Original article

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Xylan deposition and lignification in differentiating tension wood fibers in *Mallotus japonicus* (Euphorbiaceae) with multi-layered structure

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Abstract: Xylan deposition and lignification processes were examined in tension wood fibers with gelatinous layers (G-layers) in *Mallotus japonicus* (Euphorbiaceae). The cell walls consisted of a multi-layered structure of $S_1 + S_2 + G + n(L + G)$, where n indicates the number of repetitions ($n = 0-3$) and L indicates very thin lignified layers. The formation and lignification processes of the multi-layered structure of tension wood fibers were examined by light microscopy, ultraviolet microscopy, and transmission electron microscopy (TEM) following $KMnO_4$ staining. The deposition of xylan was examined by immunoelectron microscopy with a monoclonal antibody (LM11). Immunolabelling of xylan appeared in lignified cell wall layers, except in the compound middle lamella (CML), i.e., the S_1 , S_2 , and L layers but not the G-layers. The density of LM11 xylan immunogold labeling in S_2 layers increased during the formation of G-layers. This increase was due to the shrinkage of S_2 layers during development rather than intrusive deposition of xylan through G-layers. Lignification of the CML, S_1 , and S_2 layers proceeded during G-layer formation. The shrinkage of S_2 layers occurred almost simultaneously with the lignification of the S_2 layers during G-layer formation, suggesting that the S_2 layers shrank with lignification.

1 Introduction

Most angiosperm trees produce tension wood on the upper side of inclined stems (Onaka 1949; Robards 1966; Bamber 2001). In some species, tension wood contains wood fibers with gelatinous layers (G-layers) (Clair et al. 2006; Fisher and Stevenson 1981; Onaka 1949). The cell wall structure of tension wood fibers with the G-layer has been classified into three types, namely $S_1 + G$, $S_1 + S_2 + G$, and $S_1 + S_2 + S_3 + G$ (Saiki and Ono 1971; Wardrop and Dadswell 1955). In addition to the three types of G fibers, tension wood fibers with peculiar multi-layered structures have been found by Bailey and Kerr (1935) and Daniel and Nilsson (1996) in *Homalium* and *Olmediella*, in *Casearia javitensis* (Clair et al. 2006) and *Laetia procera* (Ruelle et al. 2007) in Salicaceae (former Flacourtiaceae). The occurrence of tension wood fibers with multi-layered structures in Salicaceae was reviewed in the paper by Ghislain et al. (2016). These trees produce tension wood fibers with G-layers composed of alternating thick and very thin layers. *C. javitensis* combines strong tension stress on the upper side with the presence of fibers with multi-layered secondary walls and very low stress in the opposite wood (Clair et al. 2006). In Euphorbiaceae, Encinas and Daniel (1997) found multi-layered G-layers in *Hevea brasiliensis*. Such a multi-layered structure was also reported in reaction phloem fibers in poplar (Nanko et al. 1982). Phloem fibers with a multi-layered structure were found in *Mallotus japonicus* in Euphorbiaceae and the number of layers was higher on the tension side compared with the opposite side (Nakagawa et al. 2012). This species produced tension wood fibers with a multi-layered structure, although the cell wall organization was different from phloem fibers; that is $S_1 + S_2 + G + n(L + G)$, where n indicates the number of repetitions and L indicates very thin lignified layers in the

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tension wood fibers and $S_1 + S_2 + n(G + L)$ in phloem fibers (Nakagawa et al. 2012). The role of the multi-layered structure in growth stress generation is not fully understood.

