Pergamon

0031-9422(95)00958-2

Phytochemistry, Vol. 42, No. 3, pp. 611–616, 1996

0031-9422/96 $15.00 + 0.00

PECTIC POLYSACCHARIDES FROM XYLEM-DIFFERENTIATING ZONE OF *CRYPTOMERIA JAPONICA*

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(*Received in revised form* 13 *November* 1995)

Key Word Index—*Cryptomeria japonica*; Taxodiaceae; sugi; xylem-differentiating zone; cell walls; pectic polysaccharide; rhamnogalacturonan.

Abstract—Pectin was extracted from the cell walls of xylem-differentiating zones of sugi (*Cryptomeria japonica*) with the chelating agent, *trans*-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid at 20° and Na_2CO_3 solution at 1° and 20°. The extracted pectin fractions were purified by anion-exchange chromatography and digested with pure *endo*-polygalacturonase from *Aspergillus niger.* The hydrolysates were then fractionated by gel permeation chromatography into three or four fractions. Sugar composition and glycosyl linkage composition analyses of each fraction showed that the void volume fractions contained 2- and 2,4-linked rhamnosyl, 5-linked arabinosyl, terminal galactosyl and 4-linked galacturonic acid residues, indicating that they were rhamnogalacturonan I. The fractions obtained from the included volume had rare sugar residues, such as apiosyl, 2-*O*-methyl-L-fucosyl, traces of 3-*O*-methyl-L-fucosyl, 2-*O*-methyl-D-xylosyl residues, aceric acid and thiobarbituric acid assay-positive sugar residues, showing that these fractions contained rhamnogalacturonan II-like polysaccharides. Several linear oligosaccharides composed of (1→4)-linked α-D-galacturonic acid residues were purified from the lowest M_r regions and some oligogalacturonides were characterized by methylation analysis, FAB-mass spectrometry and NMR.

INTRODUCTION

Pectin is one of the main components of primary walls of higher plants and is an extremely complex polysaccharide. Pectin in the primary walls consists of homogalacturonan and rhamnogalacturonans I and II (RG-I and RG-II) [1]. The structure of the pectin gel matrix is important in determining the strength and flexibility of plant cell walls [2]. A series of oligogalacturonides that are released by partial hydrolysis of homogalacturonan region in the pectin have been recognized as biologically active oligosaccharides (oligosaccharins) [3]. These oligomers are able to regulate a number of physiological responses in plants, including increase of phenylalanine ammonia lyase activity, proteinase inhibitor production, lignification, phytoalexin production, etc. [3].

Previous structural analyses of pectic polysaccharides have been performed using pectin fractions obtained from commercially important sources, such as apple, sugar beet and citrus pulp [4]. Since these materials contain a variety of cell types, it could not be determined which pectins were of the primary wall origin. Thornber and Northcote examined the change in the chemical composition of cambial cells in four tree species, birch (*Betula platyphylla*), pine (*Pinus poderosa*), sycamore (*Acer pseudoplatanus*) and ash (*Fraxinus eladitor*), but they did not study the properties of pectins [5–7]. Simson and Timell isolated a pectin composed of (1→4)-linked α-D-galacturonic acid residues interspersed with α-L-rhamnopyranose units from the cambial tissues of basswood (*Tilia americana*) and aspen (*Populus tremloides*) [8]. Albersheim and his co-workers have extensively studied the structure of cell wall polysaccharides using suspension-cultured cells of sycamore, Douglas fir (*Pseudotsuga menziesii*), rice (*Oryza sativa*), maize (*Zea mays*) and other species [1]. Thomas *et al.* [9] reported that glycosyl linkage compositions of the pectins isolated from the cell walls of suspension-cultured Douglas fir cells were similar to RG-I and RG-II from sycamore. However, there are few reports on the structure of pectin from xylem-differentiating zones of conifers.

