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ISOLATION AND STRUCTURAL CHARACTERIZATION OF THE PECTIC POLYSACCHARIDE RHAMNOGALACTURONAN II FROM WALLS OF SUSPENSION-CULTURED RICE CELLS*†

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ABSTRACT

Rhamnogalacturonan II (RG-II) is a structurally complex pectic polysaccharide that had previously been isolated from the primary cell-walls of a dicot (sycamore, *Acer pseudoplatanus*) and a gymnosperm (Douglas fir, *Pseudotsuga menziesii*). Herein are described the isolation and structural characterization of RG-II from the primary cell-walls of a suspension-cultured monocot (rice, *Oryza sativa*). The glycosyl-linkage composition of rice RG-II was similar to that of sycamore and Douglas fir RG-II. Partial hydrolysis with acid was used to release, from rice RG-II, two disaccharides, a heptasaccharide, and an octasaccharide; all of these oligosaccharides had previously been shown to be components of sycamore RG-II. The rice RG-II disaccharides characterized were α-rhamnosyl-(1→5)-3-deoxy-D-*manno*-2-octulosonic acid (KDO) and β-arabinosyl-(1→5)-3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA). The rice RG-II heptasaccharide contained, *inter alia*, aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) and 2-*O*-methylfucose, and was shown to have the same residue sequence and same points of attachment of glycosyl linkages (except for that to aceric acid, which was not determined) as its sycamore counterpart. Finally, an octasaccharide containing, *inter alia*, terminal 2-*O*-methylxylose, 2-linked glucuronic acid, and 3,4-linked fucose was isolated after partial hydrolysis of rice RG-II with acid. The same octasaccharide had been postulated to be a component of sycamore RG-II; conclusive evidence of its existence as a component of rice RG-II was obtained by using fast-atom-bombardment mass spectrometric analysis. The results indicated that RG-II from the primary cell-walls of a monocot is indistinguishable from the previously characterized RG-II isolated from a dicot.

*Structure of Plant Cell-Walls, Part XXIV. For Part XXIII, see ref. 1.

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INTRODUCTION

Rhamnogalacturonan II (RG-II) is a pectic polysaccharide first isolated from the primary walls of suspension-cultured sycamore *(Acer pseudoplatanus)* cells[2]. RG-II contains at least 11 different glycosyl residues, including the rarely observed aceric acid, apiosyl, DHA, KDO, 2-*O*-methylfucosyl, and 2-*O*-methylxylose[3–5]. The glycosyl residues in RG-II are connected by a wide variety of glycosyl linkages[2]. Several of these glycosyl linkages, *i.e.*, those of 3,4-linked fucosyl, 2-linked glucuronic acid, and 3-linked rhamnosyl residues, are found in the plant cell-wall only in RG-II. The unique (and unusual) monosaccharides and their distinctive glycosyl linkages are diagnostic of RG-II.

Progress has been made in determining the complete primary structure of RG-II through the isolation and characterization of oligosaccharides released from the purified polysaccharide[1,4–7]. Among these oligosaccharides are two disaccharides [β-Ara-(1→5)-DHA[4] and α-Rha-(1→5)-KDO[5]] and several larger oligosaccharides[3,6], all of which were released from RG-II by partial hydrolysis with acid. Isolation of these characteristic oligosaccharides provides another criterion by which to identify and compare RG-II polysaccharides.

Evidence of RG-II in the primary cell-walls of monocots is limited. The presence of 2-*O*-methylfucosyl and 2-*O*-methylxylose in pectic polysaccharides of *Agave sisalana*[8] and cell walls of oat *(Avena sativa)* internodes[2] has been reported. A pectic polysaccharide has been isolated from onion *(Allium cepa)* that contains 3-linked rhamnosyl residues[9]; however, it was not determined whether any of the unusual monosaccharide components of RG-II were present. The results now presented demonstrate that RG-II is present in the primary walls of suspension-cultured rice *(Oryza sativa)* cells, and that rice RG-II has the same glycosyl and glycosyl-linkage compositions, and the same oligosaccharide components released by acid as its dicot (sycamore) counterpart.