From the viewpoint of cell wall formation, walls with a multi-layered structure seem to be useful materials in considering the biosynthesis and deposition of hemicelluloses and lignin because different phases producing different cell wall components will be necessary to produce such a multi-layered structure. Xylan deposition and lignification in phloem fibers with a multi-layered structure were analyzed in *M. japonicus* (Nakagawa et al. 2014). It was found that xylan deposition occurred appositionally in lignified S_1 , S_2 , and L layers, but not in the compound middle lamella (CML) and G-layers. Lignification of the S_1 and S_2 layers continued during G-layer formation suggesting that lignin precursors might either penetrate into non-lignified G-layers or may be transported from outer parenchyma cells through the apoplast. In the L layer, lignification finished just after deposition of the L layer. In secondary phloem in *M. japonicus*, phloem fibers form tangential bands, and it is necessary to collect different sample trees in different seasons to examine the deposition processes of hemicellulose and lignin. The developmental stages of phloem fibers were variable even in trees collected in one season. In contrast, tension wood fibers with a multi-layered structure are produced from cambial cells and tension wood fibers with different degrees of differentiation are arranged radially from the cambial zone toward the annual ring boundary. This enabled us to examine the deposition process of hemicellulose and lignin more easily from one tree than in the case of phloem fibers, when several trees were artificially inclined and collected in the appropriate season. Thus, the aim of the present study was to clarify xylan deposition and lignification in differentiating tension wood fibers with a multi-layered structure.

2 Materials and methods

2.1 Plant materials

Three- to six-year-old trees of *M. japonicus* were collected at the Kitashirakawa Experimental Station of the Field Science Education and Research Center of Kyoto University, Japan. At the beginning of cambial growth at the end of March or beginning of April, sample trees were inclined artificially to either 15°, 30°, or 45° from the vertical. To prevent upward bending of stems, a rope was fixed to the lower side of each inclined stem. To examine the change in thickness of cell wall layers within an annual ring, a four-year-old tree was kept inclined at 30°–45° from vertical over one growing season and then collected. Information from the sample trees used is listed in Table 1. Wood discs were taken from each tree and small blocks containing differentiating xylem were cut from the upper side, and fixed with 2% paraformaldehyde in 0.07 M phosphate buffer (pH 7.0). The blocks were washed with phosphate buffer, dehydrated through an ethanol series and embedded in LR White resin or epoxy resin without methyl nadic anhydride (MNA) in accordance with the method of Nakagawa et al. (2014). Some of the specimens used for scanning electron microscopy were stored in 70% ethanol.

2.2 Scanning electron microscopy

To analyze the number of thin layers in multi-layered G-fibers, selected transverse sections (20 µm thick) were dehydrated through an ethanol series, transferred into tert-butyl alcohol and then freeze-dried. Dried sections were mounted on aluminum stubs with sheets of carbon double-sided tape and sputter-coated with gold with an ion sputter (JEOL JFC-1100E, Tokyo, Japan). Sections were observed using a scanning electron microscope (JEOL JSM-6060, Tokyo, Japan) at 5 kV. The proportion of cells in different numbers of thin layers in the multi-layered G-fibers was recorded at intervals of approximately 60 µm from the annual ring boundary. Because the width of a differentiating xylem ring or annual ring was variable among the sample trees, the data recorded at 60 µm intervals were gathered into 10 relative positions within a differentiating xylem ring or an annual ring. The proportion of cells in different numbers of thin layers was calculated for the 10 relative positions within a differentiating xylem ring or annual ring.

Table 1: Trees used in the present study.

No.	Inclination date	Inclination angle (°)	Sampling date	Height (cm)	DBH (cm)	Tree age	No. of thin layers ^a
1	2010.3.26	15	2010.7.5	335	2.2	3	0–1
2	2010.3.26	30	2010.7.6	280	1.9	3	1–2
3	2010.3.26	45	2010.7.8	255	1.5	3	1–2
4	2008.4.8	30	2008.7.9	512	3.8	5	1–3
5	2008.4.4	30	2009.7.10	580	4.5	4	1–2
6	2011.4.6	30–45	2011.7.8	455	4.3	4	1–2
7	2013.3.25	30–45	2013.6.11	498	3.8	4	1–2
8	2013.3.25	30–45	2013.7.26	438	3.3	4	1–3
9	2013.3.25	30–45	2013.7.26	530	2.5	6	1–2
10	2013.3.25	30–45	2013.8.16	676	4.3	6	1–2

^aNumber of thin layers in the G-layers observed in developed tension wood fibers; DBH, diameter at breast height.