In a previous paper, we isolated cell wall polysaccharides of xylem-differentiating zones of sugi (*Cryptomeria japonica* D. Don) and characterized the main structural polysaccharides using methylation analysis [10]. We now report the isolation and characterization of RG-I, RG-II and oligogalacturonides derived from homogalacturonan from the cell wall polysaccharides of xylem-differentiating zones of sugi.

RESULTS AND DISCUSSION

Extracts obtained with 0.05 M *trans*-1,2-cyclohexanediamine-*N,N,N',N'*-tetraacetic acid (CDTA-I and

Table 1. Sugar composition of CDTA and Na_2CO_3 extracts from cell walls of xylem-differentiating zones of sugi

Fractions	Yield* (%)	Glycosyl composition (mol %)†								
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A	Glc A
CDTA-I	6.8	3.4	0.2	36.6	5.5	1.5	21.4	0.5	28.7	2.5
CDTA-II	3.9	6.0	0.8	47.5	4.2	2.6	16.1	—‡	22.7	—
Na_2CO_3-I	7.1	4.9	0.5	32.0	—	—	10.8	—	51.8	—
Na_2CO_3-II	6.4	6.6	0.3	41.0	—	—	26.3	—	25.7	—

*Weight % of cell walls.

†Sugar composition obtained by hydrolysis with methanolic HCl at 80° for 16 hr.

‡Not detected.

CDTA-II) and 0.05 M Na_2CO_3 (Na_2CO_3-I and Na_2CO_3-II) (see Experimental) amounted to *ca* 24% of the cell walls of the xylem-differentiating zones of sugi (Table 1). GC analysis of the trimethylsilyl derivatives from the CDTA and Na_2CO_3 extracts showed that they were rich in arabinosyl, galactosyl and galacturonic acid residues. Glycosyl linkage composition analysis of the extracts (Table 2) revealed that they contained 2- and 2,4-linked rhamnosyl, 5-linked arabinosoyl, terminal galactosyl and 4-linked galacturonic acid residues, indicating that the extracts contained RG-I. There were some differences noted in glycosyl composition (Tables 1 and 2). Glycosyluronic acid linkages are resistant to acid hydrolysis [11]. As the sugar compositions in Table 1 were determined by hydrolysis with 5% methanolic HCl at 80° for 16 hr without reduction of uronic acid residues, uronic acid residues of pectic polysaccharides could not be hydrolysed completely. On the other hand, the glycosyl linkage compositions in Tables 2 and 3 were determined by hydrolysis with 2 M trifluoroacetic acid at 121° for 1 hr after conversion of uronic acid residues into the corresponding 6,6-dideuteriohexosyl residues, and the samples were hydrolysed completely. Differences between hydrolysis rates of uronic acid and hexosyl residues may be the cause of the discrepancies of glycosyl composition given in Tables 1 and 2.

The CDTA and Na_2CO_3 extracts were separated by anion-exchange chromatography and purified extracts were then digested with *endo*-polygalacturonase (EPGase) to degrade homogalacturonan fractions. Separation of RG-I, RG-II and oligogalacturonides from the enzymic hydrolysates by gel permeation chromatography was insufficient, if the extracts were treated with

Table 2. Glycosyl linkage composition (mol %) of CDTA and Na_2CO_3 extracts from cell walls of the xylem-differentiating zones of sugi

