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NOTE

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Isolation and structural characterization of rhamnogalacturonan II–borate complex from *Pinus densiflora*

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Abstract A boron-containing pectic polysaccharide was isolated from Driselase digests of akamatsu (*Pinus densiflora*) cell wall. The boron-containing polysaccharide was purified by successive column chromatography of Cosmosil C_{18} OPN, DEAE Sepharose FF, and Superdex 75. The complex had 0.12% (w/w) boron (B) and ^{11}B-NMR spectroscopy showed the complex to be the tetrahedral diester form of boron. The sugar composition and glycosyl linkage analyses showed that the sugar portion of the complex was rhamnogalacturonan-II (RG-II). The boron-attached glycosyl residue was identified to be 2-*O*-Me-xylose-containing apiosyl side chain in RG-II. These results established that the location of borate in the cell wall is conserved among dicots, gramineous monocots, and coniferous gymnosperm.

Key Words *Pinus densiflora* · Gymnosperm · Pectin · Rhamnogalacturonan II · Boron

Introduction

Boron is one of the essential micronutrients for higher plants.[1] Although many physiological and biological functions for boron have been suggested,[2] definitive functions remain unknown. Boron (B) deficiency, which first appears in growing tissues, results in disorganized cell expansion and formation of cell walls with abnormal morphology.[3] Studies have demonstrated that the borate, localized in the cell walls,[4] cross-links two rhamnogalacturonan II (RG-II) to form a dimer of RG-II (dRG-II–B) in radish root,[5] sugar beet,[6] bamboo shoot,[7] pea and sycamore cell walls,[8] and red wine.[9]

A structurally complex pectic polysaccharide, RG-II consists of oligogalacturonide with four different oligoglycosyl side chains.[10] We elucidated that the apiosyl residue of the 2-*O*-Me-Xyl-containing side chain, not the apiosyl residue of the aceric acid-containing side chain, is esterified with borate in the primary cell walls of dicots, monocots, and *in vitro* synthesized dRG-II–B.[11] RG-II from gymnosperms was isolated and characterized from cell walls of suspension-cultured Douglas fir[12] and the cambial zone of sugi.[13] As far as we know, there is no report on isolation of the RG-II–B complex from coniferous gymnosperms. This paper describes the structure of the RG-II–B complex isolated from the etiolated whole hypocotyls of akamatsu (*Pinus densiflora* S. et Z.) and the determination of the borate-linked glycosyl residues in RG-II–B.

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Materials and methods

Plant material and cell wall preparation

Seeds of akamatsu (*Pinus densiflora*) were germinated and grown at 25°C under white light. Whole hypocotyls were harvested 14 days after sowing. The cell wall (5 g) was isolated as alcohol-insoluble residues.[14]

Isolation of RG-II complex

Akamatsu cell walls (5 g) were suspended in 250 ml of deionized water, and the pH was adjusted to 5.0 with diluted AcOH. A solution of Driselase (750 µl, containing 75 mg Driselase in 50 mM sodium acetate buffer, pH 5.0) was added to the cell wall suspended solution, and the reaction continued at 30°C for 16 h.[15] The soluble fraction was immediately passed through a Cosmosil C_{18} OPN (Nakarai) column (1.8 × 20 cm) equilibrated with deionized water at a flow rate of 1 ml/min. The eluate was fractionated into 8-ml

portions until the sugar was not detected. The total sugar content in each fraction was measured by the phenol-sulfuric acid method.[16] The sugar-containing fraction was concentrated to 100 ml by rotary evaporator at 40°C and then centrifuged at 10000 *g* for 20 min to remove insoluble material. The supernatant was adjusted to pH 5.3 with dilute ammonia solution and put on a column (1.8 × 20 cm) of DEAE Sepharose FF (Pharmacia) equilibrated with 50 mM $HCOONH_4$ buffer (pH 5.3). The sugar in the column was eluted with a linear gradient of 50 mM to 1 M $HCOONH_4$ buffer (total volume 500 ml) at a flow rate of 20 ml/h. The eluate was fractionated into 5-ml portions. The total sugar in each fraction was measured by the phenol-sulfuric acid method. In addition, the amount of 3-deoxy-D-*manno*-octulosonic acid (Kdo) and 3-deoxy-D-*lyxo*-heptulosaric acid (Dha) in each fraction was determined by the modified thiobarbituric acid assay.[17] The Kdo-containing fraction was collected and concentrated. The presence of boron in this Kdo-containing fraction from the DEAE Sepharose FF column was analyzed by nuclear magnetic resonance (^{11}B-NMR) spectroscopy as described previously.[18] The sugar composition was also checked at this stage. The concentrate thus obtained was further purified by a Superdex 75 (Pharmacia) column equilibrated with 50 mM $HCOONH_4$ buffer (pH 5.3) using the high-performance liguid chromatography (HPLC) system of Shimazu LC 6A. The column was eluted at a flow rate of 0.6 ml/min, and the sugar was detected with a refractive index detector (Shimazu model RID-6A). The purified RG-II–B complex was concentrated, dialyzed with a mol-cutoff 1000 dialysis tube against deionized water, and lyophilized.

