

Localization of Xyloglucan Epitopes in the Gelatinous Layer of Developing and Mature Gelatinous Fibers of European Aspen (*Populus tremula* L.) Tension Wood

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There is controversy concerning the presence of xyloglucans in gelatinous (G) layers of *Populus* spp. tension wood, particularly in mature G-fibers. Transmission electron microscopy (TEM) immunogold localization combined with LM15 antibody (recognizes XXXG-motif of xyloglucans, heptasaccharide) was used to investigate the distribution of xyloglucan epitopes in both transverse and radial sections of *P. tremula* tension wood. Results provided clear evidence for the presence of xyloglucans in both mature and developing G-layers. Developmental decrease of LM15 epitope localization in G-layers was also detected during G-fiber maturation. High magnification TEM observations showed specific localization of LM15 epitopes on newly synthesized cellulose microfibrils present in the innermost layer of developing G-layers adjacent to the cell lumen, suggesting linkage between xyloglucans and cellulose microfibrils. Possible mechanisms were discussed for developmental changes of xyloglucan with respect to the different results reported in the literature.

Keywords: Gelatinous layer; Immunolocalization; LM15 antibody; *Populus tremula*; Tension wood; Xyloglucan

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INTRODUCTION

Gelatinous (G) fibers are a unique cell type found in tension woods that are formed in leaning stems and branches of hardwoods (Gardiner *et al.* 2014; Donaldson and Singh 2016). Although many hardwood species have no G-fibers in tension wood, G-fiber formation is generally thought of as a prominent feature of tension woods (Fisher and Stevenson 1981; Clair *et al.* 2006; Gardiner *et al.* 2014; Donaldson and Singh 2016). G-fibers are characterized by the formation of a G-layer adjacent to the cell lumen (Fisher and Stevenson 1981) and are typically classified into three types, S₁ + G, S₁ + S₂ + G, and S₁ + S₂ + S₃ + G (Wardrop and Dadswell 1955; Saiki and Ono 1971; Ghislain and Clair 2017). In *Populus tremula* L., the G-layer replaces part of the S₂ layer and the entire S₃ layer (Kim and Daniel 2012).

Compared to normal fibers, G-fibers are well known to contain more cellulose, but less lignin and xylans (Pilate *et al.* 2004; Gorshkova *et al.* 2010) because the G-layers are composed predominantly of cellulose. Tension wood is known to show much greater tensile stress than normal woods, which is thought developed at the tissue and fiber levels through changes in G-layer swelling (*i.e.* lateral pressure on surrounding secondary cell walls) and/or G-layer shrinkage (*i.e. via* changes in molecular interactions between cellulose and matrix polysaccharides) (reviewed by Donaldson and Singh 2016). With

advances in understanding G-layer chemistry, it has been proposed that non-cellulosic polysaccharides of the G-layer are involved in tensile stress generation in G-fibers (Mellerowicz and Gorshkova 2012). In particular, xyloglucans have frequently been implicated in the generation of tensile stress in G-fibers (Nishikubo *et al.* 2007; Mellerowicz *et al.* 2008; Baba *et al.* 2009; Kaku *et al.* 2009; Mellerowicz and Gorshkova 2012). However, the distribution and amount of xyloglucans have been reported to vary greatly between studies, depending on the methods of analysis including chemical analysis, immunofluorescence, immunogold-silver staining, and scanning electron microscopy or transmission electron microscopy (SEM, TEM) (Nishikubo *et al.* 2007; Bowling and Vaughn 2008; Mellerowicz *et al.* 2008; Baba *et al.* 2009; Sandquist *et al.* 2010; Gorshkova *et al.* 2015; Guedes *et al.* 2017). For example, Nishikubo (2007) reported the presence of xyloglucans in *P. alba*, *P. tremula*, and hybrid aspen (*P. tremula* × *P. tremuoides*) using chemical analysis (showing 10% to 15% of the total dry weight) and immunocytochemistry. In contrast, Guedes *et al.* (2017) reported no evidence of the presence of xyloglucans in hybrid poplar (*P. tremula* × *P. alba*) using an immunocytochemical study combined with various antibodies. Alméras and Clair (2016) also suggested that xyloglucan-mediated tensile stress generation in G-layers can be rejected because xyloglucans are not actually localized in G-layers. Therefore, an in-depth immunocytochemical study of xyloglucans in G-layers (*i.e.*, early to mature stages of G-layer formation) is necessary to obtain a more complete understanding of xyloglucan distribution in G-layers and thereby help understand its function in the generation of tensile stress in G-layers.