2.3 Immunogold labeling of xylan

Transverse ultrathin sections (approximately 0.1 μm thick) were cut from embedded specimens using an ultramicrotome (Reichert-Jung ULTRACUT E, Heidelberg, Germany) equipped with a diamond knife. Immunogold labeling was conducted in accordance with procedures described previously by Higaki et al. (2017). A xylan-specific monoclonal antibody, LM11 (PlantProbes, UK, McCartney et al. 2005), was used as the primary antibody. An anti-rat secondary antibody labeled with 10 nm colloidal gold particles (EMGAT10, BBI International, Cardiff, UK) was used. As a negative control, some grids were incubated with blocking buffer (1% bovine serum albumin in (tris [hydroxymethyl] aminomethane-buffered saline [TBS] at pH 8.2 containing 0.1% sodium azide) instead of LM11. The sections were observed using a transmission electron microscope (JEM1400, JEOL, Japan) at 100 kV without post staining.

To quantify labeling density, the number of gold particles and the area of the S_1 and S_2 layers were measured using Image J software (National Institutes of Health, Bethesda, MD, USA). The labeling density was calculated from the area and number of gold particles and expressed as the number of particles per unit of cell wall area. Measurement was done with 4–15 images and the results were expressed in following four stages, namely the, (1) S_1 layer forming stage, (2) S_2 layer forming stage, (3) G_1 layer forming stage, and (4) G_2 layer forming stage.

2.4 Ultraviolet microscopy

Transverse sections (3 μm thick) were cut using a rotary microtome equipped with a diamond knife. The sections were mounted on quartz slides with glycerin, covered with quartz coverslips, and observed using a microscope spectrophotometer (Carl Zeiss UMSP-80, Germany) at a wavelength of 280 nm. Ultraviolet (UV) photomicrographs were taken using a digital camera (CM-140GE-UV, JAI Corp., Japan) and JAI Control Tool software.

2.5 Confocal laser scanning microscopy after staining with acriflavine

Transverse sections (30 μm thick) were cut using a sliding microtome from differentiating xylems. Sections were stained with 0.001% acriflavine aqueous solution, which stains lignified tissue with a green fluorescence (530 nm) (Donaldson et al. 2001), for 2 h at room temperature, washed with water and mounted with glycerin:water (1:1). The sections were observed using a confocal laser scanning microscope (Fluoview FV300, Olympus, Japan).

2.6 Transmission electron microscopy after staining with potassium permanganate

Transverse sections (0.1 μm thick) were cut from the embedded specimens. The ultrathin sections were mounted on copper grids, stained with 1% potassium manganate/0.1% sodium citrate aqueous solution for 20 min at room temperature. The grids were washed with water and observed using a transmission electron microscope (JEOL JEM 1400, Japan) at 100 kV. Brightness profiles of inverted images were taken to evaluate changes in the degree of KMnO_4 staining in the

CML, S_1 , S_2 , and G-layers during the development of tension wood fibers. From the images, the thickness of the S_1 , S_2 , and G-layers was also measured with the same software as described above. To measure the distance from the cambial zone of each developing G-fibers, low magnification images were taken from sections mounted on the grid using a light microscope and a straight line was drawn in the cambial zone and the distance from the cambial zone was measured as the distance between the line and the center of developing G-fibers used for the measurements. Approximately 30 cell wall positions were measured from 15 TEM images. The thickness was measured on three positions from one cell wall layer and the data were averaged.

2.7 Activity staining of peroxidase/laccase with metal-enhanced DAB substrate

The activity of peroxidase was detected using a metal-enhanced DAB substrate kit (Thermo Scientific co. Ltd, Adams 1981). From tree 10, transverse sections (30 μm thick) were cut using a sliding microtome and washed with phosphate buffer (pH 6.2) for 10 min three times to remove soluble peroxidase/laccase. Then, the sections were treated with DAB/metal concentrate diluted at a 1 to 10 ratio with stable peroxide buffer for 30 min at room temperature. After washing with phosphate buffer and water three times, sections were put on glass slides, mounted with a Pristine Mount (Falma Co. Ltd., Tokyo, Japan), and observed using a light microscope. As a negative control, a proportion of the sections were boiled for 15 min before treatment of the substrate.