Glycosyl residue	Linkage	CDTA-I	CDTA-II	Na_2CO_3-I	Na_2CO_3-II
Rhamnosyl	Terminal-	1.0	1.5	0.4	0.3
	2-linked*	+†	+	2.1	7.2
	2,4-	+	+	3.2	8.1
Fucosyl	Terminal-*	+	+	0.9	0.9
Arabinosyl	Terminal-	3.7	8.5	3.7	15.5
	2-linked*	+	+	+	0.8
	3-*	6.5	+	3.2	7.8
	5-	5.0	4.1	6.4	29.3
	2,5-	3.5	+	+	4.8
Xylosyl	4-Linked	3.8	2.4	0.6	+
Mannosyl	4-Linked	5.5	6.6	0.6	+
Galactosyl	Terminal-	15.8	21.5	5.9	12.3
	3-linked	+	+	1.9	3.7
	4-	6.3	8.3	+	5.7
	6-	6.0	4.5	0.7	0.6
	2,4-	+	+	1.0	0.7
	3,6-	5.4	5.4	+	+
	4,6-	5.2	6.6	2.5	+
Glucosyl	3-Linked	+	0.3	+	+
	4-	+	2.1	2.0	+
	6-	1.4	+	+	+
Gal A	Terminal-	5.7	4.2	1.4	1.2
	4-linked	20.6	16.3	57.2	10.2
	2,4-	+	3.5	3.3	0.8
	3,4-	4.6	5.0	3.6	+

*Glycosyl residues detected but could not be quantified precisely because peaks overlapped contaminant peaks on GC analysis.

†Detected but not determined quantitatively.

EPGase without saponification (data not shown). The CDTA and Na_2CO_3 extracts were saponified with dilute NaOH solution to degrade ester bonds and the de-esterified extracts were separated by anion-exchange chromatography. De-esterified pectic polysaccharides were digested with EPGase, and the hydrolysates were fractionated by gel permeation chromatography (Fig. 1). The glycosyl linkage composition of these fractions is shown in Table 3. Reduction of uronic acid residues with Super-Deuteride to 6,6-dideuteriohexosyl residues was carried out in order to determine the linkage positions of uronic acid residues. Some unknown peaks, probably from contaminants in the Super-Deuteride, appeared in the regions of 2-linked rhamnosyl, terminal-fucosyl, terminal-xylosyl, and 2- and 3-linked arabinosyl residues on the chromatogram. As available samples for methylation analysis were limited, e.g., fractions A-II, B-II, C-II and D-II, some peaks of the above mentioned glycosyl residues overlapped the contaminant peaks. However, it was possible to identify the glycosyl residue peaks, but it was impossible to quantify them (peaks which could be detected but not determined quantitatively are designated '+' in Tables 2 and 3). As residual CDTA could not be removed by exhaustive dialysis [12] and the CDTA extracts (CDTA-I and -II) were not completely soluble in dimethyl sulphoxide (DMSO), complete methylation could not be performed. On the other hand, enzymic hydrolysate fractions of the CDTA extracts dissolved in DMSO completely and per-*O*-methylation could be performed. The discrepancies of glycosyl linkage compositions between CDTA extracts and their fractions in Tables 2 and 3 might be due to the difference in solubility in DMSO. The fractions at the lowest M_r region (A-III, B-III, C-IV and D-III) were composed of galacturonic acid residues and the main glycosyl linkages were terminal and 4-linked galacturonic acid residues. The results showed that these fractions were oligogalacturonides derived from homogalacturonan. Separation of the oligosaccharides from these fractions using anion-exchange and gel permeation chromatography gave a series of α-(1$\rightarrow$4)-linked oligogalacturonides from dimer to heptamer. The structures of the oligogalacturonides were characterized by FAB-mass spectrometry and NMR spectrometry (data not shown).

The void volume fractions of each extract (A-I, B-I, C-I and D-I) consisted mainly of 2- and 2,4-linked rhamnosyl, 5-linked arabinosyl, terminal galactosyl and 4-linked galacturonic acid residues and contained no glycosyl residues characteristic of RG-II [1]. These results indicated that the fractions were RG-I. Glycosyl composition analysis of the fractions, C-I, C-II and C-III, showed that C-II and C-III contained 2-*O*-methyl-L-fucosyl, a trace amount of 3-*O*-methyl-L-

