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VARIATION IN HEMICELLULOSE STRUCTURE AND ASSEMBLY IN THE CELL WALL ASSOCIATED WITH THE TRANSITION FROM EARLYWOOD TO LATEWOOD IN CRYPTOMERIA JAPONICA

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The size of cellulose microfibril (CMF) bundles varies to interact with glucomannan/galactoglucomannan (GM/GGM). Arabino-4-O-methylglucuronoxylan (AGX) bonded CMF bundles coated with GM/GGM also have important roles in elaborating the distance between these components. Since the precise roles of GM/GGM and AGX are not clear, the elution analysis to evaluate the strength of the interaction between the cell wall were tried. Earlywood (EW) and latewood (LW) were separated in a Japanese cedar. The chemical components of cellulose, hemicellulose including GM/GGM and AGX, and lignin were almost the same in EW and LW. Slight differences in GM/GGM, the side-chain substitution in AGX and the ionic bond characteristics of glucuronic acid side chains were observed. Based on measurements of GM/GGM and AGX adhering to CMFs, there were more hemicelluloses forming strong hydrogen bonds in LW than in EW. The results showed that the highly assembled hemicellulose in LW produced a strong cell wall framework.

KEYWORDS. Arabino-4-O-methylglucuronoxylan, cell wall microstructure, earlywood, glucomannan, hemicellulose, Japanese cedar, latewood

INTRODUCTION

Japanese cedar (*Cryptomeria japonica* D. Don) is one of the most important softwood species in Japan for building materials. A large amount of data regarding its physical properties has been collected. Characteristic of Japanese cedar is a different cell wall structure in cells of both earlywood (EW) and latewood (LW) depending on the growth conditions. EW cells or tracheids are produced during the spring growth period, and have expanded lumens and relatively thin cell walls. On the other hand, LW tracheids have smaller lumens with thicker cell walls that provide the mechanical strength to support the large tree size. Although EW and LW are derived from the same cambial initial cells in the cambium layer, their cell morphology and cell wall structure differ dramatically according to the season, and they affect the tree's growth and mechanical strength.[1–4] The main components of EW and LW are cellulose, hemicellulose and lignin. These polymers are synthesized under temporal and spatial regulation by their respective biosynthetic enzyme

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systems. Such polymers spontaneously interact and self-organize through weak bonds such as hydrogen bonds and electrostatic interactions, and accordingly come into contact with each other. Quantitative changes in the composition of the cell walls of EW and LW might be due to change in the chemical structure of the hemicelluloses. As hemicelluloses affect the process of interaction and self-organization for molecules in the cell wall, this could explain how differences in EW and LW are produced. The thickened cell walls of the stem xylem tissues are eventually formed and are comprised of the cell wall framework of a cellulose microfibril (CMF) core and the cell wall superstructure.

According to previous studies,[5,6] the formation of the secondary wall of tracheids in the xylem of the gymnosperm ginkgo (*Ginkgo biloba*) is believed to begin with the formation of CMFs in the presence of glucomannan/galactoglucomannan (GM/GGM), which leads to the aggregation of cellulose chains and CMF formation. As a result, the size of the CMF bundles varies according to their interactions with GM/GGM. The CMFs are laid down on the innermost layer of the cell wall at a changing cellulose microfibril angle (CMFA). Since CMFA is the orientation of the CMF with respect to the major axis of the cell, the core structure of the cell wall is formed in this way. Therefore, GM/GGM has an important part to play in the formation of the core structure of the cell wall. At the same time, it is assumed that arabinoglucuronoxylan (AGX) bonds together CMF bundles coated with GM/GGM, and elaborates the distance between them. However, the precise roles of GM/GGM and AGX in the formation of the core structure of the cell wall are not clear.[6] The present study applied the elution analysis to evaluate the strength of the interaction between the main cell wall components and their mode of assembly. We tried to understand the structure of the cell walls of EW and LW, which is expected to make a significant contribution to the process by which xylem tissues are formed.