3 Results and discussion

3.1 Structure of multi-layered cell walls of tension wood fibers

Although G-layers were detached from the outer secondary walls during cutting and/or drying, scanning electron microscopy enabled us to distinguish very thin layers in multi-layered G-fibers (Figure 1 arrows). The number of thin layers in multi-layered G-fibers was in the range of 0–3 as shown in Table 1. Scanning electron micrographs of tension wood fibers with no thin layers (Figure 1a), one (Figure 1b), two (Figure 1c), and three thin layers (Figure 1d) are shown in Figure 1. Based on observations using a scanning electron microscope, the proportion of cells in different numbers of thin layers was plotted versus the relative position of the developing (a–i) and developed (j) annual ring (indicated as 10 relative positions that were based on the distance from the annual ring boundary to the cambial zone (a–i) and the next annual ring boundary [j]) in Figure 2. The right side of Figure 2 shows the bark side and the left side is the pith side. Except for developed xylem (j), the thickness of G-layer gradually increased from the right end (close to the cambial zone) to the left end during formation of the G-layer. Since the thin layers are

Figure 1: Scanning electron micrographs of transverse sections of tension wood fibers in *Mallotus japonicus* with no thin layers (a) from tree 1, one (b), two (c), and three thin layers (d) from tree 4. Arrows indicate the thin layers located in the G-layers.

formed in the middle of G-layers as shown in Figure 1, fibers close to the right end with no thin layers can be regarded as developing G-fibers before the formation of thin layers. In a three-year-old tree inclined at 15° (tree 1), most of G-fibers did not have thin layers in multi-layered G-fibers, and only a few fibers had G-layers with one thin layer (Figure 2a), suggesting that inclination angle may affect the formation of thin layers in multi-layered G-fibers. In a 3-year-old tree inclined at 30° (tree 2), G-fibers with one thin layer were distributed throughout the differentiating xylem, and G-fibers with two thin layers were distributed close to the annual ring boundary (Figure 2b). In a three-year-old tree inclined at 45° (tree 3), G-fibers with one thin layer were widely distributed throughout the differentiating xylem, and only a very few G-fibers with two thin layers were found close to the annual ring boundary (Figure 2c). From the results obtained from different inclination angles (Figure 2a–c), at least more than 30° inclination may be necessary to induce the formation of more than one thin layer in multi-layered G-fibers. In a five-year-old tree inclined at 30° (tree 4), G-fibers with one thin layer were widely distributed throughout the differentiating xylem, and G-fibers with two thin layers and occasionally with three layers were distributed close to the annual ring boundary (Figure 2d). In developed annual rings in tree 5, which was inclined for two growing season, G-fibers with one thin layer were widely distributed throughout the annual ring and the proportion decreased just close to the terminal zone (right end) (Figure 2j). G-fibers with two thin

layers were distributed close to the annual ring boundary and almost the middle part of the annual ring and their distribution varied throughout one annual ring, suggesting that the occurrence of G-fibers with two thin layers may change during one growing season. The variation in the occurrence of these G-fibers can also be seen in trees 4, 5, 6, 9, and 10 (Figure 2d–f and i, respectively). This result suggested that tension wood fibers in *M. japonicus* basically have one thin layer in the G-layer except for tree 1 with lower inclination angle, and the distribution of tension wood fibers with more than two thin layers may be variable within an annual ring. Therefore, in the present study, the processes of xylan deposition and lignification were examined until G-layers with one thin layer formed, although the occurrence of G-fibers with one thin layer also varied between trees.