EXPERIMENTAL

Cell culture and cell-wall preparation. — Suspension cultures of rice (*Oryza sativa* cv. Yamabiko) cells derived from immature embryos were the gift of H. Lörz at the Max Planck Institute in Köln. The growth medium was that described by Chu *et al.*[10], containing 2.0 mg/L of 2,4-dinitrophenoxyacetic acid/L and 1 μg of kinetin/L.

Cell walls were isolated as described in the accompanying paper[11].

CDTA extraction of polysaccharides from cell walls. — Cell walls (25.0 g dry weight) were suspended in 2.5 L of 50mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) (prepared by titrating the acid to pH 6.5 with 5M NaOH) and stirred for 6 h at 26° (ref. 12). The suspension was centrifuged for 20 min at 20,000*g*, and the supernatant solution was passed through two layers of Whatman GF/C glass filter to remove cell-wall residue, the filtrate concentrated under di-

minished pressure at 40°, and the concentrate dialyzed extensively against H_2O using tubing having a nominal molecular-weight cutoff of 12,000 to 14,000, and lyophilized. The cell-wall residue was resuspended in fresh CDTA solution, and extracted as just described. The first and second CDTA extracts yielded 379 and 194 mg, respectively (a total of 2.3% by weight), of the starting cell-wall material.

Enzyme purification. — Purification of endo-α-1,4-polygalacturonanase (EPGase) from *Colletotrichum lindemuthianum* was performed as described[11,13].

Gel-permeation chromatography. — Columns (3.5 × 65 cm) of Bio-Gel P-10 and A-5m (Bio-Rad Laboratories) were equilibrated in, and eluted with, 50mM Na acetate (pH 5.2); 10-mL fractions were collected. Pooled fractions from the columns were passed through Dowex-50W (H^+) ion-exchange resin, and the solutions lyophilized. Void and included volumes were determined by chromatographing dextran and D-glucose, respectively.

De-esterification and enzyme treatment of polysaccharides. — Polysaccharides extracted from cell walls with CDTA were de-esterified by dissolving them in 0.1M NaOH and incubating for 24 h at 26°. The pH was adjusted to 5.2 with acetic acid, and the de-esterified polysaccharides were chromatographed on Bio-Gel P-10. All of the carbohydrate voided the P-10 column; polysaccharides in pooled, lyophilized P-10 fractions were dissolved in 50mM Na acetate (pH 5.2) containing 0.02% of thimerosal (10 mL/g of polysaccharide), and enzyme (~50 units/g of polysaccharide) was added. After 48 h, the enzyme-digested mixture was chromatographed on Bio-Gel P-10. De-esterification and treatment with EPGase were necessary in order to separate RG-I from RG-II (see Results).

Analytical techniques. — The alditol acetate derivatives[14,15] of neutral sugars and aceric acid[3] were identified by g.l.c.–m.s. and quantitated by g.l.c. A 30-m SP-2330 fused-silica column (Supelco) was used isothermally at 235°. Galacturonic and glucuronic acids were quantitated as their per-*O*-(trimethylsilyl) methyl glycosides, prepared[16] and analyzed[15] as described.

Glycosyl-linkage compositions were determined by g.l.c. and g.l.c.–m.s. of the partially *O*-methylated alditol acetate derivatives[15,17,18]. To determine the linkages of glycosyluronic acid residues, the methyl-esterified carboxyl groups of per-*O*-methylated oligo- or poly-saccharides were reduced with M Li-triethylborodeuteride in tetrahydrofuran[15] for 1 h. Prior to positive-ion f.a.b.-m.s. analysis, oligosaccharides were reduced with NaB^2H_4 and the products per-*O*-alkylated[15,18], and in some cases, carboxyl-reduced and remethylated.