In this study, the authors report the spatial microdistribution of LM15 epitopes (recognizing XXXG motifs of xyloglucans, heptasaccharide) in G-layers of *P. tremula* during G-fiber formation using TEM immunogold labeling. To evaluate the possibility of differences in antibody accessibility depending on fiber wall orientation, both transverse (cross)- and radial (longitudinal) sections were observed. This study was primarily designed to improve the understanding of inconsistency with observations of xyloglucan distribution in G-layers of *Populus* spp.

EXPERIMENTAL

Plant Materials, Fixation, and Embedding

Wood discs were taken from a stem of 10-year-old leaning poplar tree (*Populus tremula* L.) grown in the field (Uppsala, Sweden) on August 16 (Kim and Daniel 2012). A tree was naturally inclined to *ca.* 40° from the vertical and was *ca.* 7.1 cm in diameter (4.3 cm: 2.3 cm = TW: opposite wood). Small sectors were cut from the tension wood and fixed within a mixture of 2% v/v paraformaldehyde and 2.5% v/v glutaraldehyde in a 0.05 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature (Kim and Daniel 2012). After dehydration through a graded ethanol series, sectors were embedded in LR White resin (London Resin Company Ltd., Basingstoke, UK) as previously described (Kim and Daniel 2012). All chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted.

Methods

TEM and immunocytochemistry analysis

For TEM immunogold labeling, transverse- and radial ultrathin sections (*ca.* 90 nm) mounted on nickel grids were incubated with the LM15 antibody (Marcus *et al.* 2008) (PlantProbes, Leeds, UK), followed by incubation with anti-rat secondary antibody labeled with 10-nm colloidal gold particles (BBInternational, Cardiff, UK) (Kim and Daniel 2012). Grids were examined with a Philips CM12 TEM (Philips, Eindhoven, Netherlands) after post-staining with 4% w/v aqueous uranyl acetate. Negative TEM films were scanned using an Epson Perfection Pro 750 film scanner (Epson, Nagoya, Japan). Images were contrasted using Adobe Photoshop CS6 (version 13; San Jose, CA, USA). Results reflect observations on two transverse and two radial sections prepared from four different embedded blocks. For observations of tension wood (TW) formation, some semi-thin resin sections (*ca.* 1 μ m) were stained with 1% w/v toluidine blue (in 0.1% boric acid) and examined using a Leica DMLB light microscope (Leica, Wetzlar, Germany) equipped with an Infinity X-32 digital camera (Deltapix, Samourn, Denmark).

RESULTS AND DISCUSSION

Using immunolocalization methods, this study provides information on the distribution of LM15 xyloglucan epitopes in poplar TW fiber cell walls, with particular focus on G-layers. For a clearer comparison of results from other studies performed on other *Populus* spp., there are certain aspects that need to be considered in the following results and discussion. First, the TW samples studied were collected near the end of a growing season, *i.e.* TW fibers studied contain G-fibers from both latewood (1 to 2 cells from cambium) and earlywood. Second, the wood samples studied are collected from an outdoor-grown juvenile poplar tree that was naturally leaning for several years. Third, mature TW fibers in the study represent fully developed G-fibers (*i.e.* the absence of cytoplasm) within a current growth ring, *i.e.* fibers developed from previous growth rings were not studied.

Numbers marked in cells (*e.g.* GF-1 and GF-2 in Fig. 2) indicate approximate locations of G-fibers from cambium, which was identified on radial sections using TEM observations. Higher number in images indicates greater maturity of G-fibers. No contribution of non-specific background labeling was detected in control sections with the primary antibody omitted (not shown).

No noticeable difference was detected in the localization of LM15 epitopes between the transverse- and radial orientations for either developmental or mature stages of G-layers. This result reduces the possibility of differences in antibody accessibility in G-layers depending on fiber wall orientation (*i.e.*, cross vs. longitudinal).

G-layer formation was observed in G-fibers across developing and mature xylem (Fig. 1a), in which a part of S₂ including the entire S₃ layer was replaced by the G-layer (arrowheads, inset in Fig. 1a). Epitopes were first detected in G-layers at early stages of formation (Figs. 1b and 2a). During G-layer development, xyloglucan epitopes were found randomly across G-layers (Figs. 1c, d and 2b, c) but gradually decreased (Fig. 3).

