WOOD RESEARCH doi.org/10.37763/wr.1336-4561/68.3.413424
ISSN 1336-4561 e-ISSN 2729-8906
68(3): 2023 413-424 pp.

CHANGES IN HEMICELLULOSE STRUCTURE ASSOCIATED WITH THE TRANSITION FROM EARLYWOOD TO LATEWOOD AT JUVENILE WOOD IN *CRYPTOMERIA JAPONICA*

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(RECEIVED JANUARY 2023)

ABSTRACT

The chemical composition and variations in chemical structure of hemicellulose in earlywood (EW) and latewood (LW) of two individual Japanese cedar trees (C-Boka and T-Boka) were investigated. The trees were cultivated under different growth conditions: C-Boka grew slowly in a forest, while T-Boka grew rapidly in a location rich in nutrients and sunshine. For the chemical structure of hemicellulose, arabinoglucuronoxylan (AGX) showed varied side-chain substitution rates with glucuronic acid and different molecular weights in the transition between EW and LW. In contrast, the fundamental composition of glucomannan/galactoglucomannan (GM/GGM) was relatively unchanged between EW and LW. The modification of AGX and GM/GGM from EW to LW differed between C-Boka and T-Boka and might be influenced by the growth rate of the trees.

KEYWORDS: Arabinoglucuronoxylan, earlywood, glucomannan, Japanese cedar, latewood.

INTRODUCTION

Japanese cedar (*Cryptomeria japonica* D. Don) is one of the most important forest tree species in Japan with a number of cultivated varieties. The majority of the cultivars are currently propagated as clones from cuttings of trees exhibiting superior qualities. Nonetheless, even in the cultivars with preserved genetic structure and traits, strength properties, such as Young's modulus, vary markedly among individuals depending on the forest site and growth environment (Fujisawa et al. 1992, 1994, 1995, Hirakawa et al. 1997). The mechanism behind

this variability is unknown. The well-established differences in the cell wall structures between earlywood (EW, thin wall large lumens) and latewood (LW, thick wall small lumens) are also dependent on the growth conditions. One of the factors in the differences of the cell wall structure is suggested to be the variation in wood properties from the core to the outer part of stem xylems. Two different xylems including juvenile wood and mature wood are generally formed depending on the growth year, which determine the wood properties such as density, mechanical strength, and cellulose microfibril angle (CMFA). Especially, the juvenile wood is assumed to be responsible for the properties because it can have higher EW density, lower LW density, shorter tracheid and a larger CMFA (Fujisawa et al. 1993, Hirakawa and Fujisawa 1995, Hirakawa et al. 1997). Previous studies showed that the relationships among wood properties such as Young's modulus, shrinkages, and juvenile wood portions, varied among Japanese cedar varieties (Hirakawa et al. 1997, Yamashita et al. 2009).

Changes in wood properties are related to the cell wall structure and chemical components including cellulose, hemicellulose, and lignin. Terashima et al. (2009) stated that hemicellulose is essential for impacting the assembly of the chemical components, including the aggregation of CMFs and alterations to the CMFA. It has been suggested that CMF aggregation to bundles is controlled mainly by the glucomannan (GM) and galactoglucomannan (GGM), and that the distance between the bundles is determined by the arabinoglucuronoxylan (AGX) moieties of hemicellulose. EW has been shown to have higher lignin content and less cellulose content than LW (Yeh et al. 2004, Bertaud and Holmbom 2004). GM occurs in higher concentrations in thick-walled LW than in EW, whereas AGX has an inverse abundancy (Bertaud and Holmbom 2004). The thick-walled tracheids of LW in Norway spruce (*Picea abies*) also contained more holocellulose (cellulose + hemicellulose) and less lignin than EW (Bertaud and Holmbom 2004). However, investigations on the chemical changes in cell walls with the transition between EW and LW have been limited to the quantification of sugar concentration and composition ratio. Thus, the chemical components and chemical structure of hemicellulose has been poorly investigated between EW and LW of *C. japonica* clones.