3.2 Distribution of xylan and lignin in multi-layered G-fibers

Confocal laser scanning microscopy after staining with acriflavine showed green fluorescence in very thin layers in multi-layered G-fibers (Figure 3a arrows). These thin layers showed UV absorption (Figure 3b arrows), suggesting that the thin layers in multi-layered G-fibers were lignified. Thus, these thin layers in multi-layered G-fibers were called as L (lignified) layers in this paper. The cell wall structure of G-fibers can be expressed as $S_1 + S_2 + G + n(L + G)$, where n is

Figure 2: Number of thin layers in multi-layered G-fibers in developing (a–i) and developed (j) annual rings. The numbers on the horizontal axis indicate the relative position in the annual ring from the annual ring boundary to the cambial zone. The left side is the pith side and the right side is the bark side. a: tree 1, b: tree 2, c: tree 3, d: tree 4, e: tree 5, f: tree 6, g: tree 7, h: tree 8, i: tree 10, j: tree 5.

the number of repetitions. Lignin was distributed in CML, S_1 , S_2 , and L layers.

Immunogold labeling of xylan is shown in Figure 4. LM11 heteroxylan epitopes were distributed in lignified

layers except the CML, but were in the S_1 , S_2 , and L_1 layers (Figure 4a). Almost no labeling was found in the G_1 -layers and G_2 -layers. In G-fibers with two thin layers, L_2 layers also showed positive labeling (Figure 4c).

Figure 3: A confocal scanning laser micrograph (a) of a transverse section stained with acriflavine and a UV micrograph (b) of a transverse section taken at 280 nm from developed tension wood fibers of tree 2. Arrows indicate the thin layers located in the G-layers.

The distribution of xylan and lignin was examined in phloem fibers of the same species *M. japonicus* (Nakagawa et al. 2014). Although the cell wall structure was not exactly same, the xylan and lignin distribution of the multi-layered G-fibers was similar to that of phloem fibers. This colocalization of the LM11 heteroxylan epitope and lignin in lignified layers except the CML suggests that the presence of the LM11 heteroxylan epitope may have some role in the presence of lignin in the S_1 , S_2 , and L layers as suggested by Reis and Vian (2004). Terashima et al. (2009) suggested that galactoglucomannan may associate with cellulose mostly at the surface of cellulose microfibril (CMF) bundles, and that a bundle of arabinosyl-4-*O*-methylglucuronoxylan chains crosslinking between CMF bundles in the radial and tangential directions at about 16 nm intervals

Figure 4: Immunogold detection of xylan in tension wood fibers of tree 6 using the LM11 anti-xylan monoclonal antibody. Labeling was found in the S_1 , S_2 , and L layers and almost no labeling was found in the G_1 and G_2 layers (a). In a tension wood fiber with two L layers, labeling was found in the S_1 , S_2 , L1, and L2 layers and almost no labeling was found in the G_1 , G_2 , and G_3 layers. (a) and (c) are enlarged images from the square-enclosed regions shown in the low-magnification images (b and d), respectively.

may play a role as an initiation site for the lignification of ginkgo tracheid S_2 layers. Secondary wall lignin is formed as a tubular hemicellulose lignin module between narrow spaces between CMFs, whereas CML lignin is formed as a globular pectin-lignin module clustered in a grape-like supramolecular structure between CMFs (Terashima et al. 2012). The colocalization of xylan and lignin suggested that the absence of the LM11 heteroxylan epitope may be related to the absence of lignin in G-layers other than the thin layers. Another possibility is that heterogeneous lignin distribution may be related to the heterogeneous distribution of peroxidases or laccases that polymerize monolignols in the cell wall layers. Peroxidases with hydrogen

peroxide and laccases with oxygen are involved in the final step of lignin biosynthesis, and the coordination of polymerization machinery localization and monolignol supply is likely critical for the spatio-temporal patterning of lignin polymerization (Tobimatsu and Schuetz 2019). Peroxidase/laccase activity was examined using metal-enhanced DAB substrates, and unexpectedly strong activity was found throughout the G-layers (Figure 5). Similar high activity was also found in the phloem fibers (Nakagawa et al. 2014). This finding raised the question of why G-layers other than L layers are less lignified in spite of the presence of strong peroxidase activity. If it is assumed that xylan may have a role as an initiation site of lignification, the absence of the LM11 heteroxylan epitope might be one of the reasons why G-layers other than the thin layers are less lignified.