F.a.b.-m.s. was performed as described[11]. Xenon was used as the bombarding gas, and the gun was operated at 8 kV, 1 mA. Negative-ion f.a.b-m.s. of native oligosaccharides was performed by dissolving samples in 5% aqueous acetic acid (from 1 to 5 μg/μL) and loading 1 μL of the solution into 1:1 (v:v) glycerol–monothioglycerol. Positive-ion f.a.b.-m.s. of per-*O*-methylated oligosaccharides was performed by dissolving samples in methanol, and loading 1 μL of the solution into 1:1 (v:v) glycerol–monothioglycerol. The mass spectrometer was operated with an accelerating voltage of 8 kV in both the negative- and positive-ion modes. Linear-

mode, mass-controlled scans were recorded on oscillographic paper and counted manually. Scan-rate settings were varied from 300 to 100 s during scans, in order to obtain spectra having peaks suitably spaced for counting. A mass range of 1500 to 170 a.m.u. was scanned in the negative-ion mode, and 2000 to 170 a.m.u. in the positive-ion mode.

One-dimensional ^{1}H-n.m.r. spectra of native oligosaccharides were recorded with a Bruker AM-500 spectrometer operated at 500 MHz and at a temperature of 25°. Samples were dissolved in deuterium oxide, and chemical shifts are reported relative to H_2O (δ 4.75).

Isolation and characterization of Rha→KDO and Ara→DHA from rice RG-II. — The procedure was that described by Stevenson *et al.*[4]. Purified polysaccharide (2 mg) was methylated, carboxyl-reduced, remethylated, and then partially hydrolyzed with 0.1M TFA for 0.5 h at 60°. Disaccharide products were reduced with NaB^2H_4, per-*O*-methylated, and analyzed by g.l.c.–m.s. with electron-impact ionization.

Isolation and characterization of larger oligosaccharides from rice RG-II. — The conditions for partial acid hydrolysis of RG-II to yield maximum quantities of hepta- and octa-saccharides, and the conditions for ion-exchange chromatography of the oligosaccharide products, were determined by T. Stevenson (unpublished data) using RG-II isolated from Pectinol AC[4,5,7]. Purified rice RG-II (10.2 mg) was partially hydrolyzed in 2.0 mL of 0.1M TFA for 24 h at 50°. After adjusting the pH of the solution to 8 with NaOH and diluting it to 100 mL with H_2O, the acid hydrolyzate was loaded onto a column (1.2 × 13 cm) of QAE-Sephadex equilibrated in 1.0M NH_4HCO_3, and washed with H_2O. The column was first eluted with a gradient of 0 to 0.5M NH_4HCO_3 (100 mL of each), and then stepwise with 50 mL of 1.0M NH_4HCO_3 followed by 50 mL of 1.0M imidazole HCl (pH 7.0), the eluate being collected in 2.5-mL fractions. Pooled fractions were lyophilized, the solids dissolved in H_2O, and the solutions lyophilized to remove NH_4HCO_3.

RESULTS

Gel-permeation chromatography and glycosyl-linkage analysis of CDTA-extracted polysaccharides. — The fractionation by gel-permeation chromatography of CDTA-extracted polysaccharides on Bio-Gel P-10 is shown in Fig. 1. The glycosyl-linkage compositions of the material in pooled P-10 column fractions 16 to 20, and 28 to 33, are shown in Table I. The material that voided the Bio-Gel P-10 column (fractions 16 to 20) contained variously linked glycosyl residues characteristic of the pectic polysaccharide[19] RG-I, including 2- and 2,4-linked rhamnosyl, 4- and 6-linked galactosyl, and terminal and 5-linked arabinosyl residues (see Table I). The ratio of branched (2,4-linked) to linear (2-linked) rhamnosyl residues was higher in the rice RG-I extracted in CDTA than in sycamore RG-I. However, RG-I having a high ratio of branched to linear rhamnosyl residues has also been isolated from cell walls of sycamore[20] and rice endosperm[21]. The P-10 void material