The present study focused on the hemicellulose of a single cloned Japanese cedar (hereafter "C-Boka"), which was a 50 years old individual with normal growth speed, by examining the GM/GGM and AGX contents between juvenile EW and LW. For comparison, the hemicellulose of a 15 years old individual of the same clone (hereafter "T-Boka") grown in a sunny field with good nutrition was analyzed. T-Boka grew vigorously, and showed wide annual ring due to wide EW. The expectation is that new insights can be obtained concerning the role of hemicellulose in the cell wall formation by comparing the results of wood properties in *C. japonica*.

MATERIAL AND METHODS

Wood materials

Two trees of the same cloned variety of Japanese cedar (*C. japonica* D. Don), Boka-sugi, were investigated. C-Boka (50 years old, diameter at breast height (DBH) 19.7 cm, tree height 14.1 m) was grown in a forest environment in the University of Tokyo Chiba Forest, Chiba, Japan. T-Boka (15 years old, DBH 29.5 cm, tree height 10.7 m) was grown in a field with favorable nutrition and sunshine in the Forest and Forest Products Research Institute, Ibaraki, Japan. Wood disks of both trees taken at a height of 0.9–1.5 m were used to isolate the EW and LW for chemical composition analysis.

The adjacent EW and LW bands of each clone were separated into thin wafers by cutting along an annual ring with a carving knife. LW was considered the darker material in the last 1 mm of the growth ring. The ambiguous material was removed until the wafer contained only the light EW band and the dark LW band. In case of C-Boka, 12 annual rings between the 5th and 16th rings were used for EW and LW preparations. For the T-Boka juvenile wood, EW and LW samples were prepared from 11 annual rings between 3rd and 13th. Wood samples were sieved through a 40 mesh screen (422 μm pore size) using a Wiley mill before extracting with ethanol: toluene (1:2) for 6 h in a Soxhlet apparatus. The samples taken from the lower adjacent parts of the disks for chemical analysis were used for microscopic examination and physical properties.

Microscopy and mechanical analysis

Small blocks were collected from the juvenile wood in both trees. Cross-sections (10–15 μm thick) were stained with 0.1% aqueous safranin and observed under an optical microscope.

The dynamic module of elasticity (MOE_{dyn}) was measured according to the tapping method using a rectangular of 20 mm (T) x 20 mm (R) x 320 mm (L) in air-dry condition (Sobue 1986). The weights and three dimensions of the wood samples were measured to calculate the wood density. The annual ring width, air-dry density, and MOE_{dyn} are shown in Tab. 1 as basic sample data.

Chemical composition analysis

Klason lignin content of the EW and LW samples obeyed to the gravimetric method of previous reports (Yokoyama et al. 2002, Yeh et al. 2004). The relative sugar composition in the Klason hydrolysate was determined by partition chromatography by the instrument: LC-10AT HPLC, Shimadzu Corp., Japan. Buffer: 0.5% borate-1.0% ethanolamine-hydrochloric acid (pH 7.9) with an ion exchange resin (TSK-gel Sugar AX1 column, Tosoh Corp., Japan) according to a previously described method (Nakamura et al. 1999, Yamasaki et al. 2011). Relative percentages were calculated electronically from duplicate experiments. The cellulose content was determined according to a previous report, as Cellulose (%) = Glucose (Glc) – (1/3 × Mannose (Man)), while the hemicellulose content was calculated as Hemicellulose (%) = (Arabinose (Ara) + Galactose (Gal) + Glc + Man + Xylose (Xyl)) – Cellulose (Jones et al. 2006).

Preparation of AGX and GM/GGM

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 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 (Hashi et al. 1970) and with 10% aqueous potassium hydroxide in the presence of barium hydroxide to isolate AGX (Brink and Pohlman 1972, Yamasaki et al. 2011). The latter extract was adjusted to pH 6.0 with glacial acetic acid and poured into ethanol (4 vol.). The precipitate was collected; washed in succession with 80% ethanol, ethanol, and light petroleum; and dried *in vacuo* over phosphorus pentoxide. The resulting AGX was dissolved in water and titrated with 0.1 M sodium hydroxide (NaOH) to pH 8.0 in a nitrogen atmosphere to determine the equivalent weight. 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 (Nakamura et al. 1999, Yamasaki et al. 2011). The uronic acid content of AGX was determined by the carbazole reaction in sulfuric acid according to a previous study (Blumenkrantz and Asboe-Hansen 1972).

