Distribution of Hemicelluloses in Warts and the Warty Layer in Normal and Compression Wood Tracheids of Cryptomeria Japonica*1

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ABSTRACT

The distribution of arabino-4-O-methylglucuronoxylans (AGXs) and O-acetyl-galactoglucomannans (GGMs) in warts and the warty layer of tracheids in normal wood (NW) and compression wood (CW) of Cryptomeria japonica was investigated. Under field emission scanning electron microscope (FE-SEM) observation, warts and the warty layer of delignified NW and CW tracheids were degraded by xylanase treatment, indicating that warts and the warty layer contain high amounts of AGXs. However, the effect of xylanase was not observed in NW and CW tracheids before delignification, suggesting that AGXs in warts and the warty layer may be encrusted with lignin. After β-mannanase treatment, no noticeable changes were observed in warts and the warty layer of NW tracheids, indicating that warts and the warty layer contain either no or very few GGMs. Similar results to FE-SEM observations were also observed with immunogold labeling. AGX labeling was observed in warts and the warty layer of NW and CW tracheids, while GGM labeling was not detected. NW tracheids showed a much stronger density of AGX labeling than did CW tracheids in warts and the warty layer, indicating differences in the chemical compositions of warts and the warty layer between NW and CW tracheids.

Keywords: O-acetyl-galactoglucomannans (GGMs), arabino-4-O-methylglucuronoxylans (AGXs), compression wood, Cryptomeria japonica, wart, warty layer

1. INTRODUCTION

According to the definition by the International Association of Wood Anatomists (IAWA Committee, 2004), warts are small, unbranched protuberances on the inner layer of the secondary wall (S3 or tertiary wall) of tracheids in gymnosperms, and of vessel elements and fibers in dicotyledons. In contrast, Ohtani (1984) suggested that the term ‘warts’ be changed to ‘vestures’ on the basis of similarities in morphology, origin, and chemical composition of warts and vestures, but this change is still disputed.

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Warts are generally developed in the innermost layer of the cell wall, called the warty layer, as distinguished from the S₃ layer. In contrast, despite the lack of an S₃ layer, compression wood (CW) tracheids develop warts and a warty layer in the innermost part of the S₂ layer (Timell, 1986). Some early studies suggested that warts and the warty layer are developed before complete degradation of the cytoplasm (Cronshaw, 1965; Baird et al., 1974a). However, the formation of warts and the warty layer is still poorly understood. In particular, the accumulation of cell wall components in warts and the warty layer is little understood.

In regard to the chemical composition of warts and the warty layer, several early studies suggested that warts and the warty layer are very resistant to chemical treatments because they are composed of high concentrations of lignin (Liese, 1963; Baird et al., 1974b; Côté and Day, 1962). Some studies also reported that warts were composed of hemicelluloses in addition to lignin (Kuo and Manwiller, 1985; Takabe et al., 1989). Recently, Hosoo et al. (2006) also reported that the warty layer contains high amounts of xylans (arabino-4-O-methylglucuronoxylans, AGXs) and gluco-mannans (O-acetyl-galactoglucomannans, GGMs). However, here we report some different results from those by Hosoo et al. (2006).

In the present work, we investigated the distribution of AGXs and GGMs in warts and the warty layer of normal wood (NW) and CW tracheids using field emission scanning electron microscopy (FE-SEM) in combination with enzymatic degradation and immunogold transmission electron microscopy (TEM). To clarify the hemicellulose composition of warts and the warty layer in relation to lignin, delignified samples were also investigated using FE-SEM.

2. MATERIALS and METHODS

2.1. Wood Material

Small pieces of wood blocks were collected on 9 June 2009 from 32-year-old NW and on 18 May 2009 from 10-year-old artificial CW of Japanese cedar (Cryptomeria japonica) grown at the Kyoto University Forest Station, Japan. For the immunochemical study, the blocks were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 4 h at 4°C, dehydrated through a graded ethanol series, and then embedded in LR-white resin. Some blocks were also stored in 70% ethanol before use for FE-SEM observations.

2.2. Delignification with Sodium Chlorite

Delignification was performed according to procedures described in our previous report (Kim et al., 2010a) with minor modification. Longitudinal sections (100 µm thick) of NW and CW were delignified in 8% NaClO₂ (in 1.5% acetic acid) at 40°C for 72 h, and then washed several times with distilled water.