Boron determination

The boron concentration was determined using an inductively coupled plasma mass spectrometer SII SPQ 9000 (Seiko Instruments).[6]

Molecular weight determination of RG-II

The molecular weight of RG-II monomer and dRG-II–B complex was determined using Superdex 75. Pullulan narrow molecular weight (M*w*) standards (P-5, M*w* 5800; P-10, 12200; P-20, 23700) (Showa Denko K. K.) were used for the calibration.

Glycosyl composition and glycosyl linkage analyses

Glycosyl residue compositions were determined as trimethylsilyl methyl ester methyl glycoside derivatives by GC-14A GLC (Shimazu) equipped with a DB-1 column (0.25 mm × 30 m). Combined neutral sugars and apiose were analyzed as alditol acetate derivatives by gas-liquid chromatography (GLC) equipped with a SP-2330 column (0.25 mm × 30 m).[14]

Glycosyl linkage compositions were analyzed by GLC and GLC-MS (mass spectrometry). The sample was methylated by a modified method[19] of Hakomori.[20] The methyl esters of uronic acid residues in the per-*O*-methylated sample were reduced with Super-Deuteride (Aldrich) as described.[14] The reduced partially *O*-methylated sample was hydrolyzed and converted to their alditol acetate derivatives. GLC-MS was carried out as described previously with a JEOL JMS-DX303HF mass spectrometer coupled with a Hewlett-Packard 5890 gas chromatograph with a SP-2330 column interfaced to an MS-MP7000/MP7010 work station.[21]

Determination of borate esterified side chain

Partial acid hydrolysis of per *O*-methylated and carboxy-reduced RG-II was done with 88% HCOOH at 70°C for 2 h.[11] After removal of acid by co-evaporation with *iso*-propanol, the hydrolyzate was reduced with $NaBD_4$. The partial methylated glycosyl alditols thus obtained were acetylated and analyzed by chemical ionization (CI) and electron impact (EI)-mode GLC-MS.[21]

Results and discussion

Isolation of akamatsu boron–polysaccharide complex

Akamatsu cell wall, prepared as alcohol-insoluble residue, contained boron at 20.8 μg/g. The wall was digested with Driselase, and the borate complex was isolated from the digest by sequential column chromatography of Cosmosil C_{18} OPN and DEAE Sepharose FF. ^{11}B-NMR spectroscopy showed a sharp peak at δ-9.45, indicating that borate was present as tetrahedral borate-diol diester.[18] No other substantial peak, such as δ-0.00, which indicates the presence of free boric acid, was observed (data not shown). The boron–polysaccharide complex was further purified by Superdex 75 column chromatography to exclude a small amount of contamination by the RG-II monomer.

Characterization of akamatsu boron–polysaccharide complex

The sugar composition of the purified boron–polysaccharide complex from akamatsu cell wall is summarized in Table 1. It contained 2-*O*-Me-Xyl, 2-*O*-Me-Fuc, Api, aceric acid, Kdo, and Dha in addition to Gal, Rha, Fuc, GalA, and GlcA residues. The mol% of each glycosyl residue resembled those of dRG-II–B isolated from sugar beet[6] and bamboo shoot.[7] Glycosyl linkage analysis (Table 2) showed the presence of RG-II characteristic linkages, such as 3-linked and 2,3,4-linked Rha, 3,4-linked Fuc, and 2-linked GlcA.[10] These results showed the polysaccharide moiety of akamatsu boron–polysaccharide complex to be RG-II.

The akamatsu dRG-II–B contained 0.12% boron (w/w). This value is almost the same as those of sugar beet[6] and bamboo shoot[7] dRG-II–B.