MATERIALS AND METHODS

Wood Materials and Microscopy

A Japanese cedar tree (named Ryuunohige, 49 years old) was grown in a forest environment in Kumamoto, Japan. Sample logs were 1.0 m long at 1.0 m above the ground. Two adjacent 100-mm-thick disks were cut from the center of each sample log and air-dried. The upper disc was used for microscopic examination with 0.1% aqueous safranin. The lower disc was used for isolation of EW and LW to analyze their chemical components. Five annual rings between ring numbers 35 and 39 were used for EW and LW preparations. The EW and LW bands were separated with a carving knife along an annual ring. LW was considered as the darker-colored material in the last 1 mm of the growth ring. After ambiguous material was removed, the light-colored EW and the dark-colored LW were separated. Wood samples were milled to pass a 40-mesh screen (422-μm pore size) in a Wiley mill before extracting with ethanol: benzene (1:2) for 6 h in a Soxhlet apparatus to remove extractives.

Chemical Component Analysis

Determination of the Klason lignin content of EW and LW samples followed the gravimetric method of Yokoyama et al.[7] and Yeh et al.[8] Holocellulose was determined with the method of Yokoyama et al.[7] and Yeh et al.[8] The total sugar content in the Klason hydrolysates was determined by the phenol-sulfuric acid colorimetric method according to Rao and Pattabiraman.[9] For calibration, a standard curve obtained from known amounts of glucose (Glc) was used.

The relative sugar composition in the Klason hydrolysate was determined by partition chromatography on an ion exchange resin (TSK-gel Sugar AX1 column, Tosoh Corp., Japan) according to Nakamura et al.[10] and Yamasaki et al.[11] on an LC-10AT HPLC instrument (Shimadzu Corp., Japan) with the buffer 0.5% borate-1.0% ethanolamine-hydrochloric acid (pH 7.9). Relative percentages were

calculated electronically from duplicate experiments.

The cellulose content was determined according to Easty et al.[12] and Jones et al.[13] as Cellulose (%) = Glc − (1/3 × Mannose (Man)), while the hemicellulose content was calculated as Hemicelluloses (%) = (Arabinose (Ara) + Galactose (Gal) + Glc + Man + Xylose (Xyl)) − Cellulose.

The GM/GGM and AGX content of holocellulose in EW and LW was calculated by assuming a fixed ratio of the relevant sugar units in each type of polysaccharide.[14–16]

Preparation of AGX

AGX was prepared according to Yamasaki et al.[11] Extractive-free wood meal samples (20.0 g) were treated at 80°C for 1 h with sodium chlorite (8.0 g) and acetic acid (1.6 mL) with gentle stirring. After four successive treatments, the solid residue was recovered by filtration, washed with water and acetone, and air-dried. The resulting holocellulose was extracted successively with hot water to remove GGM[17] and with 10% aqueous potassium hydroxide in the presence of barium hydroxide to isolate AGX.[18] The latter extracts were adjusted to pH 6.0 with glacial acetic acid and poured into ethanol (4 vol.). The precipitates were collected, dialyzed against deionized water, evaporated, and freeze-dried. The natural sugar composition of AGX was determined by means of partition chromatography on a TSK-gel Sugar AX1 column after hydrolysis with 2 M trifluoroacetic acid at 120°C for 2 h.[10,11] The uronic acid content of AGX was determined by the carbazole reaction in sulfuric acid according to Blumenkrantz and Asboe-Hansen.[19]

Preparation of GM

GM was prepared according to published methods.[11,20] During AGX preparation as described earlier, the resulting precipitate after addition of 10% aqueous potassium hydroxide in the presence of barium hydroxide was collected by centrifugation.[11]