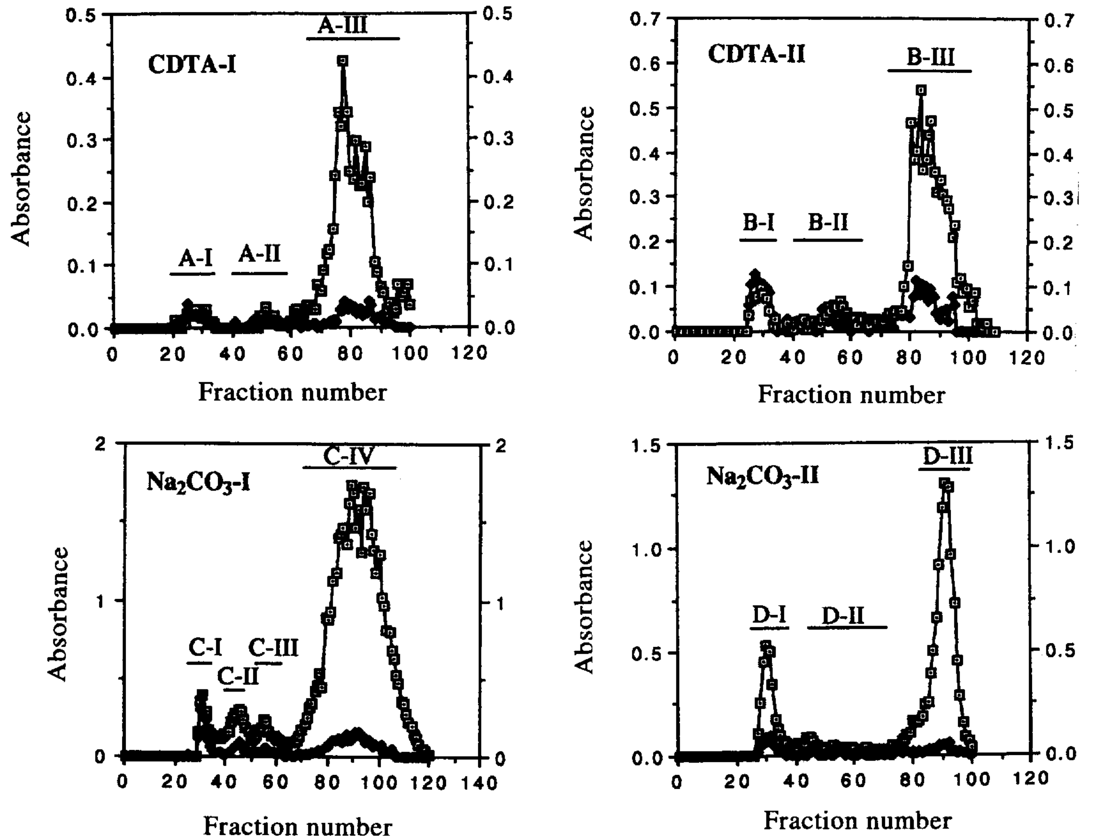

Fig. 1. Gel permeation chromatography on Bio-Gel P-30 of EPGase-treated pectic polysaccharide fractions. Hexosyl and uronic acid residues determined by anthrone (■) and *m*-hydroxybiphenyl (□) assays, respectively.

Table 3. Glycosyl linkage composition (mol %) of fractions from enzymic hydrolysates of pectic polysaccharides separated by Bio-Gel P-30 gel permeation chromatography