GM/GGM was prepared according to published methods **(**Thornber and Northcote 1962, Kurata et al. 2019). During the AGX preparation, the resulting precipitate in 10% aqueous potassium hydroxide in the presence of barium hydroxide was collected by centrifugation (Yamasaki et al. 2011).

Ion exchange chromatography of AGX

Ion exchange column chromatography was conducted according to a previous study (Kato et al. 1988). AGX (150 mg) was dissolved in 50 mL deionized water and centrifuged. The supernatant was placed on a column (3.0 × 25.0 cm) of a 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 a previous method (Rao and Pattabiraman 1989).

Size-exclusion chromatography (SEC) of AGX and GM/GGM

SEC analysis of hemicellulose was performed according to a previous method (Brown et al. 2011). AGX and GM/GGM were individually analyzed by SEC using a Superose 6 10/300 column (GE Healthcare, USA) at flow rate of 0.5 mL min^{-1} in 50 mM NaOAc using refractive index detection. The column was calibrated using dextran molecular markers corresponding to 410, 50, and 12 kDa.

RESULTS AND DISCUSSION

Tissue morphology and physical properties

As shown in Fig. 1a, the LW from C-Boka was narrow, at less than 10% of the annual ring. In contrast, the mean annual ring width of T-Boka (18.8 mm) was approximately seven times greater than that of the C-Boka juvenile wood. Accordingly, the extremely vigorous growth diminished the transition zone between EW and LW. Therefore, the large reduction of the density and MOE data of T-Boka is not surprising (Tab. 1).

Based on microscopy observation, the transition from EW cells with expanded lumens and thin cell walls to LW cells with narrow lumens and thickened cell walls was clearly visible within a single annual ring (Fig. 1b). Furthermore, the proportion of LW cells in a given annual ring was estimated to be 20 to 30%, which is comparable to that observed in a typical Japanese cedar (Fujisawa et al. 1995). In contrast, the EW cells of T-Boka showed more expanded lumens and thin cell walls occupying a greater proportion of the annual ring, and the transition to LW cells was not pronounced. Even in the LW zone, there were only a few typical LW cells with narrow lumens and thickened cell walls.

Density data and other material properties are also shown in Tab. 1. The density of T-Boka was lower than that of C-Boka. The dependency of anatomical and mechanical properties of Japanese cedar on the growth condition is clear. The juvenile wood of Japanese cedar is suggested to determine the properties because it can have higher density in EW, lower density in LW and shorter tracheids with larger CMFA (Fujisawa et al. 1993, Hirakawa and Fujisawa 1995, Hirakawa et al. 1997). Previous studies reported that the timing of ring formation and development patterns of EW and LW of *Abies balsamea* and variations at the beginning of the growing season, the EW-LW transition, and the end of the growing season were dependent on climate or environmental conditions (Oleksyn and Fritts 1991, Deslauriers et al. 2003).

