with previous reports, as shown in Figs. 2C-2D. In contrast, labeling with FXG-14b was recorded not only on the primary cell wall but also on the developing g-layer, as shown in Fig. 2E. FXG-14b labeling was most intense in the primary cell wall, followed by the developing zone in the g-layer, and only occasionally recorded in the secondary S1 and S2 layers.

In general, we found the labeling patterns to be slightly inconsistent between fluorescence and TEM. There were also indications that sample processing (i.e. fixation, resin embedding) may affect labeling. In order to obtain higher resolution with reduced processing, we adopted silver enhancement of immunogold labeled poplar wood samples for SEM.

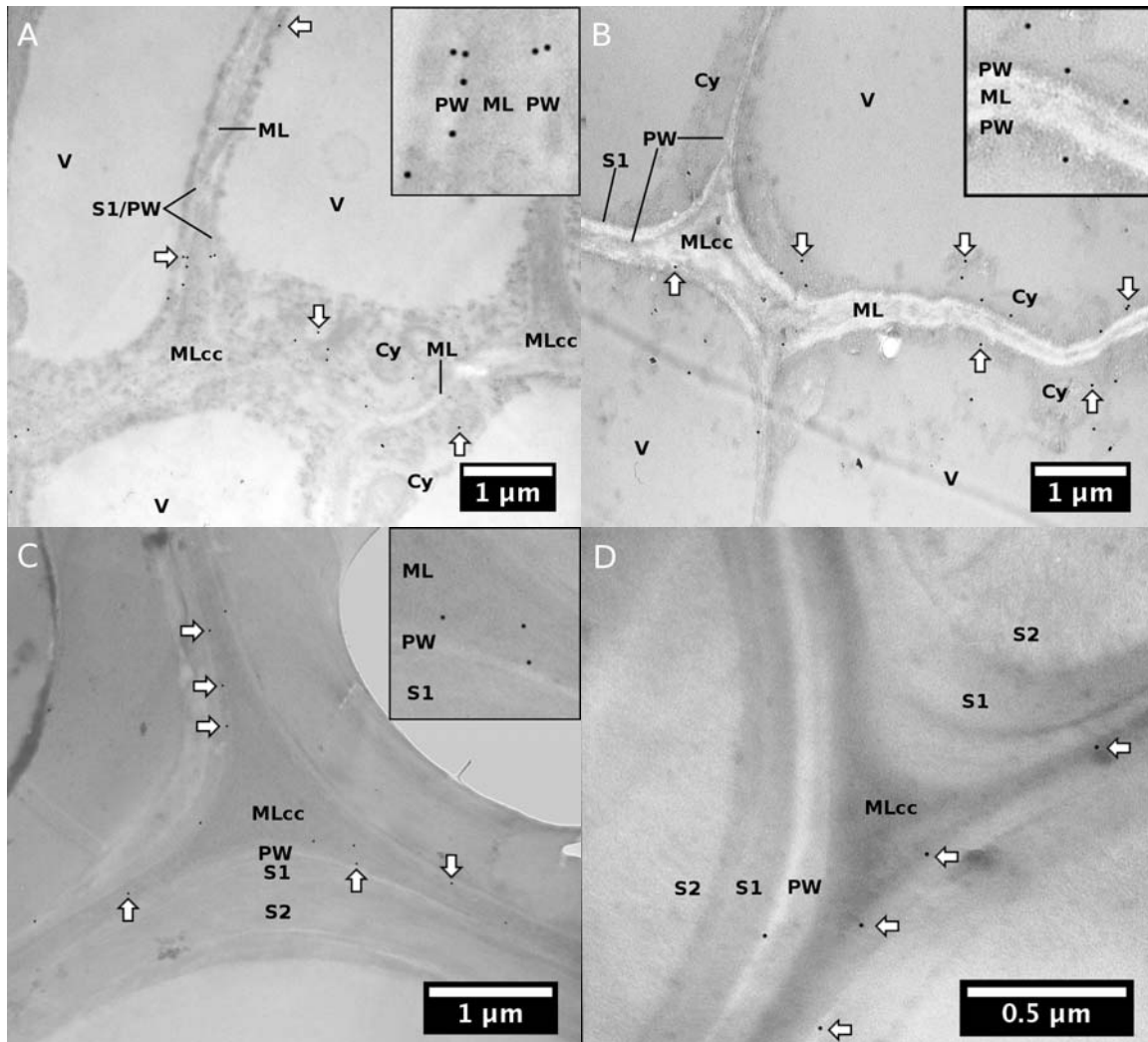


Fig. 2. Labeling of fusiform initial cells with CCRC-M1 (A) and CBM FXG-14b (B). Labeling for xyloglucan was mainly found in cytoplasmic vesicles (arrows) with sporadic labeling of the outer fiber cell wall. In mature normal wood cells labeling was mainly found on the primary cell wall with CCRC-M1 (C) and CBM FXG-14b (D) (arrows). Insets in A, B and C show higher magnifications of labeled areas. Note, the images reflect observations on a minimal of 30 sections per label type on representative developing fiber cells. Abbreviations: ML (middle lamella), MLcc (middle lamella cell corner), PW (primary cell wall), S1, S2 (secondary cell wall layers), Cy (cytoplasm) and V (vacuole).