The molecular weight of akamatsu RG-II–B complex determined by size exclusion chromatography (SEC) was

Table 1. Glycosyl residue composition of dRG-II–boron from akamatsu, sugar beet, and bamboo shoot cell walls

Glycosyl residue	Composition of dRG-II–boron (mol%)		
	Akamatsu	Sugar beet[a]	Bamboo[b]
Rha	13.3	11.3	18.6
Fuc	2.2	1.6	3.2
2-*O*-Me-Fuc	5.8	3.3	4.6
Ara	12.2	10.9	12.1
2-*O*-Me-Xyl	5.3	4.9	4.5
Api	6.7	4.5	6.7
Gal	5.5	12.4	8.3
GlcA	2.2	7.0	3.2
GalA	37.7	37.7	28.7
Aceric acid	–	–	–
3-Deoxy sugar[c]	5.6	5.3	8.3

–, present but not quantified
[a]Ishii and Matsunaga[6]
[b]Kaneko et al.[7]
[c]Kdo and Dha

Table 2. Glycosyl linkage composition of dRG-II–boron from akamatsu, sugar beet, and bamboo shoot cell walls

Glycosyl residue[a]	Composition of dRG-II–boron (mol %)		
	Akamatsu	Sugar beet[b]	Bamboo[c]
Rha			
T-Rha	4.0	4.5	4.6
2-Rha	3.2	4.3	2.3
3-Rha	4.7	5.3	5.1
2,3-Rha	ND	0.8	1.6
2,4-Rha	ND	0.9	0.8
2,3,4-Rha	11.3	6.5	5.2
Fuc			
3,4-Fuc	5.4	3.3	5.8
2-*O*-Me-Fuc			
T-Fuc	3.0	5.6	6.7
Api			
3′-Api	7.8	5.0	5.2
2,3,3′-Api	5.8	ND	ND
Ara			
TAra*f*	6.6	6.3	6.4
T-Ara*p*	1.3	2.5	ND
2-*O*-Me-Xyl			
T-Xyl	4.4	4.1	ND
Gal			
T-Gal	4.1	5.0	11.0
2,4-Gal	4.0	5.2	7.6
3,4-Gal	5.1	4.3	ND
3,6-Gal	ND	0.3	ND
GalA			
T-GalA	5.9	10.0	7.2
4-GalA	6.6	14.3	5.6
3,4-GalA	3.7	ND	10.7
2,4-GalA	3.4	ND	4.8
2,3,4-GalA	5.5	4.7	7.8
GlcA			
2-GlcA	4.4	7.1	3.1

ND, not determined
[a]T, nonreducing terminal
[b]Ishii and Matsunaga[6]
[c]Kaneko et al.[7]

9200. It was decreased to half by acid treatment with 0.5 N HCl at 30°C for 30 min, suggesting that 1 mol of boron cross-linked 2 mol of RG-II to form the dimer RG-II.

Borate location of akamatsu dRG-II–B

The molar ratio of 3′-linked and 2,3,3′-linked apiosyl residues was almost equal (Table 2), indicating that 3'-linked apiosyl was the borate-linked glycosyl residue in RG-II.

To determine the borate-esterified side chains, akamatsu dRG-II–B, which has four apiosyl residues, was *O*-methylated, carboxyl-reduced, and then partially hydrolyzed by HCOOH.[11] Because the borate-diol ester linkages are stable during methylation and carboxyl reduction of dRG-II–B, borate-esterified apiosyl residues were not methylated whereas nonesterified apiosyl residues were *O*-methylated. Formolysis generated a mixture of partially methylated oligoglycoses, including the partially methylated disaccharide Rha-(1 → 3′)-Api. The methylated oligoglycoses were converted to their corresponding partially methylated oligoglycosyl alditols, and newly exposed hydroxyl groups were *O*-acetylated. The partially *O*-methylated alditol from a borate esterified 3′-linked Api residue is acetylated at *O*-1, *O*-2, *O*-3, and *O*-4, whereas the partially *O*-methylated alditol from an unesterified 3′-linked Api residue would be acetylated at *O*-1 and *O*-4 and *O*-methylated at *O*-2 and *O*-3. The Rha residue originating from 2-*O*-Me-Xyl-containing the apiose side chain would be acetylated at *O*-2, *O*-3, and *O*-4. The Rha residue originating from aceric acid-containing the apiose side chain is methylated at *O*-2 and *O*-4 and acetylated at *O*-3. These Rha-(1 → 3′)-apitol derivatives differ in the number and positions of *O*-methyl and *O*-acetyl groups and are distinguished by using GC-CI-MS to monitor their $[M + NH_4]^+$ ions. The location of the *O*-methyl and *O*-acetyl groups are defined by the primary fragment ions in the GC-EI mass spectrum of the partially *O*-methylated and partially *O*-acetylated derivatives. As shown in Fig. 1, quasimolecular ions $[M + NH_4]^+$ of *m/z* 611 and 499 were detected, whereas the ion of *m/z* 555 was not shown. The quasimolecular ion of *m/z* 611 is 2,3,4-linked Rha-(1 → 3′)-apiitol derived from the borate-linked 2-*O*-Me-Xyl-containing side chain. The quasimolecular ion of *m/z* 499 is 3-linked-Rha-(1 → 3′)-apiitol derived from the nonborated aceric acid-containing side chain. On the other hand, the ion of *m/z* 555 is 3-linked Rha-(1 → 3′)-apiitol from the borate-linked aceric acid-containing side chain or 2,3,4-linked Rha-(1 → 3′)-apiitol from the nonborated 2-*O*-Me-Xyl-containing side chain. The GC-EI mass spectra confirmed the structure of these partially methylated and partially acetylated Rha-(1 → 3′)-apitol derivatives (data not shown). Consequently, only the apiosyl residue of the 2-*O*-Me-Xyl-containing side chain of akamatsu dRG-II–B is esterified with borate.