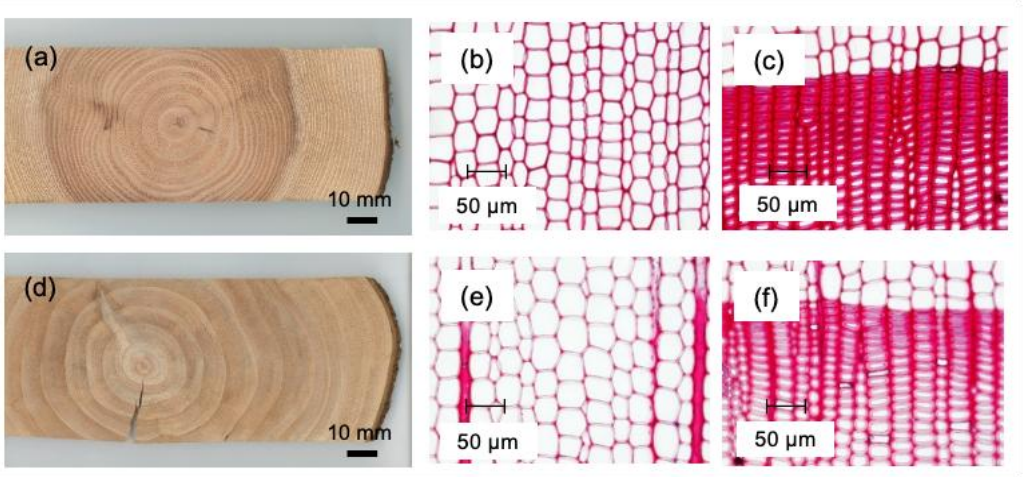

Fig. 1: Wood samples and tissue observation of EW and LW cell walls. (a) C-Boka wood disk, (b) EW cell walls from C-Boka, (c) LW cell walls from C-Boka, (d) T-Boka wood disk, (e) EW cell walls from T-Boka and (f) LW cell walls from T-Boka.

Tab. 1: Sample basic data.

Parameter	C-Boka	T-Boka
Ring width (mm)	2.8	18.8
Air-dry density ($kg \cdot m^{-3}$)	414	280
MOE_{dyn} (GPa)	5.8	1.6

Changes in chemical compositions of the cell wall

To confirm the chemical compositions of EW and LW cell walls in C-Boka and T-Boka, we determined the content of lignin, cellulose, and hemicellulose (Tab. 2). For C-Boka, the proportion of cellulose was approximately 45% for both EW and LW cell walls. The proportion of hemicellulose in C-Boka slightly increased from 22.6% in EW to 23.3% in LW. The lignin content of EW (32.0%) was similar to that of LW (31.6 %). The lignin moiety (31.7% in EW, and 30.4% in LW) did not change significantly. In contrast to C-Boka, the cellulose content for EW in T-Boka was 39.8% and increased to 43.1% in LW. In a similar manner, the proportion of hemicellulose in T-Boka EW (25.7%) increased in LW to 26.7%. The hemicellulose content of LW was higher than that of EW in T-Boka, and the lignin content (34.4% in EW) reduced to 30.3% in LW.

Tab. 2: Chemical compositions of EW and LW in C-Boka and T-Boka.

Polymer in the cell wall	C-Boka		T-Boka	
	EW	LW	EW	LW
Cellulose (%)	45.4 ± 1.1	44.7 ± 1.4	39.8 ± 0.3	43.1 ± 0.9
Lignin (%)	32.0 ± 0.4	31.6 ± 0.5	34.4 ± 0.3	30.3 ± 0.1
Hemicellulose (%)	22.6 ± 1.4	23.3 ± 1.4	25.7 ± 0.0	26.7 ± 0.8
GM/GGM[a] (%)	65.6 ± 1.0	65.1 ± 0.6	67.9 ± 0.0	69.1 ± 1.2
AGX[a] (%)	34.4 ± 1.0	34.9 ± 0.6	32.1 ± 0.0	30.9 ± 1.2

Results represent the average ± standard deviation from three independent experiments. a: percentage in hemicellulose.

Sugar compositions in hemicellulose of EW and LW

We investigated the sugar compositions of GM/GGM and AGX in the hemicellulose of EW and LW in the clones. In C-Boka, the proportion of AGX of hemicellulose was 34.4% in EW and 34.9% in LW (Tab. 2), while T-Boka showed 32.1% in EW and 30.9% in LW. In C-Boka, the proportion of GM/GGM in hemicellulose was 65.6% in EW and 65.1% in LW, while T-Boka juvenile wood presented 67.9% (EW) and 69.1% (LW). A previous study reported notable differences between EW and LW chemical compositions in Norway spruce, where LW contained greater amounts of GM/GGM than EW (Bertaud and Holmbom 2004). Considering the thicker secondary wall (S2) in LW, a higher mannan content can be expected because this compound is preferentially incorporated in the S2 layers.