Ion Exchange Chromatography of AGX

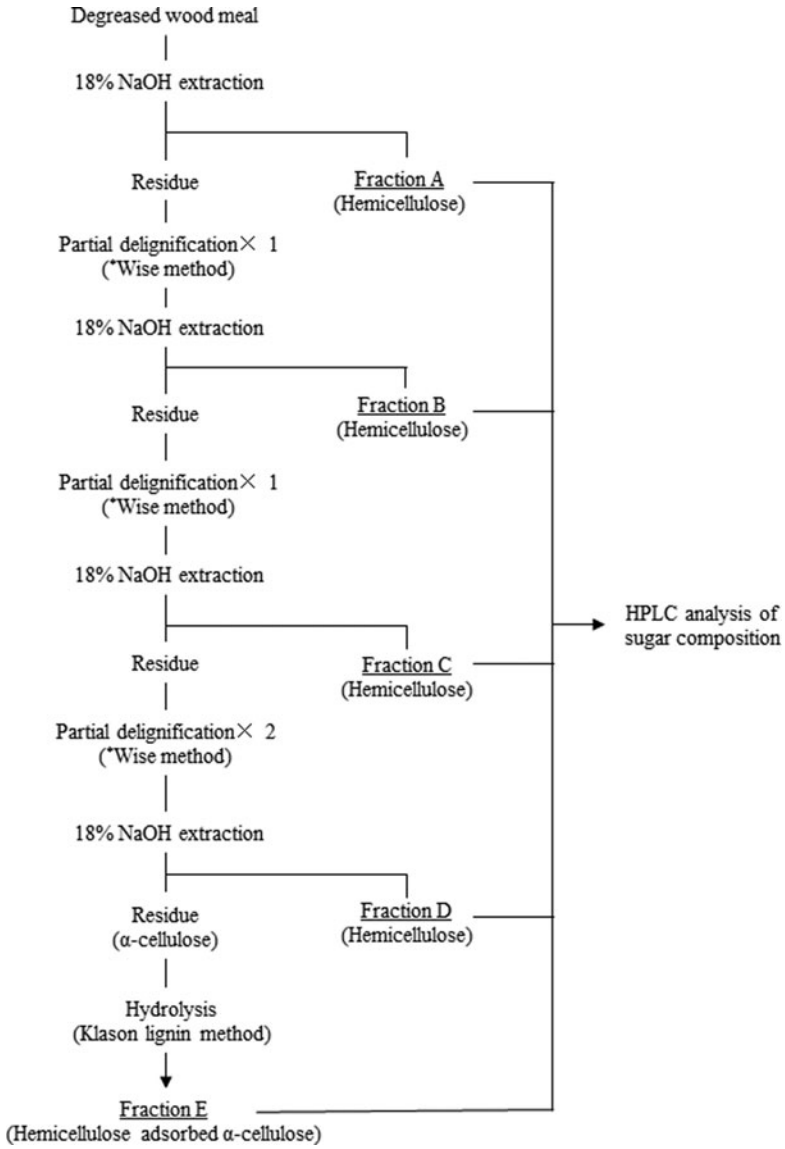
Ion exchange column chromatography was carried out according to published method.[21] AGX (500 mg) was dissolved in 50 mL deionized water and centrifuged. The supernatant was put on a column (3.0 × 25.0 cm) of DEAE-Sephadex A-25 (GE Healthcare, USA) and eluted stepwise with deionized water (450 mL), 0.2 M sodium acetate (NaOAc) (500 mL), 1.0 M NaOAc (500 mL), 2.0 M NaOAc (500 mL), and 1.0 M NaOH (500 mL). These fractions were separately neutralized, dialyzed against deionized water, evaporated, and freeze-dried. The sugar content in each fraction was determined by the phenol-sulfuric acid colorimetric method according to Rao and Pattabiraman.[9]

Size-Exclusion Chromatography of AGX and GM/GGM

Size-exclusion chromatography (SEC) analysis of AGX and GM was carried out according to previously published methods.[22,23] AGX and GM were individually analyzed by SEC using a Superose 6 10/300 column (GE Healthcare, USA) at a flow rate of 0.5 mL min^{-1} in 50 mM NaOAc using refractive index detection. The column was calibrated using blue dextran molecular markers corresponding to 410, 110, and 12 kDa.

Elution Analysis of Cell Wall Hemicellulose Assembly Mode Section

The elution analysis of this study was a combination of processing to partially decompose the lignin and extraction with 18% aqueous NaOH solution, which renders hemicellulose fully soluble. Extraction with 18% aqueous NaOH solution can elute hemicellulose by dissociating hydrogen bonds and ionic bonds between hemicellulose molecules, between hemicellulose and cellulose molecules, and between hemicellulose and lignin molecules. As in Scheme 1, the soluble fraction A was obtained from degreased wood powder before lignin decomposition by extraction with 18% aqueous NaOH solution. The other soluble fraction B, C, and



SCHEME 1. The elution analysis method for cell wall hemicellulose assembly mode. *Wise method: Sodium chlorite (0.8 g) and acetic acid (160 μL) was added into deionized water, and agitated at 70°C for 1 h.