In some cases, small amounts of lignin or lignin-like materials may be present throughout the G-layer or on its lumen surface (Donaldson and Singh 2016; Gierlinger and Schwanninger 2006). Joseleau et al. (2004) and Pilate et al. (2004) suggested that the presence of syringyl lignin in G-layers in poplar. Using multivariate image generation and cluster analysis by Raman microscopy in poplar tension wood, Gierlinger et al. (2012) showed the average spectrum from the cluster corresponds to the G-layer with very small peak at 1600 cm^{-1} , suggesting the presence of

small amounts of lignin in G-layer. In the $S_1 + G$ type G-fibers in *Fraxinus excelsior*, Kim and Daniel (2019) found the presence of syringyl lignin in G-layers. Because the UV absorptivity is much lower in syringyl lignin than guaiacyl lignin, the sensitivity for detecting lignin in the present study may not be enough to detect lignin in G-layers. The peroxidase/laccase activity found throughout G-layers may be related to the formation of small amounts of syringyl lignin or syringyl lignin-like materials in G-layers.

It is suggested that xylan has two domains (major domain and minor domain). The major domain is accommodated on vacancies on the hydrophilic surface of cellulose, and the minor domain cannot bind to the hydrophilic surface of cellulose (Busse-Wicher et al. 2014). Ruel et al. (2006) examined the mode of deposition of xylan and lignin in the differentiating walls of poplar normal wood and showed the high degree of mutual relations between xylan and lignin during secondary wall assembly. They suggested that the first deposited condensed lignin is more likely to interact with a certain type of xylan coating the cellulose microfibril surface, and as the space between the loose microfibrils becomes narrower because of the first deposited condensed lignin, these conditions favor the polymerization of non-condensed lignin together with another type of xylan. In softwood, an NMR study elucidated that D-mannose units in glucomannan is linked to the alpha position of the beta-O-4 lignin subunits through an ether bond (Nishimura et al. 2018). Because only one type of monoclonal antibody against xylan (LM11) that binds to various types of substituted xyans was used, the relationship between xylan structure and the structure of lignin (condensed or non-condensed) in the L layer and G-layer remains unclear. On this point, further studies using well-defined monoclonal antibodies against xylan (Peralt et al. 2017) and lignin substructures (Kiyoto et al. 2013) will be necessary to discuss the relationship between xylan and lignin structures during tension wood formation in multi-layered tension wood fibers.

3.3 Deposition of xylan in S_1 and S_2 layers during development of G-fibers

Figure 6 shows the change in density of immunogold labeling in the S_1 layers and S_2 layers with the LM11 anti-xylan monoclonal antibody. Labeling density in the S_1 layers increased from the S_1 layer forming stage to the S_2 layer forming stage and also from the S_2 layer forming stage to the G_1 layer forming stage. The density in the S_2 layers also increased from the S_2 layer forming stage to the G_1 layer forming stage (Figure 6).

Figure 5: Light micrographs of transverse sections stained with DAB showing peroxidase/laccase activity in tension wood fibers of tree 10 (a and c) and control (after boiling of sections, b and d). Strong activity was found in the G-layers and L layers.

Figure 6: Change in density of immunogold labeling in the S_1 layers and S_2 layers with an LM11 anti-xylan monoclonal antibody during four stages of cell wall formation (tree 6). Error bars indicate standard deviation. Numbers in parentheses indicate the number of images used for the measurement for each stage.