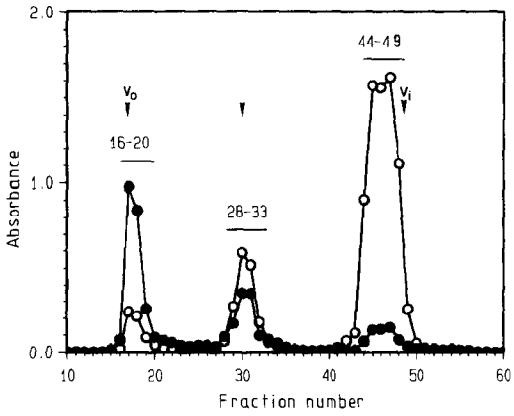


Fig. 1. Bio-Gel P-10 fractionation of polysaccharides extracted from rice cell-walls into 50mM CDTA. A portion (379 mg) of the material from the CDTA extract was de-esterified, treated with EPGase, and chromatographed as described in the Experimental section. Column fractions were assayed for neutral sugars by the anthrone method[26] (A_{620}, closed circles) and for uronic acids by the *m*-hydroxybiphenyl method of Blumenkrantz and Asboe-Hansen[27] (A_{520}). Sycamore RG-II was eluted mainly in fraction 30 (arrowhead). Column fractions 16 to 20 and 28 to 33 were combined for further chromatography; fractions 44 to 49 contained only material constituted of galactosyluronic acid residues.

was chromatographed on Bio-Gel A-5m (see Fig. 2). The two major peaks of carbohydrate-containing material eluted from the A-5m column (fractions 28 to 33, and 34 to 42) both had glycosyl-linkage compositions (data not shown) very similar to that of the material in P-10 fractions 16 to 20 (see Table I). The elution volumes of both peaks from the A-5m column indicated that rice RG-I extracted into CDTA was about the same size as sycamore RG-I (see Fig. 2).

The material eluted in the partially included volume (fractions 28 to 33) from the Bio-Gel P-10 column (see Fig. 1) contained glycosyl linkages characteristic[1,2] of RG-II, including 3′-linked apiosyl, 2-linked arabinopyranosyl, 3,4-linked fucosyl, 2-linked glucuronic acid, and 3-linked rhamnosyl residues (see Table I). In addition, the glycosyl composition of the material in fractions 28 to 33 included the aceric acid, 2-*O*-methylfucosyl, and 2-*O*-methylxylose that are diagnostic[2] of RG-II. Based on the similarity of the glycosyl-linkage compositions of the CDTA-extracted rice polysaccharides to those of sycamore RG-I and RG-II, the polysaccharides in Bio-Gel P-10 fractions 16 to 20 will hereinafter be referred to as rice RG-I, and those in fractions 28 to 33, as rice RG-II.

Bio-Gel A-5m fractions 28 to 33 and 34 to 42 (RG-I) and P-10 fractions 16 to 20 (RG-II) contained 7, 10, and 20 mg, respectively. Thus, the RG-I and RG-II purified from CDTA extracts of rice cell-walls accounted for 0.10 and 0.12%, respectively, of the starting cell-wall material. These values do not include all of the pectic polysaccharides in rice primary cell-walls, because more RG-I and RG-II can be released from the CDTA-treated walls by treatment with EPGase (unpublished data).

TABLE I

GLYCOSYL-LINKAGE COMPOSITION OF POLYSACCHARIDES EXTRACTED FROM RICE CELL-WALLS WITH CDTA[a]

Glycosyl residue	*Linkage*[b]	*Fractions 16 to 20*	*Sycamore RG-I*[c]	*Fractions 28 to 33*	*Sycamore RG-II*[d]
		mol%			
Rhamnosyl	terminal	0	1.8	8.5	5.2
	2-linked[e]	1.1	7.8	0	1.8
	3-	0	0	5.8	6.0
	2,3-	0.3	0	0	0
	2,4-	5.2	8.0	0	0
	2,3,4-[f]	0.4	0.6	9.1	5.5
Fucosyl	terminal	1.4	1.4	6.2[g]	4.6[g]
	3,4-linked	0	0	4.9	2.3
Apiosyl	3-linked	0	0	8.9	7.8
Arabinosyl	terminal	16.2	9.5	5.7	7.7
	2-linked pyranose	0	0	4.1	5.0
	2-linked furanose[e]	3.2	2.7	0	0
	3-	2.3	2.7	0	0
	5-	13.3	11.2	0	0
	2,5-	1.2	1.0	0	0
	3,5-	5.1	3.5	0	0
Xylosyl	terminal	4.0	2.0	7.1[g]	4.6[g]
	2-	0.4	0	0	0
	4-	0.9	0	0	0
	2,3-	1.0	0	0	0
	2,4-	1.0	0	0	0
	3,4-	7.0	0	0	0
Galactosyl	terminal	5.9	6.3	6.7	6.8
	2-linked	0.5	0.6	0	0
	3-	1.9	2.7	0	0
	4-	5.8	8.4	0	0
	6-	2.0	7.5	0	0
	2,4-	0	0.5	5.4	5.7
	2,6-	0	1.2	0	0
	3,4-	1.0	0	0	0
	3,6-	7.5	1.2	0	0
	4,6-	0	2.4	0	0
Glucosyl	terminal	1.3	0	0	0
	4-linked	1.1	0	0	0
GalA	terminal	1.0	1.6	10.0	11.1
	4-linked	7.2	15.2	7.8	5.6
	2,4-	0	1.0	2.8	6.4
	3,4-	2.3	0	1.8	9.1
GlcA	2-linked[f]	0	0	6.2	4.6