Boron is localized in RG-II to form dimer RG-II of radish root,[5] sugar beet,[6] bamboo shoot,[7] pea and sycamore,[8] and red wine.[9] Recently, the borate location of dRG-II–B from sugar beet, potato, red wine, and bamboo shoot was identified to be a 2-*O*-Me-Xyl-containing apiose

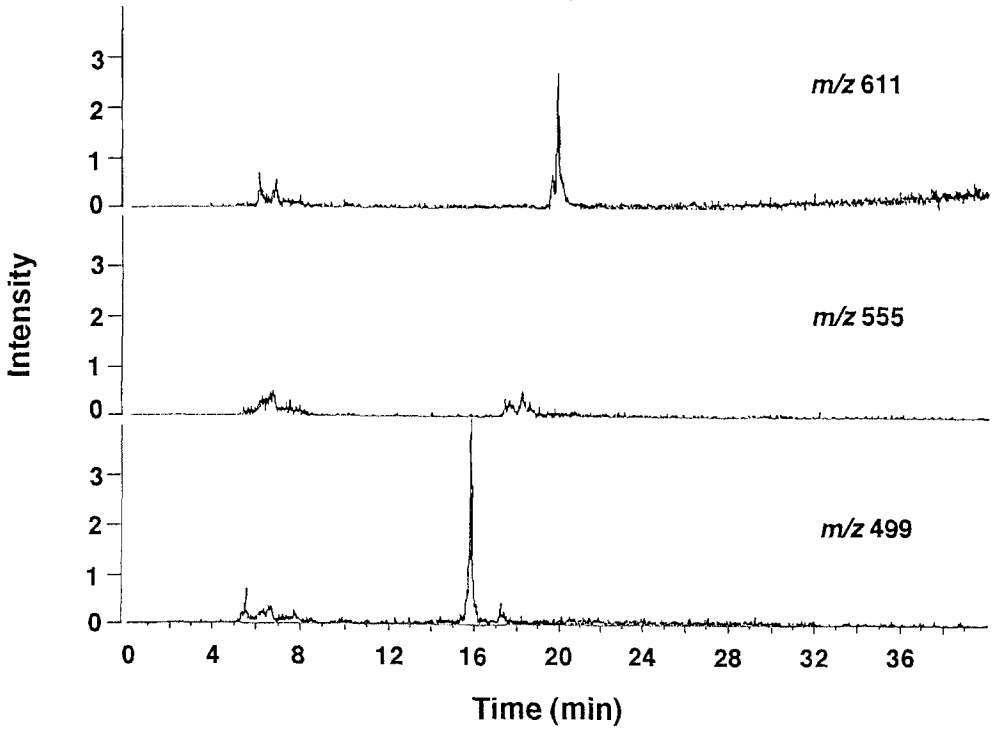

Fig. 1. Total ion chromatograms of chemical ionization (CI) mass spectra of akamatsu dRG-II–B derivatives. Selected quasimolecular ions $[M + NH_4]^+$ of *m/z* 611, 555, and 499 were monitored

side chain of RG-II.[11] In this paper we showed that the borate location site of akamatsu dRG-II–B was exactly the same as that of dicots and monocots. RG-II was isolated and characterized from Douglas fir[12] and sugi.[13] The structure of these two gymnosperm RG-IIs was similar to that of sycamore. Although dRG-II–B was not isolated from these two gymnosperm species, our present results provide evidence that the borate location of dRG-II–B was the same among dicots, gramineous monocots, and gymnosperms.

Conclusion

Akamatsu cell wall was enzymatically digested with Driselase, and a boron–polysaccharide complex was isolated. The complex is a borate cross-linked RG-II dimer. The borate-located glycosyl residue in akamatsu dRG-II–B was identified as the apiosyl residue, and only the apiosyl residue in the 2-*O*-Me-Xyl-containing side chain is esterified with boron. The structure of akamatsu dRG-II–B is the same as that isolated from sugar beet (dicots) and bamboo shoot (monocots). These results support the universality of a borate location in the cell wall among dicots, gramineous monocots, and coniferous gymnosperm.

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