Glycosyl residue	Linkage	CDTA-I (A†)			CDTA-II (B†)			Na_2CO_3–I (C†)				Na_2CO_3–II (D†)		
		A-I	A-II	A-III	B-I	B-II	B-III	C-I	C-II	C-III	C-IV	D-I	D-II	D-III
Rhamnosyl	Terminal-	0.2	3.4	–§	0.2	3.9	–	0.2	3.7	4.5	0.8	–	2.5	0.9
	2-linked*	9.6	+‡	–	9.9	+	–	5.7	+	1.3	–	+	+	–
	3-*	+	+	–	+	+	–	+	+	1.2	–	+	+	–
	2,3-	3.4	–	0.1	trace	–	–	0.9	–	3.3	–	–	–	–
	2,4-	9.1	–	–	9.8	2.2	–	9.9	5.6	5.3	–	7.6	9.4	–
Fucosyl	Terminal-*	0.6	+	–	0.6	1.2	–	0.5	+	4.0	–	+	4.4	–
Arabinosyl	Terminal-	5.6	11.7	0.1	11.8	4.6	0.8	11.9	8.1	6.0	–	10.9	7.7	0.6
	3-linked*	+	+	–	+	+	–	+	+	+	–	+	+	–
	5-	24.6	trace	trace	26.2	15.6	trace	25.7	21.2	9.3	–	20.8	16.8	2.5
	2,5-	3.7	–	–	2.9	–	–	4.5	–	1.8	–	4.3	–	–
Xylosyl	Terminal-*	+	+	–	+	+	–	0.2	0.8	1.9	–	+	3.0	–
	2-linked	trace	–	–	–	5.7	–	trace	–	–	–	–	–	–
	4-	0.1	–	trace	–	–	–	0.1	trace	4.9	–	–	2.9	–
	2,3-	–	–	–	–	–	–	–	1.0	–	–	–	–	–
	3,4-	–	–	–	–	–	–	–	1.0	–	–	–	–	–
Mannosyl	4-Linked	–	–	0.4	–	–	–	–	–	–	1.0	–	–	–
Galactosyl	Terminal-	11.4	–	–	11.5	–	–	12.8	8.1	7.7	–	14.5	6.6	trace
	3-linked	4.4	–	–	3.1	–	–	4.0	–	1.2	–	4.5	3.2	2.1
	4-	3.6	10.3	0.8	2.4	9.1	0.5	4.4	3.9	3.0	10.0	4.0	8.3	7.4
	6-	0.5	–	–	0.4	–	–	1.1	–	–	–	–	–	–
	2,4-	1.0	–	–	0.7	13.7	–	–	–	–	–	–	7.1	–
	3,6-	1.5	–	–	trace	–	–	0.2	–	–	–	–	–	–
	2,4,6-	0.7	–	–	0.3	2.8	–	0.2	–	0.3	0.3	–	–	–
Glucosyl	4-Linked	–	2.3	0.1	0.7	3.3	–	–	2.9	–	0.2	–	4.4	1.1
	2,4-	–	–	–	–	–	–	1.0	7.3	5.8	2.0	–	–	–
Gal A	Terminal-	1.3	25.1	30.1	1.1	2.1	16.5	0.7	6.6	7.3	35.3	2.8	7.1	33.8
	2-linked	–	–	2.1	–	–	10.6	–	–	4.3	1.5	–	–	9.2
	3-	–	–	3.4	trace	–	16.2	–	–	trace	trace	–	–	14.9
	4-	12.7	47.3	49.4	16.2	30.6	21.2	14.3	14.6	18.4	47.3	26.7	8.7	19.1
	2,4-	2.1	–	5.4	0.6	–	14.3	0.8	4.2	2.9	0.1	4.0	3.6	4.6
	3,4-	2.0	–	4.4	1.0	–	19.8	0.8	1.5	–	–	–	–	3.8
Glc A	Terminal-	–	–	3.5	–	–	–	–	–	–	–	–	–	–
	2-linked	–	–	–	–	5.1	–	–	9.6	6.1	–	–	–	–
	4-	1.7	–	trace	0.3	–	–	–	–	–	–	–	–	–

*See footnote to Table 2.
†See Fig. 1.
‡Detected but not determined quantitatively.
§Not detected.

fucosyl, 2-*O*-methyl-D-xylosyl, apiosyl residues and aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) and thiobarbituric acid assay-positive sugar residues [probably 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) and 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha)], which are known as characteristic sugar residues of RG-II [1] (Table 4). The fractions A-II, B-II and D-II also contained 2-*O*-methyl-L-fucose, a trace amount of 3-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, apiose, aceric acid and thiobarbituric acid assay-positive sugars. As available samples of these fractions (A-II, B-II and D-II) were limited, the glycosyl composition of the fractions could not be determined. The results suggested that these fractions also contained RG-II.