D were yielded from residues after partially lignin decomposition by extraction with 18% aqueous NaOH solution. The soluble fraction E was finally obtained from the α-cellulose adsorption fraction.

The partially delignification process in this method was according to Wise method.[10,11] A degreased wood powder (2.0 g) or residual sample was added into sodium chlorite (0.8 g) and acetic acid (160 μL) in deionized water (66 mL), and the mixture was agitated for 1 h. This process was repeated four times for complete decomposition of lignin.

In preparation of fraction A–D, a degreased wood powder (2.0 g) or residue following lignin decomposition was added into 18% aqueous NaOH solution (120 mL), and agitated at room temperature under a nitrogen atmosphere for 40 min. The residue was filtered with glass fiber paper, and washed in deionized water (150 mL). The soluble fraction was neutralized, dialyzed with deionized water, and freeze-

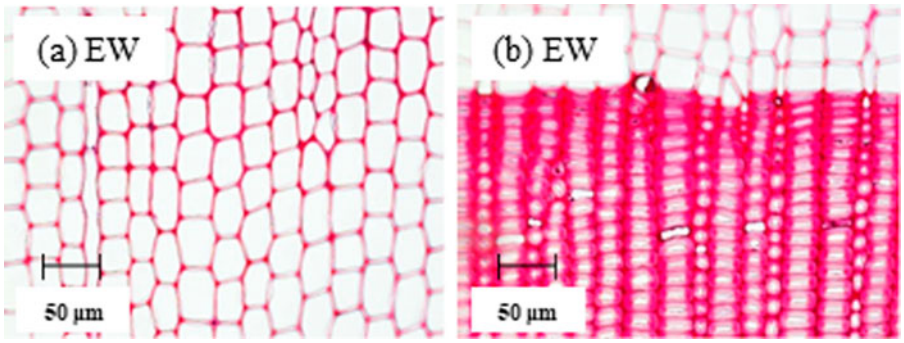


FIGURE 1. Tissue observation of (a) EW and (b) LW cell wall from 35-year-old Ryuunohige sapwood. EW, earlywood; LW, latewood.

dried. The residue was serially washed with deionized water and acetone, and was then used for the subsequent lignin decomposition reaction.

The compositional and quantitative analysis of the monosaccharides in fraction A–D were determined by using HPLC system with TSK-gel Sugar AX1 column after hydrolysis with 2 M trifluoroacetic acid at 120°C for 2 h.[10,11]

Analysis of Hemicelluloses in the α-Cellulose Adsorption Fraction

Using approximately 800 mg of the α-cellulose adsorption fraction, acid hydrolysis of the carbohydrate chains was carried out according to the Klason lignin quantification method. Compositional and quantitative analysis of the monosaccharides included in the reaction liquid was carried out.

RESULTS AND DISCUSSION

Tissue Morphology

Figure 1 shows the transition from EW to LW in a section of 35-year-old Ryuunohige. EW cells had an expanded lumen and thin cell wall. LW cells had a narrow lumen and markedly thickened cell walls. Furthermore, the proportion of LW cells in a given annual ring was estimated to be 20–30% by image analysis, which was comparable to the proportion of LW cells observed in typical Japanese cedar.[25,25]

TABLE 1. Chemical components (%) of EW and LW in Ryuunohige.

Polymers in the cell wall	EW	LW
Cellulose (%)	45.0 ± 1.2	48.3 ± 1.7
Lignin (%)	32.5 ± 0.3	29.5 ± 0.5
Hemicellulose (%)	22.2 ± 1.7	21.8 ± 1.6
GM[a] (%)	68.1 ± 1.5	68.6 ± 2.4
AGX[a] (%)	31.9 ± 1.5	31.4 ± 0.8

Results represent the average ± standard deviation from three independent experiments.
[a]Percentage in hemicellulose. EW, earlywood; LW, latewood; AGX, arabinoglucuronoxylan; GM, glucomannan.

Changes in Chemical Components of the Cell Wall

Chemical components of EW and LW in Ryuunohige are shown in Table 1. The proportion of cellulose was 45.0% in EW and 48.3% in LW. In contrast to cellulose, the proportion of lignin was 32.5% in EW and 29.5% in LW. On the other hand, the proportion of hemicellulose

TABLE 2. Neutral sugar composition (%) in holocellulose of EW and LW.