Structure of AGX in EW and LW

The chemical structure of AGX in the S2 layer of softwoods consists of a linear backbone with β-(1-4)-linked β-D-xylopyranosyl units, some of which are substituted at C-2 with a single 4-*O*-methyl-α-D-glucopyranosyluronic acid (4-*O*-Me-GlcA) or α-D-glucopyranosyluronic acid (GlcA) (Yamasaki et al. 2011). AGX also contains α-L-arabinofuranosyl residues directly

linked to C-3 of xylosyl residues. AGX comprises approximately 30% of the hemicellulose content in Japanese cedar, and generally contains one 4-*O*-Me-GlcA or GlcA side chain per 4-6 xylosyl residues and one arabinosyl residue per 5-12 xylosyl residues. However, the distribution of these side chains along the backbone of softwood is somewhat contentious.

AGX was isolated from the EW and LW of C-Boka juvenile wood, and the mean rate of GlcA (plus 4-*O*-MeGlcA) and Ara side-chain substitution in AGX were ascertained. The mean rates of GlcA and 4-*O*-Me-GlcA decreased from 36.9 units per 100 Xyl backbone units in EW AGX to 29.0 units in LW AGX. The mean rate of Ara side-chain substitution, determined by sugar analysis, was 12.1 units per 100 Xyl units in EW and 8.7 units in LW (Fig. 2a). AGX was isolated from the EW and LW of C-Boka juvenile wood, and the mean rate of GlcA was 32.1 and 29.1 units per 100 Xyl units in EW and LW AGX, respectively. In T-Boka juvenile wood, the GlcA amounts did not change between EW and LW (Fig. 2b) and the Ara side-chain substitution increased slightly from 16.2 to 16.5 units in EW and LW AGX, respectively.

The results of AGX fractionation were also conclusive (Fig. 3), with (1) the low-substitution AGX fraction eluted with 0.2 M NaOAc exhibiting the lowest degree of ionic bonding, followed by (2) the intermediate-substitution AGX fraction eluted with 1.0 M NaOAc, and finally (3) the high-substitution AGX fraction eluted with 2.0 M NaOAc + 1.0 M NaOH. For the C-Boka juvenile wood, the transition from EW to LW was accompanied by an increase in low-substitution AGX and a decrease in both intermediate- and high-substitution AGX (Fig. 3a). For T-Boka juvenile wood, the transition from EW to LW was also accompanied by an increase in low-substitution AGX and a decrease in both intermediate- and high-substitution AGX (Fig. 3b). In addition, AGX isolated from the T-Boka contained a fourth AGX fraction comprising AGX with even higher GlcA side-chain substitution degree, which was eluted with 2.0 M NaOAc + 1.0 M NaOH.

The AGX accumulated in the S2 layer presumably contains not only varying degrees of substitution, but also chains with differing lengths. As illustrated in Fig. 4, the AGX accumulated in juvenile wood consisted of long-chain molecular assemblies with a maximum at approximately 250 kDa and short-chain molecular assemblies with a maximum near 12 kDa. In addition, the transition from EW to LW was accompanied by a decline of short-chained AGX with a maximum close to 12 kDa. This decline was observed with both trees. In C-Boka juvenile wood, the long-chain short-chain molecular AGX was unchanged between EW and LW (Fig. 4a). However, the total share of AGX with short-chain molecular assemblies was clearly higher in T-Boka juvenile wood (Fig. 4b). Clearly, the degree of side-chain substitution and the change in chain length of the juvenile wood AGX backbone was affected by the transition from EW to LW.