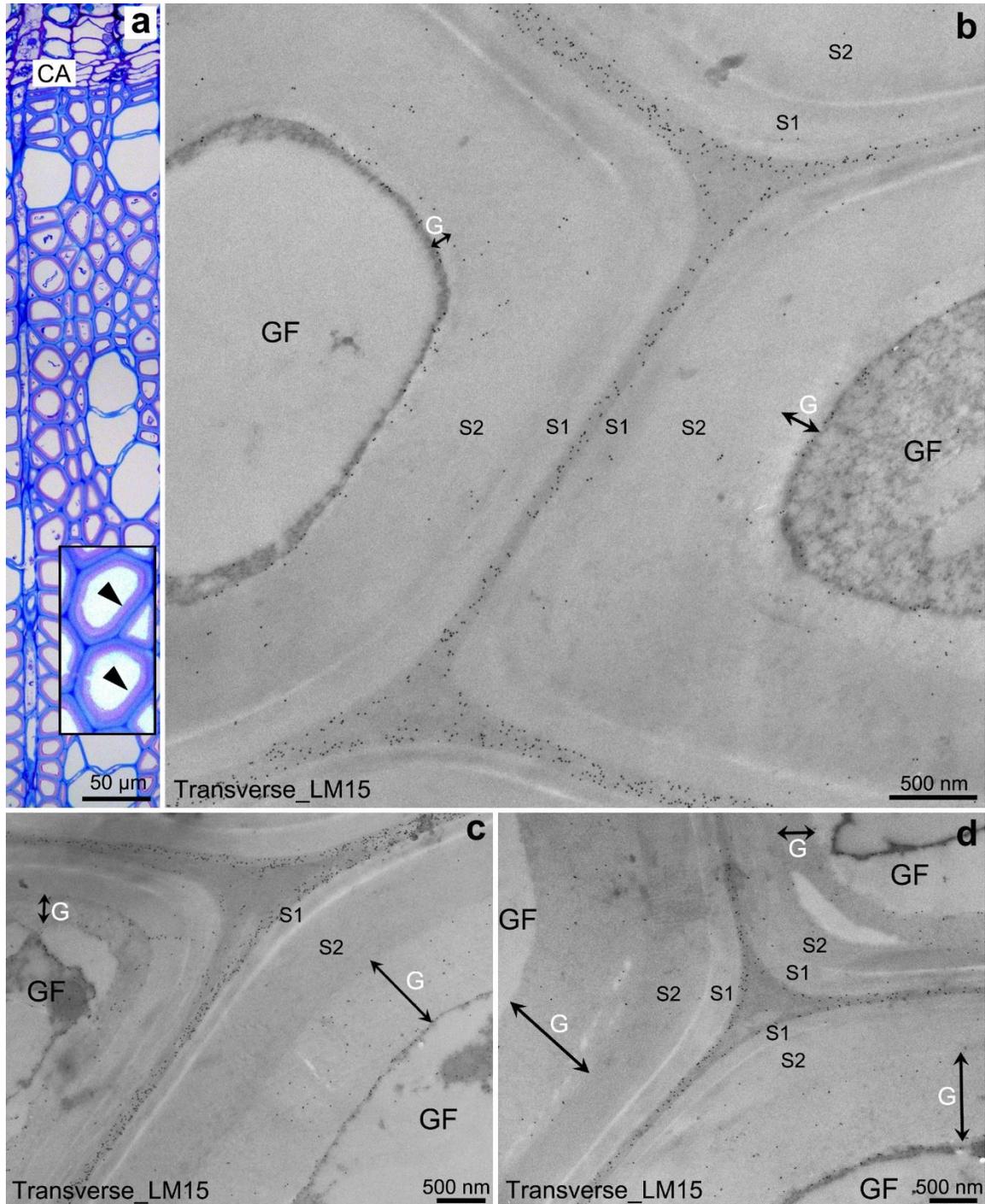


Fig. 1. Immunogold localization of LM15 epitopes on transverse sections of developing and mature G-fibers: a) *P. tremula* tension wood showing the formation of G-layers stained pink with toluidine blue. Arrowheads in inset indicate G-layer formation in mature G-fibers; b) distribution of epitopes in G-layers (G, double headed arrows) at early stages of G-layer formation. Note: epitope distribution in S₁ and S₂ layers of G-fibers (GF); c) and d) Distribution of epitopes in G-layer (G) at advanced stages of G-layer formation development. Note: epitope distribution in the boundary between the G-layer and the cytoplasm (or innermost G-layer), CA= cambium cell