[a]Polysaccharides extracted from isolated cell-walls into CDTA were partially purified by using gel-permeation chromatography (Fig. 1). Per-methylated alditol acetates of polysaccharides in the pooled column-fractions listed above were prepared, identified, and quantified as described in the Experimental section. For comparison, the glycosyl-linkage compositions of sycamore RG-I and RG-II are included. [b]Where ambiguous, or unless otherwise specified, all hexosyl, deoxyhexosyl, xylosyl, and glycosyluronic acid residues were assumed to be in the pyranose ring-form and arabinosyl residues in the furanose ring-form. [c]See York *et al.*[15]. [d]See Stevenson *et al.*[15]. [e]Relative amounts of these coeluted derivatives were determined from ratios of fragment-ions observed upon g.l.c.–m.s. analysis. [f]These derivatives are coeluted during g.l.c. The relative amount of 2,3,4-linked rhamnose was determined in a separate sample in which glycosyluronic acids were not carboxyl-reduced. The g.l.c. peak area contributed by 2,3,4-linked rhamnose was subtracted from the total, and the remainder was attributed to 2-linked glucosyluronic acid residues. [g]Derived from endogenous 2-*O*-methyl derivatives.

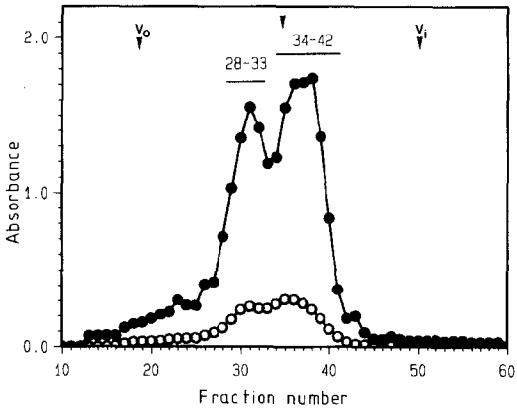
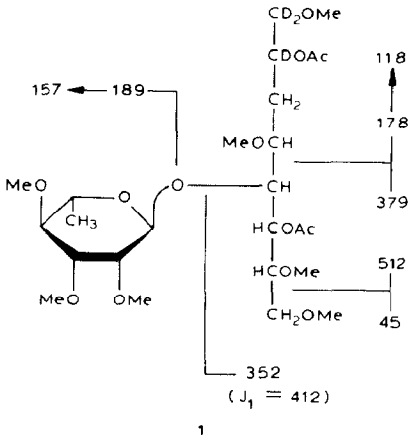


Fig. 2. Bio-Gel A-5m fractionation of material in fractions 16 to 20 (33 mg) from the Bio-Gel P-10 column (see Fig. 1; symbols as in Fig. 1). Details of chromatography are given in the Experimental section. Sycamore RG-I was mainly eluted in fraction 35 (arrowhead). Column fractions 28 to 33 and 34 to 42 were combined for analysis.