The overall labeling pattern in SEM was consistent with both fluorescence and TEM. Because of the lack of resin embedding, labeling was much more intense than in TEM with a declining trend from the primary cell wall to the S2 or g-layer, as shown in Figures 3A-3F. In particular, strong labeling was recorded in the interface between the S2 and g-layer and minor labeling on the inner wall of the g-layer.

The strong signal found on the interface between the g-layer and the S2 layer (Figures 3E-F) supports the hypothesis of Mellerowicz et al. (2008). They postulated that xyloglucan functions as a bridge between the g-layer and the rest of the secondary cell wall; this hypothesis should prove interesting for future study.

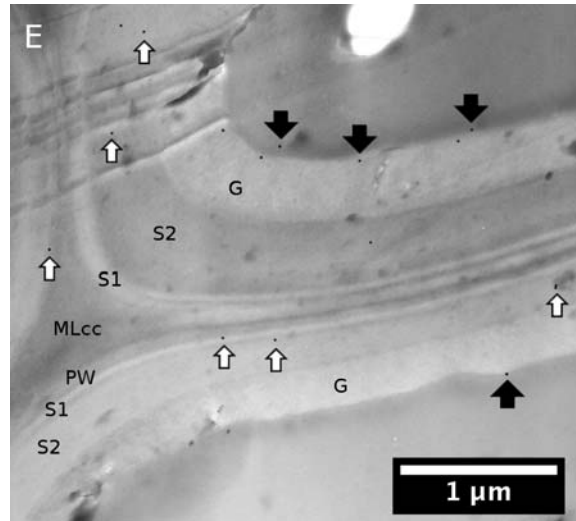


Fig. 2E. Labeling of developing or almost mature g-layer with CBM FXG-14b. Labeling was found throughout the secondary cell wall (white arrows). Note labeling along the developing g-layer (black arrows). The image reflects observations on a minimal of 30 sections on representative developing tension wood fiber cells. Abbreviations: G (g-layer), S1 and S2 (secondary cell wall layers), MLcc (middle lamella cell corner).

Minimal labeling was detected on the inside of the g-layer in SEM samples as exemplified in Figure 4. This may indicate that the labeling found on the inside of the g-layer in fluorescence microscopy may be an optical effect caused by the increased surface area along the thickness of the section. Alternatively, it may also indicate that the xyloglucan found here is easily extractable and removed during processing.

Table 1. Summary of Relative Labeling Intensities for Fucosylated Xyloglucan

Cell Wall Region	CCRC-M1			FXG-14b		
	F.	SEM	TEM	F.	SEM	TEM
Primary wall	+	++++	++	+	+++	++
S1 (S1/S2 interface)	-	++	+	-	++	+
S2 (S2/g-layer interface)	-	++	-	-	++	-
Lumen wall	+	-	-	+	+	+
Isolated g-layer	-			-		