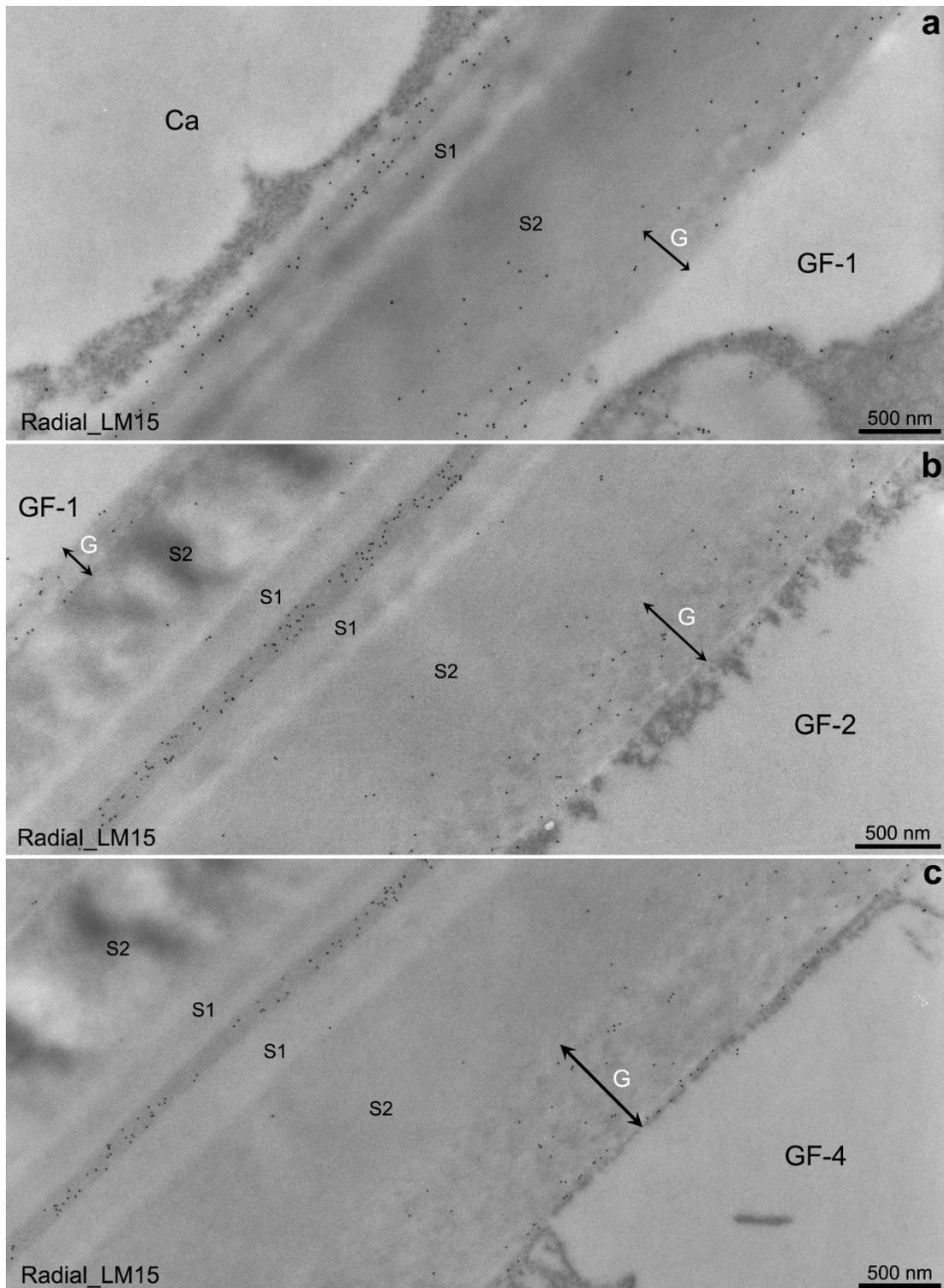


Fig. 2. Immunogold localization of LM15 epitopes on radial sections of early stages of G-fiber formation: a) through c) each image indicates first (GF-1), second (GF-2), and fourth (GF-4) G-fibers from cambium cells (Ca), respectively (see Fig. 1a). The LM15 epitopes were detected across developing G-layers (G, double headed arrows). Note: epitope distribution in S₁ and S₂ layers of G-fibers (a, b) and in the boundary between the G-layer and the cytoplasm (c).