The EPGase-released galactosyluronic acid residues in P-10 column fractions 44 to 49 are likely to have been covalently attached to RG-I or RG-II, or both. After de-esterification and EPGase treatment, both rice RG-I and RG-II were approximately the same size as their counterparts isolated from walls of suspension-cultured sycamore cells[2,6], as judged by their elution volumes from a Bio-Gel A-5m and a P-10 column (see Figs. 2 and 1, respectively). However, without EPGase treatment, all of the CDTA-extracted polysaccharides voided the Bio-Gel P-10 column (data not shown). The fact that EPGase treatment was required in order to separate RG-I from RG-II and from α-1,4-linked homogalacturonan suggests that the RG-I and RG-II were covalently connected *via* homogalacturonan when they were extracted from rice cell-walls into CDTA.

Identification of α-Rha-(1→5)-KDO and β-Ara-(1→5)-DHA as components of rice RG-II. — Isolation of α-Rha-(1→5)-KDO and β-Ara-(1→5)-DHA from rice RG-II is described in the Experimental section. Derivatives of these disaccharide fragments of rice RG-II, identical to those previously described[4,5], were produced and characterized. The absolute configurations of the component sugars were assumed to be the same as those in sycamore RG-II. The g.l.c. retention times of both derivatives were identical to those of the corresponding compounds derived from sycamore RG-II. Furthermore, all of the expected[4,5] electron-impact fragment-ions were observed in their mass spectra, except for one at *m/z* 512 (probably due to its low abundance) (see formulas **1** and **2**). Thus, it was concluded that these disaccharides are components of both rice and sycamore RG-II.

Identification of hepta- and octa-saccharides characteristic of sycamore RG-II as components of rice RG-II. — A portion (10.2 mg) of rice RG-II was partially hydrolyzed with acid as described in the Experimental section, and the resulting mixture of oligosaccharides was chromatographed on QAE-Sephadex (see Fig. 3).



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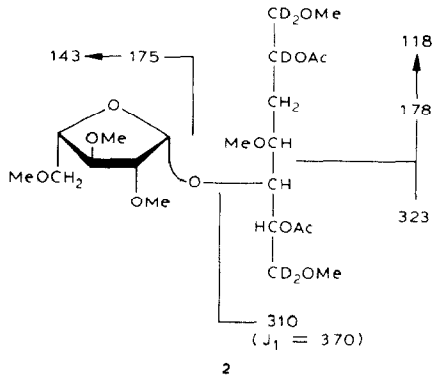

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TABLE II

GLYCOSYL COMPOSITION OF OLIGOSACCHARIDES OBTAINED FROM RICE RG-II AFTER PARTIAL HYDROLYSIS WITH ACID[a]

Glycose	*QAE-Sephadex fractions*				
	Sycamore hepta-saccharide[b]	*34–40*	*41–48*	*62–70*	*111–117*
	mol%				
Aceric acid[c]	11	7.3	6.1	0	0
Apiose[d]	12	9.7	4.6	12.2	2.7
Arabinose	17	17.9	13.8	4.7	8.2
Fucose	0	0	0	7.1	1.6
Galactose	17	18.9	13.3	19.9	4.3
Galacturonic acid	0	0	0	23.5	68.5
Glucuronic acid	0	0	0	11.1	9.5
2-*O*-Methylfucose	15	15.6	10.2	0	0
2-*O*-Methylxylose	0	0	0	13.5	3.0
Rhamnose	28	30.6	52.0	7.9	22.2

[a]Polysaccharides that were eluted from the Bio-Gel P-10 column in fractions 28 to 33 (Fig. 1) were treated with 0.1M TFA for 24 h at 50° and chromatographed on QAE-Sephadex (Fig. 3). Glycosyl compositions of the pooled column fractions indicated were determined by both preparing and analyzing alditol acetates and per-*O*-trimethylsilyl methyl glycosides, as described in the Experimental section. When glycuronic acids were present, the ratio of glycuronic acids to neutral sugars (rhamnose, fucose, arabinose, and galactose) was determined by analyzing per-*O*-trimethylsilyl methyl glycosides. This ratio was then used to combine the data from the two techniques into the composition data given. Effective carbon-response factors[25] were used to calculate relative amounts of alditol acetates from g.l.c. peak areas[15]. [b]The glycosyl composition of the aceric acid-containing heptasaccharide of sycamore RG-II characterized by Spellman *et al.*[7] is included for comparison. [c]The effective carbon-response factor was estimated to be 0.7. [d]Amounts were calculated by combining peak areas attributed to both the fully acetylated and under-acetylated derivatives (W. S. York, A. G. Darvill, and M. McNeil, unpublished data).