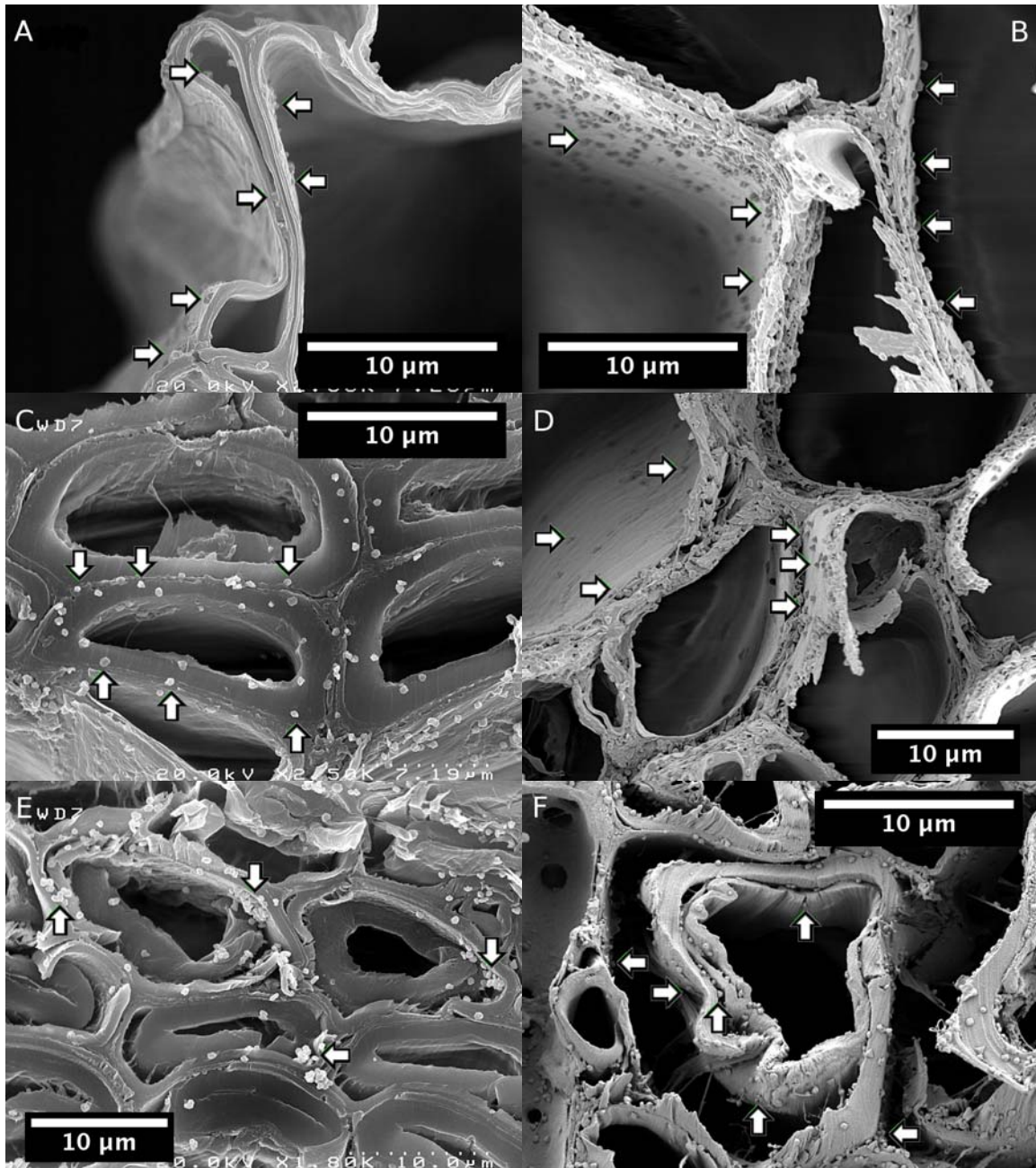


Fig. 3. Labeling of developing primary wall/S1 layer with CBM FXG-14b (A) and CCRC-M1 (B). Strong labeling was recorded throughout the wall at this early stage of development (arrows). Less labeling was detected on the developing S2 layer than the primary wall with CBM FXG-14b (C) and CCRC-M1 (D) (arrows). The developing g-layer showed the least amount of labeling with CBM FXG-14b (E) and CCRC-M1 (F), with the exception of the interface between the S2 and g-layer (arrows), where labeling was often abundant. Note, the images reflect observations on a minimal of 5 wood blocks per label type.

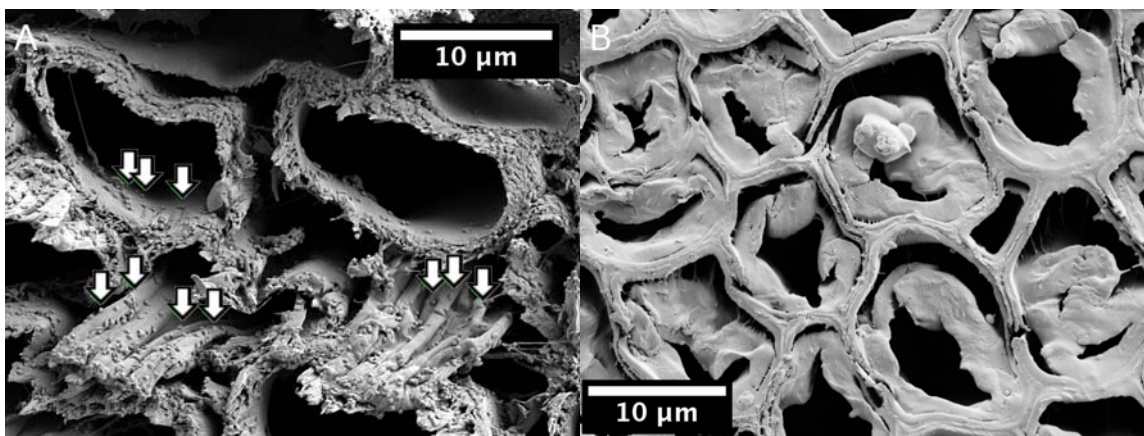


Fig. 4. Intense labeling with CCRC-M1 of g-layer drawn out during sectioning of the poplar blocks (A) (arrows). Control sample with omission of the primary antibody/CBM (B).

CONCLUSIONS

1. Using two complementary antibody and CBM probes, we have shown evidence for the presence of xyloglucan in the g-layer of tension wood in poplar.
2. Labeling of xyloglucan was absent with extracted g-layer, indicating a loss of available xyloglucan for labeling, either through masking or extraction.
3. The main concentration of xyloglucan was found in the primary cell wall, with a declining concentration of xyloglucan through the developing secondary wall layers.
4. One exception to the declining trend of xyloglucan was a higher concentration at the interface between the S2 and the g-layer, supporting the hypothesis that xyloglucan acts as an anchor for the g-layer to the inner S2 layer and rest of the secondary cell wall.

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