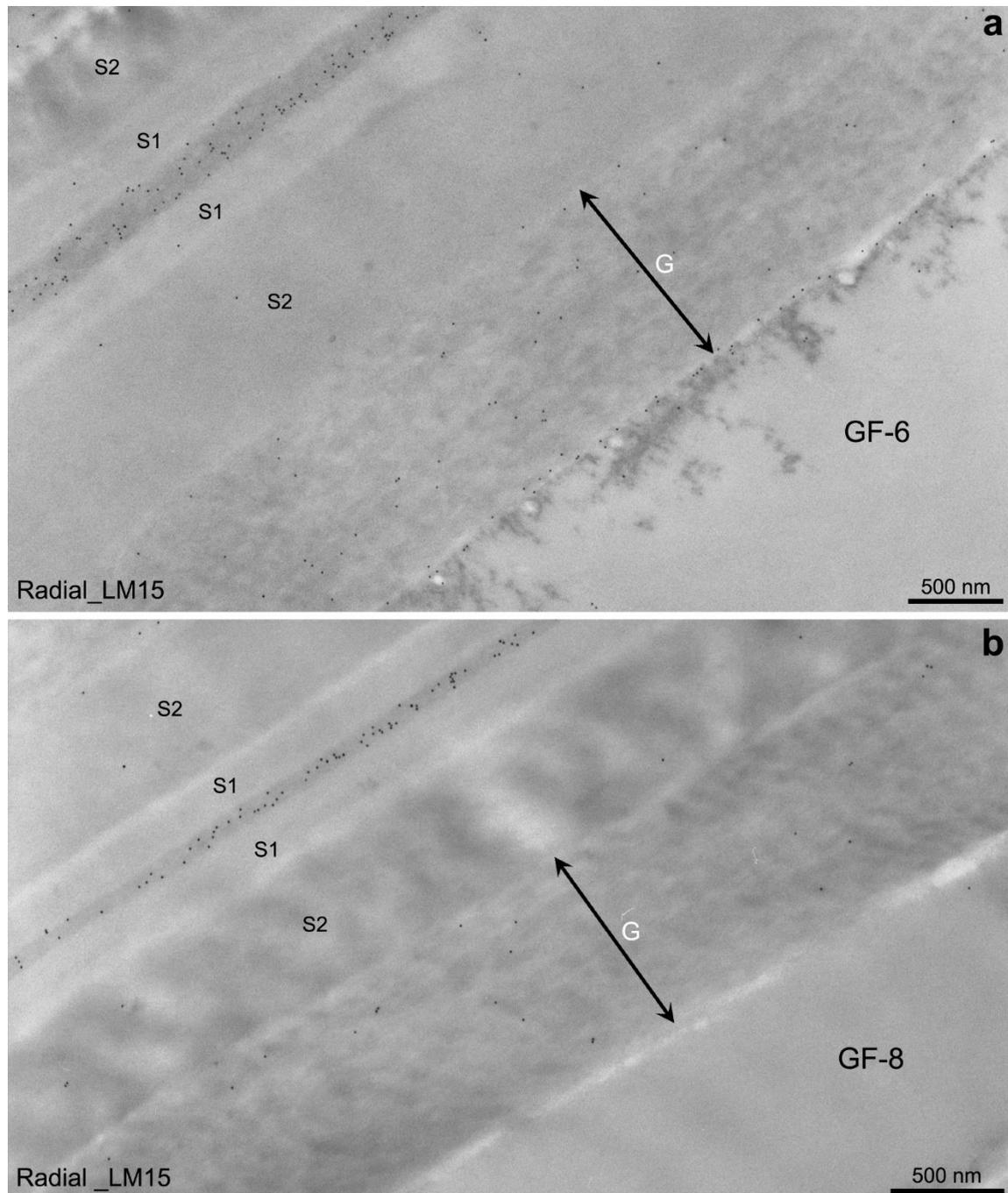


Fig. 3. Immunogold localization of LM15 epitopes on radial sections of late stages of G-fiber formation: a) and b) each micrograph indicates sixth (GF-6) and eighth (GF-8) G-fibers from cambium cells, respectively (Fig. 1a). The amount of LM15 epitopes detected gradually decreased in G-layers (G, double headed arrows) at late stages of G-layer formation. Note: epitope distribution in the boundary between the G-layer and the cytoplasm (a)