Considering the labeling density in developing cell walls, the density was based on the cell wall area. Labeling density may change when the cell wall thickness changes during cell wall formation. Therefore, it is important to confirm whether the cell wall thickness changes or not during differentiation. Figure 7 shows the change in thickness of the S_1 , S_2 , and G_1 layers during development. The thickness of the S_1 layer decreased from about $0.6 \mu\text{m}$ to about $0.3 \mu\text{m}$ between the S_1 layer forming stage to the S_2 layer forming stage. Of note, the thickness of the S_2 layers decreased drastically from about $1.4 \mu\text{m}$ to approximately $0.6 \mu\text{m}$ between the middle stage of the S_2 layer forming stage and the G_1 layer forming stage (200–300 μm from cambial zone, tree 6, Figure 7a). Although the degree of decrease varied slightly, the decrease in S_2 layer thickness was also found in other three trees (tree 7, tree 8, and tree 9). Figure 7b shows the result of tree 9. This decrease in thickness suggested the following three possibilities, namely, (1) S_1 layers and S_2 layers, especially S_2 layers, shrank during formation of the inner G_1 layers, or (2) the thickness of the S_1 and S_2 layers are variable within an annual ring, or (3) non-lignified S_1 and S_2 layers swelled during sample preparation.

To confirm the variation in thickness of the S_1 , S_2 , and G_1 layers within one annual ring, the measurement of cell wall thickness was also done on fully developed xylem in a tree 5 inclined for two growing seasons. The thickness of the S_1 layer was approximately $0.3 \mu\text{m}$ and that of the S_2 layer ranged from approximately 0.5 – $0.8 \mu\text{m}$ both in earlywood side and latewood side in one annual ring (Figure 7c). This suggested that the decrease in thickness of the S_1 and S_2 layers may be due to shrinkage occurring

Figure 7: Change in the thickness of the S_1 , S_2 , and G -layers during development in tree 6 (a), tree 9 (b), and in a developed annual ring in tree 5 (c). Measurements of the thickness were done at the centers between two cell corners of TEM micrographs. a, b, c, g, h, and i in an indicate the positions of tension wood fibers where TEM micrographs were taken (Figure 8a–c, g–i, respectively). The thickness of G -layers indicating the total thickness of the G -layer including data on G_1 , L_1 and G_2 layers.

during formation rather than thickness variation of the cell wall layers within an annual ring.

Although the thickness of the S_2 layers decreased to less than half during the formation of G -layers, the labeling density did not increase more than twofold (about 1.7 times). If it is assumed that xylan deposition occurred completely appositionally, the labeling density should increase more than twofold when the cell wall thickness

decreased to less than half. This lower increase might be due to either (1) underestimation of cell wall thickness from measurement of thinnest position in the S_2 layers and/or (2) the masking of epitopes of the LM11 antibody occurred due to lignification or structural change in epitopes in the cell wall. In normal wood fibers in *Fagus crenata*, the labelling density of xylan in each secondary wall layer (S_1 , S_2 , and S_3) increased during cell wall formation, suggesting that the deposition of xylan occurs in a penetrative manner (Awano et al. 1998). If it is assumed that xylan deposition occurred in a penetrative manner, the degree of increase in the labeling density should be more than that expected due to the shrinkage of S_2 layers. Actually, the degree of increase in labeling density was lower than that expected due to the shrinkage of S_2 layers. Therefore, xylan deposition in the S_2 layers should occur appositionally rather than in a penetrative manner. The appositional deposition of xylan was

also found in phloem fibers in *M. japonicus* (Nakagawa et al. 2014).

3.4 Relationship between the shrinkage of S_2 layers and lignification during the development of G-fibers

Lignification processes were examined using the brightness profiles from inverted transmission electron microscopy (TEM) images during the development of G-fibers (Figure 7). About 80 images were examined and representative patterns for different developmental stages were selected for Figure 8. Although cell wall components other than lignin (proteins and acidic polysaccharides) may also be stained with KMnO_4 (Bland et al. 1971; Hoffmann and Parameswaran 1976), this technique is useful to examine lignification processes of

Figure 8: Transmission electron micrographs of transverse sections from differentiating xylem of tree 6 following staining with KMnO_4 (a, b, c, g, h, and i) and the brightness profiles of inverted images (d, e, f, j, k, and l) showing the lignification process of the CML, S_1 , S_2 , and L layers.