Table 4. Sugar composition of pectic polysaccharide fractions isolated from enzymic hydrolysate of Na_2CO_3 extract from cell walls of xylem-differentiating zones of sugi

	Glycosyl composition (mol %)											
Fractions	Rha	Fuc	Ara	Xyl	Gal	Glc	2-*O*-MeFuc	3-*O*-MeFuc	2-*O*-MeXyl	Api	Aceric acid	3-Deoxysugar*	Uronic acid
C-I†	15.0	1.3	22.5	2.3	18.3	6.9	—	—	—	—	—	—	33.8
C-II†	14.4	1.3	32.4	—‡	18.0	1.7	2.1	trace	3.0	5.3	0.8	0.9	20.9§
C-III†	23.0	3.7	22.1	—	20.4	1.7	4.1	trace	5.3	7.7	2.0	0.8	10.0§
Sycamore‖	12.4	2.8	10.0	—	9.0	—	3.5	—	4.8	12.2	3.5	7.0	34.4

2-*O*-MeFuc = 2-*O*-methyl-L-fucose; 3-*O*-MeFuc = 3-*O*-methyl-L-fucose; 2-*O*-MeXyl = 2-*O*-methyl-D-xylose; Api = apiose.

*3-Deoxysugar (Kdo and Dha) content determined by thiobarbituric acid assay [18].

§Uronic acid content was determined by *m*-hydroxybiphenyl assay [17].

†See Fig. 1.

‡Not detected.

‖Sycamore RG-II [13].

From our results, it could be concluded that pectic polysaccharides of cell walls of the xylem-differentiating zones of sugi were composed of RG-I, RG-II and homogalacturonan, and that the chemical structures of the pectic polysaccharides were very similar to those of sycamore [13] and bamboo [14].

EXPERIMENTAL

Preparation of pectin. Cell walls of xylem-differentiating zones of sugi were prepd as described in ref. [10]. Cell walls were extracted with 50 mM CDTA (pH 6.5) and then 50 mM Na_2CO_3 soln to solubilize pectic polysaccharides. CDTA was used to solubilize pectic polysaccharides by removal of Ca^{2+} ions. About 1 g of cell walls was treated with 100 ml 50 mM CDTA with magnetic stirring at 20° for 6 hr (CDTA-I) and then at 20° for 2 hr (CDTA-II). Residual cell walls were then treated with 50 mM Na_2CO_3 containing 20 mM $NaBH_4$ under N_2 at 1° for 16 hr (Na_2CO_3-I) and then with 0.05 M Na_2CO_3 under N_2 at 20° for 3 hr (Na_2CO_3-II). After each extraction, insoluble material was collected by centrifugation and subjected to the next extraction. The Na_2CO_3 extracts were adjusted to pH 5 with HOAc. Each extract was dialysed exhaustively against deionized H_2O at 4°, concd to small vol. under red. pres. and kept at −20°.

Purification of pectic polysaccharides. Crude pectin frs (CDTA-I, -II, Na_2CO_3-I and -II) were de-esterified with dilute alkali soln. The extract was dissolved in 0.01 N NaOH and the pH was adjusted to 12 with 0.1 N NaOH. After 2 hr at 4°, the soln was adjusted with HOAc to pH 5, dialysed exhaustively against deionized H_2O, concd to small vol. under red. pres. and then lyophilized. The de-esterified pectin was dissolved in a small vol. of 10 mM imidazole–HCl buffer (pH 7) and loaded on to a DEAE-Sepharose Fast Flow (80 × 1.5 cm i.d.) column equilibrated with 10 mM imidazole–HCl buffer (pH 7). The column was washed with 50 ml imidazole–HCl buffer (pH 7) and then eluted with a linear gradient of imidazole–HCl buffer (pH 7) from 10 mM to 1.5 M. Frs (1.5 ml) were collected and the contents of acidic sugar were determined colorimetrically. Frs enriched in acidic sugar were collected, dialysed exhaustively against deionized H_2O, concd to small vol. under red. pres. and lyophilized.