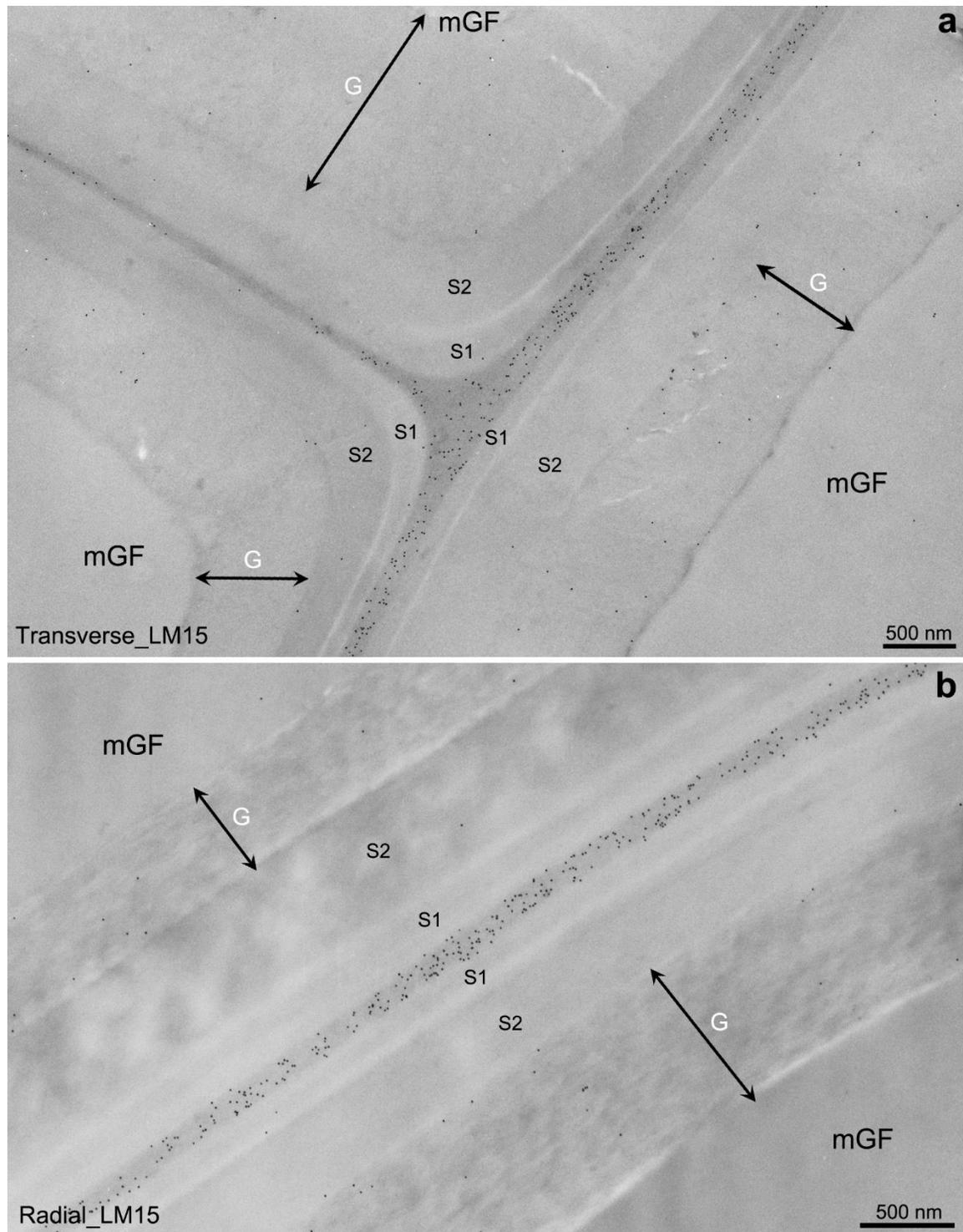


Fig. 4. Immunogold localization of LM15 epitopes in mature G-fibers (mGF): a) and b) transverse and radial sections, respectively. Epitopes were sparsely distributed in mature G-layers (G, double headed arrows) with considerable variation in amounts of epitopes labeled. Note: absence of epitope labeling in S₁ and S₂ layers of mature G-fibers