Neutral sugar composition (%)	Rha	Man	Ara	Gal	Xyl	Glc
EW	Trace	13.9 ± 0.8	2.4 ± 0.2	4.1 ± 0.2	8.7 ± 0.8	71.0 ± 0.5
LW	Trace	13.2 ± 1.2	1.8 ± 0.6	2.8 ± 0.6	8.7 ± 1.6	73.4 ± 2.1

Results represent the average ± standard deviation from three independent experiments.
EW, earlywood; LW, latewood; Rha, rhamnose; Man, mannose; Ara, arabinose; Gal, galactose; Xyl, xylose; Glc, glucose.

was 22.2% in EW and 21.8% in LW. GM/GGM and AGX were almost the same in EW and LW.

We also investigated the sugar units in holocellulose of EW and LW, cell walls, and the results are shown in Table 2. The Ara and Gal content were differed only minor change between EW and LW. The proportion of GM/GGM and AGX making up the total hemicellulose showed almost no change in the transition between EW and LW. Given this result, it is difficult to explain the apparent difference in cell wall structure and properties of EW and LW based on the change in hemicellulose content.

GM/GGM Structure in EW and LW

We isolated GM/GGM from EW and LW cell walls and measured the mean rate of Man and Glc. The sugar composition ratio (Man:Glc:Gal) of GM/GGM differed very slightly between EW (2.7:1:0.1) and LW (2.6:1:0.1). In addition, as shown in Figure 2, very little differences were observed in the molecular chain length of GM between EW and LW. The cell walls of EW and LW differ markedly in morphology, so the GM/GGM composition might be expected to differ between the two. However, this method of analysis did not reveal any clear difference in the molecular structure of GM/GGM. It was also unable to detect changes in the sequence of Glc and Man making up the main chain or the status of acetyl group substitution in Man. Determining the role these differences in GM/GGM play in the formation of EW and LW is an issue remaining for future work.

Changes in AGX Structure in EW and LW

AGX was isolated from EW and LW cell walls, and the mean rate of glucuronic acid (GlcA) (plus 4-O-MeGlcA) and Ara side-chain substitution in AGX were determined. The mean rate of GlcA and 4-O-Me-GlcA side-chain substitution decreased from 33.9 units per 100 Xyl backbone units in EW to 28.6 units in LW. The mean rate of Ara side-chain substitution, determined based on neutral sugar analysis, was 12.1 units per 100 Xyl units in EW and 10.6 units in LW AGX (Figure 3), indicating that the AGX side-chain substitution rate declined with the transition from EW to LW. These results suggested that there is a control mechanism that elaborate between EW and LW for reducing the AGX side-chain substitution rate.

In addition, we investigated the ionic bond characteristics of the AGX GlcA side-chain substituents isolated from EW and LW using anion exchange chromatography (Figure 4). The results of AGX fractionation were conclusive, leading to (1) a low-substitution AGX fraction eluted with 0.2 M NaOAc exhibit-

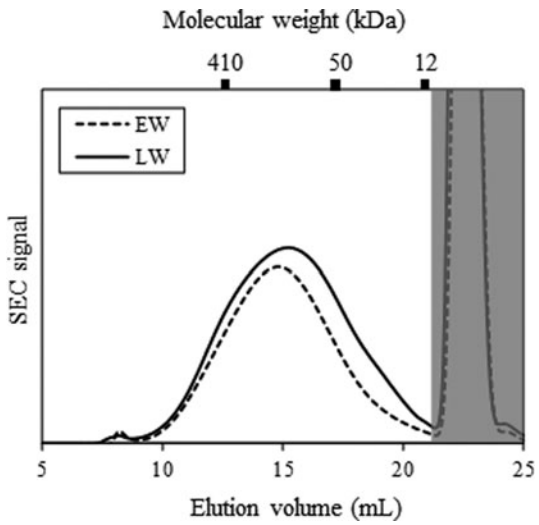


FIGURE 2. SEC of GM from EW and LW. The gray zone was indicated a salt in exclusion volume. GM, glucomannan; EW, earlywood; LW, latewood.