2.3. Enzyme Treatment and FE-SEM Observations

Longitudinal sections (100 µm thick) of NW and CW before and after delignification were incubated in 17.2 U xylanase (Sigma, USA) or 12.5 U β-mannanase (Megazyme, Ireland) in 1 ml of 0.1 M acetate buffer (pH 4.5) for 1 week at 35°C. For the control, some sections were also incubated only with acetate buffer. After washing several times with distilled water, sections were post-fixed with 2% osmium tetroxide (OsO₄) for 2 h at room temperature. They were then dehydrated through a graded ethanol series, substituted with t-butyl alcohol, and freeze-
dried. Thereafter, sections were coated with approximately 2-nm-thick platinum with an ion sputter coater (E-1045; Hitachi, Japan) and examined under a FE-SEM (S-4800; Hitachi, Japan) at an accelerating voltage of 1.5 kV and around 2.5 mm working distance.

2.4. Immunogold Labeling

Immunogold labeling was conducted according to procedures described in our previous reports (Kim et al., 2010b, c). Briefly, transverse ultrathin sections (90-nm thick) prepared from LR-white resin-embedded NW and CW blocks were incubated with LM10 or LM11 monoclonal antibodies specific to (1→4)-β-linked xylopyranosyl residues (McCartney et al., 2005). In our previous work, LM10 and LM11 antibodies showed some differences in AGX localization (Kim et al., 2010c). Some sections were also incubated with BGM C6 monoclonal antibody specific to (1→4)-β-linked manno-pyranosyl residues (Pettolino et al., 2001). Thereafter, grids were incubated with goat anti-rat (LM10 and LM11) or anti-mouse (BGM C6) secondary antibody labeled with 10-nm colloidal gold particles for 1 h at room temperature. The sections were examined under the JEOL1220 TEM after staining with 2% uranyl acetate for 20 min.

3. RESULTS and DISCUSSION

3.1. FE-SEM Observations of Warts and the Warty Layer of NW and CW Tracheids

3.1.1. Appearances of Warts and the Warty Layer before and after Delignification

The innermost layer of mature NW and CW tracheids is shown in Figs. 1A and 2A, respectively. Warts and amorphous materials including small globular structures were observed in the innermost layer of NW and CW tracheids. No cellulose fibrils were observed in the innermost layer of NW and CW tracheids, indicating the presence of a warty layer distinct from the S3 or S2 layer, respectively. After delignification, amorphous materials including small globular structures disappeared in the innermost layer of NW and CW tracheids, and warts having spherical shape became clearly visible (Figs. 1B, 2B). As in lignified tracheids, cellulose fibrils were not clearly observed in the innermost layer of delignified NW tracheids, indicating the presence of the warty layer even after delignification (Fig. 1B). However, it was not clear whether the innermost layer of delignified CW tracheids was the warty layer or the innermost S2 layer because some structures resembling cellulose fibrils were visualized after delignification (Fig. 2B).

3.1.2. Changes in Warts after Enzyme Treatment

After xylanase treatment, warts were completely degraded in delignified NW and CW tracheids (Figs. 1E, 2E), indicating that warts contain high amounts of AGXs. However, no significant differences were observed in warts of lignified NW and CW tracheids after xylanase treatment (Figs. 1C, 2C). This result suggests that AGXs in warts may be encrusted with lignin, which blocks access of xylanase to AGXs. It is generally accepted that warts contain high amounts of lignin, and therefore show strong resistance to chemical treatments (Liese, 1963; Baird et al., 1974a; Côté and Day, 1962).

After β-mannanase treatment (Figs. 1D, 1F), warts of NW tracheids were almost identical to those before treatment (Figs. 1A, 1B) regardless of delignification, indicating that warts of NW tracheids contain either no or a very small amount of GGMs. In contrast, although lignified
CW tracheids showed no notable changes in warts after β-mannanase treatment as in NW tracheids (Fig. 2D), delignified CW tracheids showed either a decomposed or an intact appearance of warts (Fig. 2F). Unfortunately, a similar phenomenon was also observed in delignified CW sections only incubated with acetate buffer as a control (not shown). These results indicate that the chemical composition of warts may differ between NW and CW tracheids, even though it is not clear whether warts of CW tracheids contain GGMs or not under...
Fig. 2. FE-SEM observations of warts (arrows) and the warty layer in mature CW tracheids. A, B. Tracheids before (A) and after (B) delignification. Note the spherical shape of warts and the disappearance of amorphous materials including small globular structures in the warty layer after delignification. C, D. Tracheid before delignification, followed by either xylanase (C) or \( \beta \)-mannanase treatment (D). Note almost identical appearance of warts and the warty layer before and after enzyme treatments (A). E. Tracheid after delignification, followed by xylanase treatment. Note the smooth appearance of cellulose fibrils. F. Tracheid after delignification, followed by \( \beta \)-mannanase treatment. Note either the degraded (insert) or intact warts. Some decomposition was also observed between cellulose fibrils (insert). Bar = 250 nm.