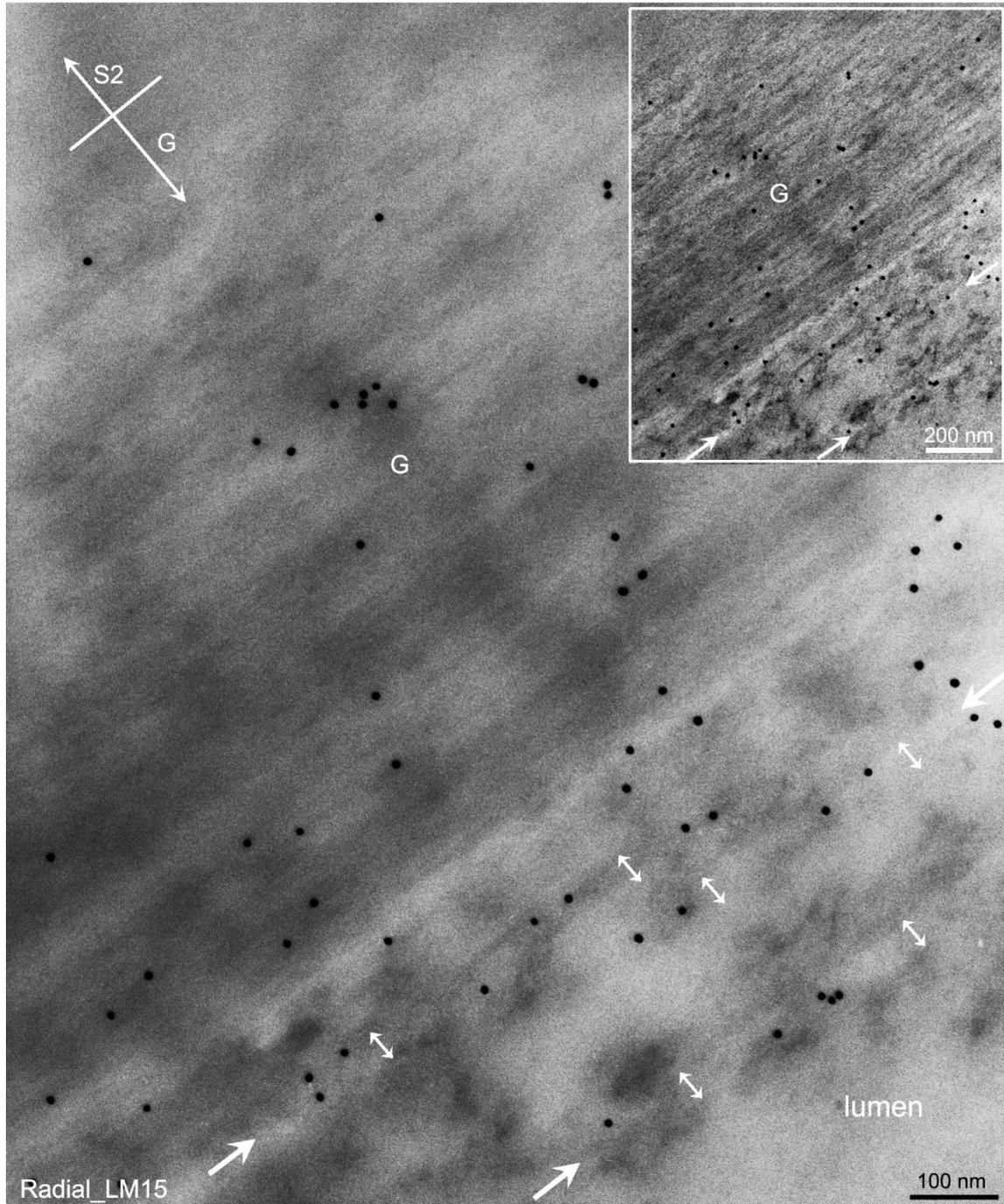


Fig. 5. High magnification (60,000 \times) image of LM15 epitope localization on radial section of a developing G-fiber (GF-6); epitopes specifically localized on the newly synthesized cellulose macrofibrils (arrows) of ca. 40 nm diameter (double headed arrows). Note: inset shows highly contrasted images using Photoshop.

These results were consistent with previous immunocytochemical studies of G-layers in *Populus* spp. including *P. tremula* (Nishikubo *et al.* 2007) and *P. alba* (Baba *et al.* 2009) by CCRC-M1 and hybrid aspen (*P. tremula* \times *P. tremuoides*) using CCRC-M1 and CBM FXG-14b (Sandquist *et al.* 2010). However, results differ from the study of hybrid poplar (*P. tremula* \times *P. alba*) (Guedes *et al.* 2017) that show an absence of

xyloglucan epitopes in developing G-layers labeled with different antibodies, including LM15 used in this study. With maturity of G-fibers, epitopes were sparsely distributed in G-layers with considerable variations observed between the G-fibers (Fig. 4). This result differs from previous studies that report an absence of xyloglucan epitopes in G-layers of mature G-fibers *P. tremula* (Nishikubo *et al.* 2007) and hybrid poplar (Guedes *et al.* 2017). Unlike previous results (Nishikubo *et al.* 2007; Sandquist *et al.* 2010), no specific (or abundant) epitope distribution was detected in the boundary between the S2- and G-layers during G-layer development (Figs. 1 through 4).