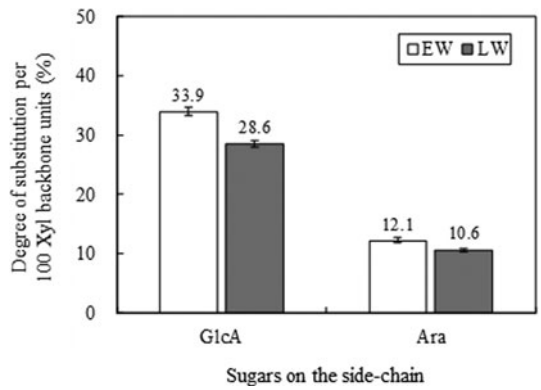


FIGURE 3. Comparison of the average substitution degree of GlcA and Ara for 100 Xyl units in EW and LW. Results represent the average ± standard deviation from two independent experiments. GlcA, glucuronic acid; Ara, arabinose; EW, earlywood; LW, latewood; Xyl, xylose.

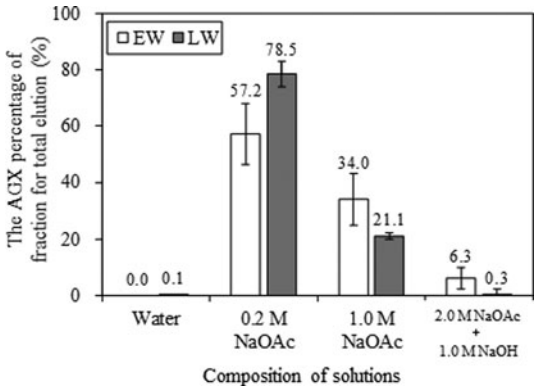


FIGURE 4. Ionic characteristics of AGX from EW and LW. Each fraction was successively obtained in order of water, 0.2 M NaOAc, 1.0 M NaOAc and 2.0 M NaOAc. The percentage of each fraction was recalculated as 100% for total elution sugar contents. Results represent the average ± standard deviation from two independent experiments. AGX, arabinoglucuronoxylan; NaOAc, sodium acetate; EW, earlywood; LW, latewood.

ing the lowest degree of ionic bonding, (2) an intermediate-substitution AGX fraction eluted with 1.0 M NaOAc, and (3) a high-substitution AGX fraction eluted with 2.0 M NaOAc and 1.0 M NaOH. The transition from EW to LW was accompanied by an increase in low-substitution AGX and a decrease in both intermediate-substitution AGX and high-substitution AGX (Figure 4).

The AGX accumulated in the secondary walls of Japanese cedar presumably is comprised of molecules with not only varying rates of substitution, but also chains of varying

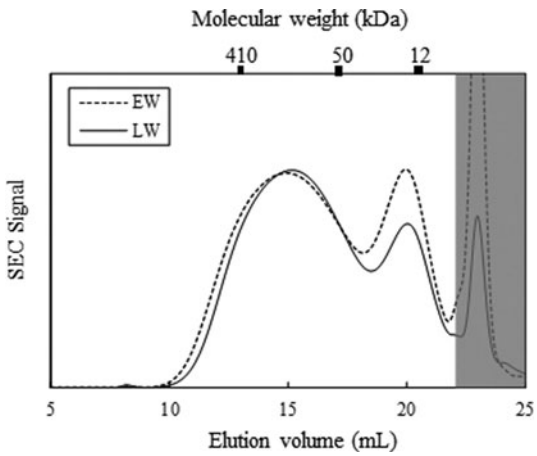


FIGURE 5. SEC of AGX from EW and LW. The gray zone was indicated a salt in exclusion volume. AGX, arabinoglucuronoxylan; EW, earlywood; LW, latewood.

lengths. We investigated the chain-length composition of AGX. As shown in Figure 5, the AGX that accumulated in both EW and LW consisted of long-chain molecular assemblies with a peak at around 250 kDa and short-chain molecules of around 30 kDa. The total share of AGX comprised by short-chain molecular assemblies was clearly higher in EW than LW. The results suggested that the decrease in the side-chain substitution rate and the change in chain length composition of AGX in LW cell walls might be involved in transition from EW to LW.