different cell wall layers in higher resolution than UV microscopy. This technique was also used to examine lignification processes in poplar tension wood fibers (Yoshinaga et al. 2012). The positions of the cells measured are shown in Figure 7a. In the S_2 layer forming stage when the thickness was increasing (Figure 7a), only weak staining was found in the CML, S_1 , and S_2 layers (Figure 8a, d), suggesting that the degree of lignification was low in this stage. In S_2 layers whose thickness reached a maximum value (Figure 7b), lignification of the CML and S_1 layers and the outer part of S_2 layers (CML side) started, whereas the degree of lignification of the inner part of the S_2 layers (lumen side) was lower than the outer part (Figure 8b, e). When the shrinkage of the S_2 layers started just before G_1 layer formation (Figure 7c), lignification of the CML proceeded, and the degree of lignification was slightly higher in the outer part of the S_2 layers (Figure 8c, f). At the early stage of the G_1 layer formation with thin G_1 layers (Figure 7g), shrinkage of the S_2 layers proceeded and the degree of lignification in S_2 layers increased, whereas that of the CML was still low (Figure 8g, j). When shrinkage of the S_2 layers finished after G_1 layer formation was almost completed (Figure 7h), the degree of lignification in the S_2 layers and CML increased at the same time as shrinkage of the S_2 layers occurred (Figure 8h, k), suggesting that shrinkage of the S_2 layers may be related to lignification that may displace water to the inner G-layers. *In situ* Raman spectroscopy has demonstrated that G-layers contain more water than the adjacent lignified S-layers in poplar (Gierlinger and Schwanninger 2006). The degree of lignification in the S_1 and S_2 layers and the CML did not change after formation of the L_1 and G_2 layers (Figures 7i and 8i, l).

In the present study, the thickness of S_2 layers decreased during the development of G-fibers. The strong reduction of secondary wall layers during the thickening of the G layer was also reported in two poplar trees studied by Chang et al. (2014); however, the authors did not comment on this phenomenon. In contrast, Abedini et al. (2015), examining the detailed process of cell wall thickening in developing poplar tension wood in various conditions, showed a slight decrease in secondary wall thickness all along the growing period, attributed to a seasonal effect but did not observed a steep reduction of the secondary layer during the G-layer thickening period. Their data on cell wall thickness was based on light microscopic measurement of the average cell wall thickness including the S_1 and S_2 layers, whereas we measured S_2 layer thickness directly from TEM micrographs from the center of the S_2 layer (the thinnest position in S_2 layers) excluding cell corners. In the data of the present study, the thickness of

the S_1 layer also slightly decreased or in some samples did not alter. Therefore, the data in the present study emphasized a decrease in thickness of the S_2 layers at the thinnest position and their data were underestimated by the presence of data on S_1 layers with smaller changes in thickness than in S_2 layers and the data in the present study on the cell corners of S_1 and S_2 layers. Another possibility is that the decrease in the thickness of the S_2 layers may be due to the swelling of non-lignified S_2 layers in the early stage of development during sample preparation. Further studies using freeze fixation techniques e.g. high-pressure freezing and freeze substitution, will be necessary to clarify this phenomenon.

The evaluation of lignification in the S_1 , S_2 , and CML using TEM suggested that lignification of these layers proceeded during the formation of G-layers. Similar results were found in poplar tension wood fibers of $S_1 + S_2 + G$ type (Yoshinaga et al. 2012) and *M. japonicus* multi-layered phloem fibers (Nakagawa et al. 2014).

4 Conclusions

Tension wood fibers in *M. japonicus* showed a multi-layered structure ($S_1 + S_2 + G + n(L + G)$, where n is the number of repetitions, $n = 0-3$). Except for the tree inclined at 15° , the structure basically had one thin layer in the G-layers, and the distribution of tension wood fibers with more than two thin layers was variable within an annual ring. The LM11 heteroxylan epitope was distributed in lignified cell wall layers except the CML, namely the S_1 , S_2 , and L layers. Xylan deposition in these layers occurred appositionally similar to multi-layered cell walls of phloem fibers in the same species. Lignification of the CML, S_1 , and S_2 layers proceeded during the formation of G-layers. With the course of G-layer formation, secondary wall layers, especially S_2 layers, shrank dramatically during lignification.

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