3.1.3. Changes in the Warty Layer after Enzyme Treatment

As in warts, no significant changes were observed in the warty layer of lignified NW and CW tracheids after enzyme treatment (Figs. 1C, 1D; 2C, 2D). However, delignified NW and CW tracheids showed some changes in the warty layer after enzyme treatment. In NW tracheids, cellulose fibrils in the \( S_1 \) layer were exposed on the innermost surface of NW tracheids.
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Fig. 3. Immunogold labeling of AGXs and GGMs in warts (arrows) and the warty layer (arrowhead) in mature NW tracheids. B, D, F. Enlargement of images in A, C, E. A-D. AGX labeling with the LM10 (A, B) or LM11 (C, D) antibody. Labeling was observed in warts and the warty layer. Note no labeling in some warts (long arrows in A, C). E, F. GGM labeling with the BGM C6 antibody. No labeling was detected in the warts and the warty layer. Staining with uranyl acetate. Bar = 500 nm (A, C, E), 250 nm (B, D, F).

after xylanase treatment (Fig. 1E), whereas the warty layer treated with β-mannanase (Fig. 1F) was almost identical to that before treatment (Fig. 1B), indicating that the warty layer contains high amounts of AGXs and either no or very few GGMs as shown in warts. In the case of delignified CW tracheids, smooth cellulose fibrils were exposed after xylanase treatment (Fig. 2E), indicating the presence of AGXs in the warty layer. However, as mentioned for warts, the presence of GGMs in the warty layer of CW tracheids was not clearly clarified by β-mannanase treatment (Fig. 2F) because change patterns in the control sections (not shown) were similar to those in sections treated with β-mannanase. Additionally, as mentioned above, it
is not clear whether the innermost layer of the CW tracheid cell wall after delignification is the warty layer or the innermost S₂ layer (Fig. 2B).

3.2. Immunogold Labeling of AGXs and GGMs in Warts and the Warty Layer of NW and CW Tracheids

The FE-SEM observations described above were confirmed and further advanced by immunogold labeling of AGXs and GGMs in warts and the warty layer. The LM10 and LM11 antibodies showed strong labeling in warts of NW tracheids (Fig. 3A, 3B), whereas the BGM C6 antibody showed no labeling in warts (Fig. 3C), indicating that warts contain AGXs but not GGMs. Compared to NW trache-
ids, CW tracheids also showed similar patterns of labeling in warts, but the labeling density of AGXs was much weaker than that in NW tracheids, indicating that AGX concentrations in warts may differ between NW and CW tracheids (Fig. 4). Additionally, although it is not clear whether chemical composition varies between warts, some warts of NW and CW tracheids showed no AGX labeling (Figs. 3A, 3C; 4C, 4D).

LM10 and LM11 antibodies also showed some labeling in the innermost layer of NW and CW tracheids (arrowheads in Figs. 3B, 3D), while the labeling was absence in this layer after treatment with the BGM C6 antibody (Fig. 3F), indicating that the warty layer may contain AGXs, but does not contain GGMs, as shown in warts. These results are slightly different from previous reports. From FE-SEM observations after immunogold labeling of AGXs and GGMs, Hosoo et al. (2006) suggested that the warty layer contains high amounts of AGXs and GGMs in NW tracheids of Cryptomeria japonica. However, our results suggest that the warty layer may contain either no or very few GGMs in NW and CW tracheids.

4. CONCLUSIONS

Present work clearly shows that warts and the warty layer of NW and CW tracheids contain high amounts of AGXs and either no or very few GGMs. This result suggests that AGXs may have important roles to maintain the structure of warts and the warty layer in combination with lignin. Our observations also indicate that AGXs in warts and the warty layer of NW and CW tracheids may be encrusted with lignin, and therefore they are not easily degraded by enzymatic treatments without delignification. The lower concentration of AGXs in warts and the warty layer of CW tracheids than NW tracheids and some unexpected degradation of warts and the warty layer of CW tracheids by the enzyme omitted solution indicate that chemical compositions of warts and the warty layer may differ between NW and CW tracheids. Hypothetically, CW tracheids may contain a higher amount of lignin in warts and the warty layer than NW tracheids to compensate lower amounts of AGXs in warts and the warty layer than NW tracheids because warts and the warty layer are mostly composed of lignin and hemicelluloses, specifically AGXs in this work.

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