Changes in Assembly Mode of GM/GGM and AGX in EW and LW

Figure 6 shows the amounts of GM/GGM and AGX sequentially eluted from the cell wall superstructure that was deposited after the formation of the cell wall framework. The hemicelluloses included in fractions A and B are those that would have interacted weakly with other cell wall components, and were in easily

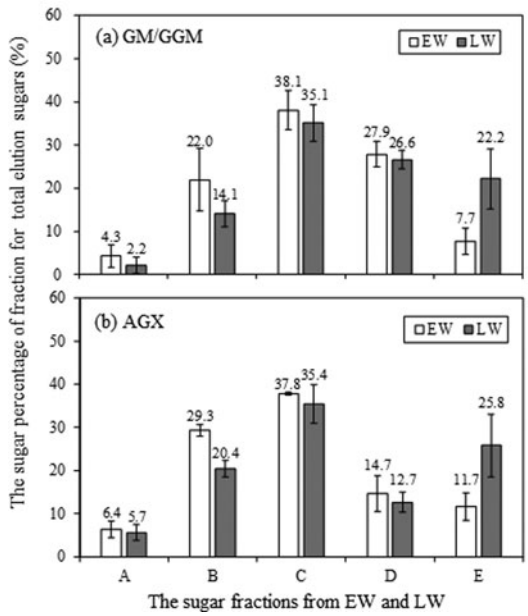


FIGURE 6. Analysis GM and AGX including α-cellulose adsorbing fraction obtained using 18% aqueous NaOH solution from EW and LW. The fractions of A-D were extracted with 18% NaOH solution. The fraction E was hydrolyzed using 72% H_2SO_4 (Klason lignin method). The percentage of each fraction was recalculated as 100% for total sugar contents. Results represent the average ± standard deviation from two independent experiments. GM/GGM, glucomannan/galactoglucomannan; AGX, arabinoglucuronoxylan; EW, earlywood; LW, latewood

released forms. The amounts of GM/GGM and AGX included in fractions A was no difference, whereas fraction B was clearly more abundant GM/GGM and AGX in EW than in LW. The hemicelluloses in fraction C had stronger bonds than those in fractions A and B. In fraction C, the differences in elution quantities between EW and LW was not observed. In fraction D, containing the hemicelluloses presumably assembled through strong interactions between hemicellulose molecules or between hemicellulose and other cell wall components, there was no difference of GM/GGM and AGX between EW and LW. On the other hand, in fraction E, the hemicelluloses that bonded most strongly to the surface of CMFs to form the cell wall framework, the GM/GGM percentage in fraction E was 22.2% in LW and 7.7% in EW. GM/GGM is believed to play an important role in the initial stage of cell wall formation, and there was a marked change in the amount bonding to CMFs in the cell walls of EW and LW. Also, the percentage of AGX included in fraction E was 25.8% of the total for LW, while the percentage for EW was 11.7%. AGX is believed to be involved in the arrangement of CMFs in the cell wall, and thus plays an important role in elaborating the ultrastructure of the cell wall. Like GM/GGM, AGX also is assembled at high density on the surface of CMFs. The AGX included in fraction E bonded to the surface of CMFs through the mediation of GM/GGM. The GM/GGM that bonded to CMFs and was subsequently assembled with AGX thus increased greatly with the transition from EW to LW. These studies showed that hemicellulose assembled at high density at latewood, producing a strong cell wall framework.

CONCLUSIONS

We focused on the differences in the cell walls of EW and LW in Japanese cedar, Ryuunohige, and measured the main chemical components, especially hemicelluloses, using the elution analysis based on the expected strength of the interaction between the main cell wall components. Chemical components of cellulose, hemicelluloses including GM/GGM and AGX, and lignin were almost the same in EW and LW of Ryuunohige. Slight differences in the monosaccharide composition ratio of GM/GGM, the side-chain substitution in AGX and the ionic bond characteristics of AGX GlcA side chains were observed with transition from EW to LW. In contrast, the measurements of GM/GGM and AGX adhesion with CMFs between EW and LW showed that the LW cell walls had more hemicelluloses forming the cell wall framework assembly through strong hydrogen bonds and electrostatic interactions than EW cell walls. The results of the present study indicated that the characterization of GM/GGM and AGX in EW and LW changed greatly. The cell wall structure determines the growth and mechanical strength of the stem xylem in Japanese cedar, so this study is expected to contribute greatly to the understanding of the process of wood formation.

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