Separation of RG-I, RG-II and oligogalacturonides. Pectin frs purified from the extract were dissolved in 50 mM NaOAc buffer (pH 5.2) and EPGase purified from pectinase of *Aspergillus niger* (Sigma, EC 3.2.1.15, 5 units per mg of sample) [15] was added and incubated at 35° for 48 hr. After inactivation of enzyme (5-min boiling), the solns were heated at 100° for 5 min and then centrifuged to remove insoluble material (20 000 *g*, 10 min). The supernatant solns were concd to small vol. under red. pres. The resulting concd samples were loaded onto a Bio-Gel P-30 column (80 × 1.5 cm i.d.) and eluted with 50 mM NaOAc acid buffer (pH 5.2). Frs (1.5 ml) were collected and their acidic and neutral sugar contents determined colorimetrically.

Colorimetric assay. Hexose and uronic acid contents were determined by the anthrone [16] and *m*-hydroxybiphenyl assays [17], respectively. Contents of 2-keto-3-deoxysugars (Kdo and Dha) were measured by thiobarbituric acid assay [18].

Glycosyl composition analysis. Neutral and acidic glycosyl compositions were determined as TMSi ethers of Me glycosides after methanolysis with 5% methanolic HCl at 80° for 16 hr [19, 20]. Derivatives were analysed by GC using a fused silica DB-1 capillary column (25 m × 0.25 mm i.d.) operated with a temp. programme starting at 140° for 2 min, increasing to 200° at 2° min^{-1}, then increasing to 275° at 30° min^{-1} and kept for 10 min. Injector and detector temps were 240°. The split ratio was 50 : 1 and the injection vol. 1 μl. Characteristic sugars of RG-II, such as 2-*O*-methylfucose, 2-*O*-methylxylose and apiose, were detected by GC and GC-MS analysis of the corresponding alditol acetate derivatives. Alditol acetates were prepd as described in ref. [20] and analysed by GC using a SP-2330 capillary column (30 m × 0.25 mm i.d.) operating at 220°. Injector and detector temps were 240°. The split ratio was 50 : 1 and a 1-μl sample was injected. GC-MS analysis was performed on a quadrupole instrument equipped with a same capillary column operating at 220°. Ionization energy was 70 eV.

Methylation analysis. Glycosyl-linkage composition was determined by GC and GC-MS analysis of the partially *O*-methylated alditol acetates. Per-*O*-methylation was performed by a modification [21] of the Hakomori procedure [22]. Per-*O*-methylated polysaccharides were purified using a Sep-Pak C_{18} cartridge (Waters) [23]. Me esters of per-*O*-methylated galacturonic acid residues in pectic polysaccharides were reduced with a 1 M soln of Li triethylborodeuteride in THF (Super-Deuteride, Aldrich) at room temp. for 1 hr after per-*O*-methylation [20]. The per-*O*-methylated, carboxyl-reduced polysaccharides were hydrolysed with 2 M TFA at 121° for 1 hr and then converted into their alditol acetate derivatives [20]. GC-MS analysis of partially *O*-methylated alditol acetates was performed in the splitless mode using a SP-2330 capillary column (30 m × 0.25 mm i.d.) and a temp. programme starting at 50° for 2 min, increasing to 170° at 30° min^{-1}, then increasing to 235° at 4° min^{-1}, isothermal at 235° for 10 min. Ionization energy was 70 eV. Composition of partially *O*-methylated alditol acetates was determined by GC in the split mode (split ratio, 50 : 1) and the response factors reported in ref. [20] were used. The GC conditions were the same as those used for GC-MS, except for the starting temp. (170° for 2 min).

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