Partial hydrolysis with 1.0M TFA for 24 h at 50° cleaved the acid-labile glycosidic bonds of apiosyl residues, releasing oligosaccharides having an apiose residue at their reducing terminus[6,7]. The determination of the structures of these oligosaccharides is described later.

Aceric acid-containing heptasaccharide. — The glycosyl and glycosyl-linkage

TABLE III

GLYCOSYL-LINKAGE COMPOSITION OF OLIGOSACCHARIDES OBTAINED FROM RICE RG-II AFTER PARTIAL HYDROLYSIS WITH ACID[a]

Glycosyl residue	*Linkage*	*Sycamore hepta-saccharide*[b]	*QAE-Sephadex fractions*			
			34–40	*41–48*	*62–70*	*111–117*
		mol%				
Rhamnosyl	terminal	19	15.7	38.3[c]	0	0
	3-linked	18	21.1	12.7	0	0
	2,3,4-		0	0	15.5[d]	6.4[d]
Fucosyl	terminal[e]	17	18.3	13.2	0	0
	3,4-linked		0	0	15.4	6.5
Apiitol	3′-linked	5	t[f]	t	t	(4.4)[g]
Arabinosyl	terminal		0	0	0	9.6
	2-linked pyranose	19	18.9	16.3	0	0
Xylosyl	terminal[e]	0	0	0	13.9	6.3
Galactosyl	terminal		0	0	14.2	4.6
	4-linked		4.7	5.3	0	0
	2,4-	15	22.1	14.3	0	0
GalA	terminal		0	0	25.5	13.8
	4-linked		0	0	0	33.7
	2,4-		0	0	0	1.2
	3,4-		0	0	0	6.9
GlcA	2-linked		0	0	15.5[d]	6.4[d]

[a]Polysaccharides that were eluted from the Bio-Gel P-10 column in fractions 28 to 33 (Fig. 1) were treated with mild acid and chromatographed on QAE-Sephadex (see Fig. 3). Glycosyl-linkage compositions of the pooled column-fractions listed were determined as described in the Experimental section. The linkage of aceric acid was not determined. [b]The glycosyl-linkage composition of the aceric acid-containing heptasaccharide of sycamore RG-II characterized by Spellman *et al.*[7] is included for comparison. Not included is the 6 mol% of 2-linked aceric acid residues. [c]Some of the terminal rhamnose in fractions 41 to 48 was associated with KDO (see Results). [d]Calculated by arbitrarily assuming that the coeluting derivatives of these residues were present in equal amounts. [e]Derived solely from endogenous 2-*O*-methyl derivatives; no xylose was present in the samples, and no fucose was detected in fractions 34 to 40 or 41 to 48. [f]Indicates that traces of 3′-linked, pre-reduced apiose derived from the reducing termini of oligosaccharides were detected. [g]Apiosyl residues in fractions 111 to 117 were glycosidically linked, not pre-reduced.