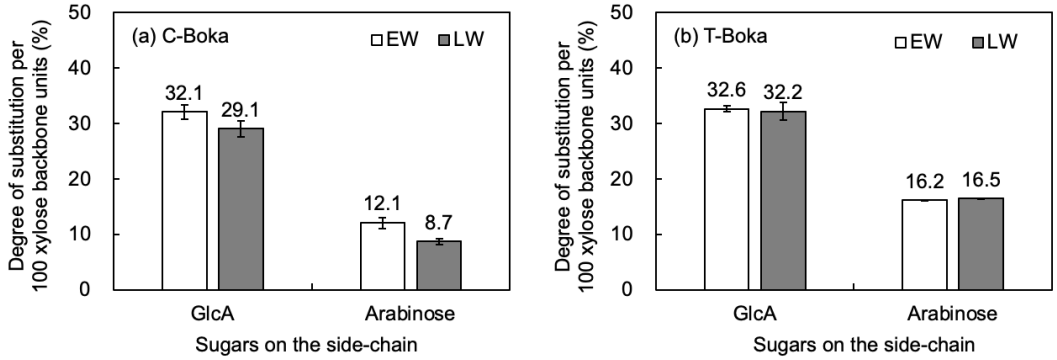

Fig. 2: The average degree of substitution of GlcA and Arabinose per 100 Xyl molecules between EW and LW in (a) C-Boka and (b) T-Boka. Results represent the average ± standard deviation from two independent experiments.

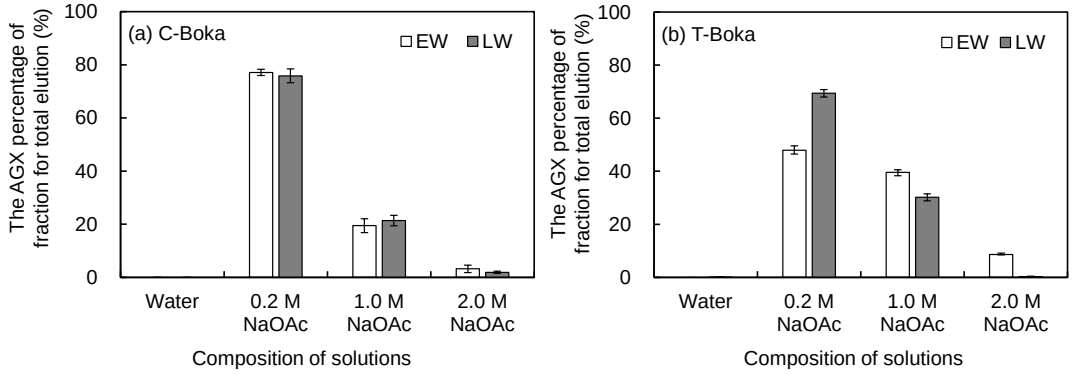

Fig. 3: Ionic characteristics of AGX from EW and LW in (a) C-Boka and (b) T-Boka based on DEAE-Sephadex A-25 column chromatography. Each fraction was successively obtained in order of water, 0.2 M NaOAc, 1.0 M NaOAc, and 2.0 M NaOAc with 1 M NaOH. The AGX percentage of the fraction was recalculated as 100%. Results represent the average ± standard deviation from two independent experiments. AGX, arabinoglucuronoxylan; NaOAc, sodium acetate; EW, earlywood; LW, latewood.

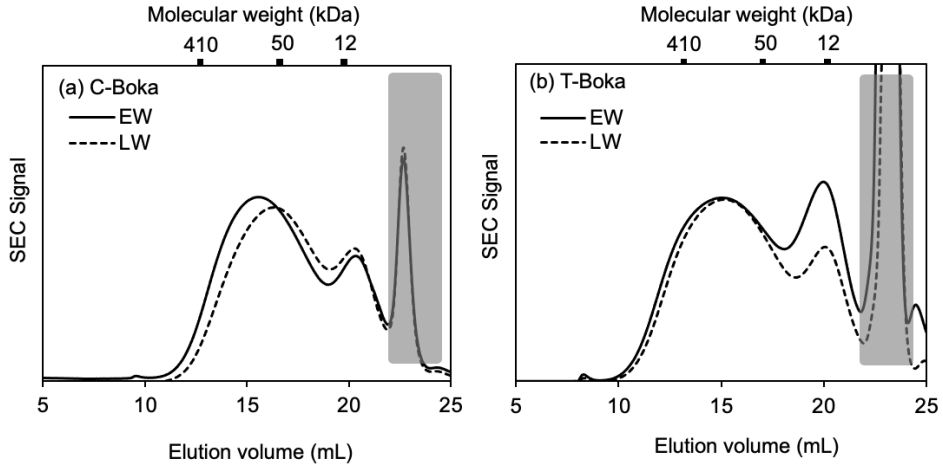

Fig. 4: SEC of AGX from EW and LW of (a) C-Boka and (b) T-Boka. The grey zone is neutral salt from potassium hydroxide. AGX, arabinoglucuronoxylan; EW, earlywood; LW, latewood.