During G-layer formation, epitopes were readily detected in the cytoplasm, particularly at the boundary with G-layers (or the innermost layer of G-layers) (Figs. 1b to 1d, 2c, and 3a). At high magnification, these epitopes were specifically localized on newly synthesized cellulose macrofibrils (*i.e.*, microfibril aggregations) of *ca.* 40 nm diameter (Fig. 5). This result clearly illustrates the association between xyloglucans and cellulose macrofibrils (*i.e.*, surface binding) during G-layer formation. Baba *et al.* (2009) proposed that xyloglucans bound to cellulose macrofibrils allow cross-linking of newly synthesized cellulose macrofibrils to pre-existing macrofibrils during development of the G-layer.

Consequently, the results indicated that xyloglucans were a component in both developing and mature G-layers of *P. tremula*. Observations also demonstrated that there were developmental changes of xyloglucan epitopes in G-layers during G-fiber maturation, *i.e.*, gradual decrease of LM15 xyloglucan epitopes in G-layers. There are several possible explanations for these developmental changes. Considering the authors' results and the available literature, the following possibilities were considered:

1) Migration of xyloglucans that were deposited initially in G-layers to compound middle lamellae (CML) during G-fiber maturation and thereby xyloglucan was decreased in G-layers. In normal fibers of hybrid aspen, Bourquin *et al.* (2002) proposed that xyloglucan deposition in CML continued during formation of a secondary cell wall, *i.e.*, transportation of xyloglucans from the cytoplasm to CML *via* secondary cell walls. This study also showed specific localization of LM15 epitopes in S₁ and S₂ layers of G-fibers and its decrease during G-fiber formation (Figs. 1b through 1c, and 2). Yoshinaga *et al.* (2012) reported that lignification of the CML continues progressively during G-layer formation, *i.e.*, penetration of monolignols through the S₁ and S₂ layers during G-fiber formation is possible.

2) A decrease in antibody accessibility following xyloglucan endo-transglycosylase (XET) mediated xyloglucan rearrangement in G-layers during G-fiber maturation. Nishikubo *et al.* (2007) reported activity of XET in developing G-layers of several *Populus* spp. Because several different types of motifs (oligosaccharides) have been demonstrated in the structure of xyloglucans (Fry *et al.* 1993), it can be expected that xyloglucan rearrangement in G-layers followed by changes in the chemical structure of xyloglucans, which will be less labeled by LM15 (*i.e.*, it only recognizes XXXG motif xyloglucans, heptasaccharides). However, the 30 commercial antibodies specific to xyloglucans, including LM15, were recently tested by Guedes *et al.* (2017), but none of these antibodies labeled mature hybrid poplar G-layers.

3) Masking of epitopes following the entrapment of xyloglucans during cellulose aggregation in G-layers. Mellerowicz *et al.* (2008) proposed that xyloglucan may be entrapped inside nascent cellulose macrofibrils of the G-layer during maturation, thereby inducing longitudinal tensile stress within macrofibrils. This entrapment can possibly make xyloglucans inaccessible to antibodies (Mellerowicz *et al.* 2008).

Although there is controversy for such an entrapment model (Alméras and Clair 2016) and the presence of xyloglucans in G-layers of *Populus* spp. (Guedes *et al.* 2017), this study provided microscopy evidence that xyloglucans are a component of G-layers of *P. tremula* both in developing and mature G-fibers.

4) Finally, there is possibility of enzymatic degradation of xyloglucans in G-layers during G-fiber maturation. Enzymatic degradation of cell wall components including pectins, xyloglucans, and galactoglucomannans by activity of hydrolases in bordered pit membranes during maturation has been proposed in several tree species including *Populus* spp. (O'Brien and Thimann 1967; O'Brien 1970; Morrow and Dute 1998; Kim *et al.* 2011; Kim and Daniel 2013). The only difference from pit membranes is that xyloglucans are still present even in fully matured G-layers of *P. tremula*. No pectin, xyloglucan, and galactoglucomannan epitopes are detected in fully matured bordered pit membranes even though epitopes were present in developing stages (Kim *et al.* 2012; Kim and Daniel 2013).

CONCLUSIONS

1. The TEM immunolocalization of LM15 epitopes indicated that xyloglucans were a component of G-layers in both developing and mature G-fibers of *P. tremula* tension wood.
2. High resolution TEM images indicated that xyloglucans are probably linked to cellulose microfibrils during G-layer formation.
3. Results indicated a developmental decrease of LM15 epitopes in G-layers. At present, the mechanism of this decrease is unclear, and it could be a key factor to clarify whether xyloglucans are involved in the generation of tensile stress in G-layers of poplar tension wood.

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