GM/GGM structure in EW and LW

In Japanese cedar, GM/GGMs comprise approximately 70% of hemicellulose and consist of three mannosyl residues per glucosyl residue, with an occasional galactosyl residue linked to the backbone of the GM/GGM (Kim et al. 2010). The ratio of Man to Glc was determined in the two isomers. The sugar composition ratios (Man:Glc:Gal) of GM/GGM in C-Boka juvenile wood differed slightly between EW (3.1:1:0.2) and LW (3.2:1:0.2). For T-Boka juvenile wood, the ratio of Man:Glc:Gal in GM/GGM was 4.3:1:0.4 in EW and 2.8:1:0.4 in LW. Only small differences were observed between EW and LW in C-Boka, whereas the relative proportion of Man, which forms the main chain of GM/GGM, was significantly higher in the EW than in the LW in T-Boka. As observed in Fig. 5, the chain lengths in the EW and LW GM/GGM were also similar. The substitution rates of Gal side chains in both of the EW and LW were higher in T-Boka than that in C-Boka. Accordingly, the side-chain composition was affected similarly in both the slow and rapid growing *C. japonica* trees.

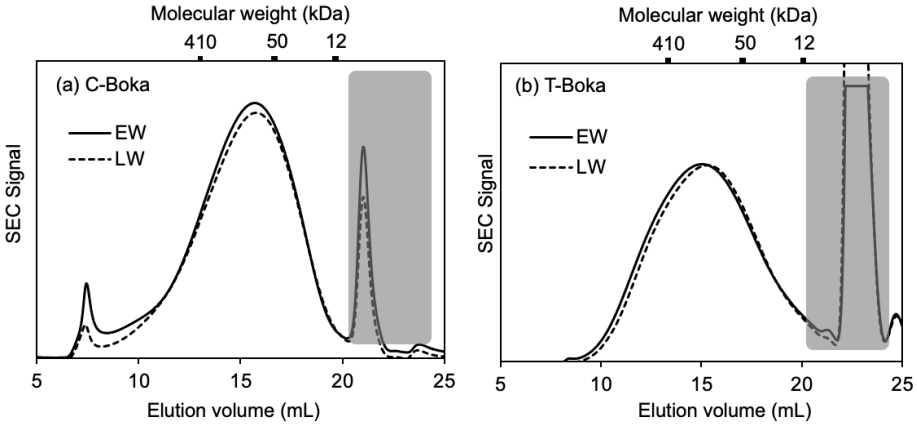

Fig. 5: SEC of GM/GGM from EW and LW in (a) C-Boka and (b) T-Boka. The grey zone is neutral salt from potassium hydroxide. GM/GGM, glucomannan/galactoglucomannan; EW, earlywood; LW, latewood.

CONCLUSIONS

In order to clarify the effect of different growth rates in Japanese cedar, we investigated the chemical composition and hemicellulose structure between EW and LW of juvenile wood. In the slow-growing Japanese cedar (C-Boka), the changes of various cell wall components during the transition from EW to LW were almost unchanged. AGX showed varied side-chain substitution rates with Ara and glucuronic acid in the transition between EW and LW. In contrast, very little change was observed in the fundamental composition of GM/GGM between EW and LW cell walls. T-Boka, with high growth rates, showed the following changes in the course of EW-LW transition: lignin accumulation, change of AGX side-chain substitution and chain length of AGX backbone, and changes in Gal side-chain substitution of GM/GGM main-chain. The modification of AGX and GM/GGM with the transition EW to LW differed between C-Boka and T-Boka and might be influenced by the growth rate of the trees. The expectation is that new insights can be obtained concerning the role of hemicellulose in the cell wall formation by comparing the results of wood properties in *C. japonica*.

ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Number JP26450244. We would like to thank the University of Tokyo Chiba Forest for providing the sample. We would like to thank editage (https://www.editage.jp) for English language editing.

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