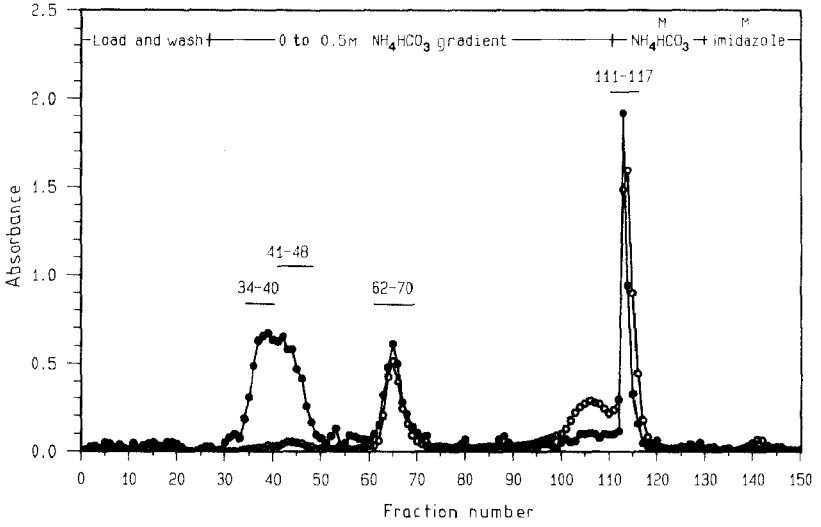
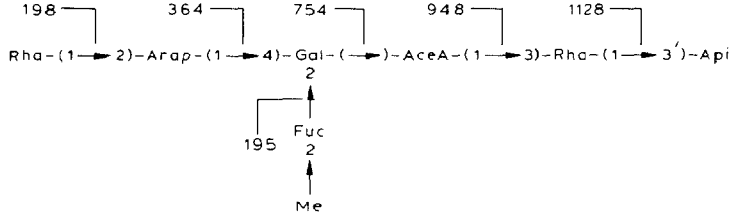


Fig. 3. QAE-Sephadex ion-exchange chromatography of material in fractions 28 to 33 from the Bio-Gel P-10 column (Fig. 1; symbols as in Fig. 1). Material in P-10 fractions 28 to 33 (10.2 mg) was partially hydrolyzed with acid and chromatographed as described in the Experimental section. Column fractions 34 to 40, 41 to 48, 62 to 70, and 111 to 117 were combined for analysis.

compositions (see Tables II and III) of the material in fractions 34 to 40 (see Fig. 3) are the same as those of an aceric acid-containing heptasaccharide isolated from sycamore RG-II by Spellman *et al.*[7] (see formula **3**). The acid lability of apiosyl



3

residues may account for its low yield as the alditol acetate derivative, and low yields of aceric acid had previously been observed[7]; the cause is still unknown. Pre-reduced, 3′-linked apiitol, derived from reducing-terminal apiose, was detected, in addition to the linkages included in Table III. The linkage of aceric acid residues could not be determined by the method used here; aceric acid residues are 2-linked in[7] sycamore RG-II. The points of attachment of glycosyl linkages to neutral glycosyl residues in the aceric acid-containing oligosaccharide derived from rice RG-II (see Table III) were the same as those in the heptasaccharide derived from[7] sycamore RG-II.

TABLE IV

FAST-ATOM-BOMBARDMENT MASS SPECTROMETRY OF OLIGOSACCHARIDES IN QAE-SEPHADEX FRACTIONS[a]

QAE-Sephadex fractions	*Pseudomolecular ions*					*A^+-Type fragment-ions*[b]	*Oligosaccharide proposed to account for the observed ions*[c]
	$(M-H)^-$	$(M+H)^+$	$(M+NH_4)^+$	$(M+Na)^+$	$(M+K)^+$		
34 to 40 and 41 to 48	1055 1097	1349	1366	1371	1387	195, 198, 364, 754, 948, 1128	AceA-containing heptasaccharide Endogenously monoacetylated heptasaccharide[d]
	911	1172	1189	1194	1210	198, 364, 771, 951	Heptasaccharide *minus* methyl-Fuc
62 to 70	[e]	1554	1571	1576		219, 221, 425	2-*O*-Methylxylose-containing octasaccharide
		1394	1411	1416		219, 221, 425	Octasaccharide *minus* methyl-Xyl
		1348	1365	1370		219, 221, 425	Octasaccharide *minus* GalA
		1188	1205	1210		219, 221, 425	Octasaccharide *minus* methyl-Xyl and GalA
		1144	1161	1166		221	Octasaccharide *minus* Gal and either GalA or GlcA

[a]Oligosaccharides that were eluted from the QAE-Sephadex column (see Fig. 3) were analyzed by f.a.b.-m.s. as described in the Experimental section. [b]Fragment ions may be derived from any or all parent compounds. [c]Structures of the oligosaccharides are shown in Formulas **3** and **4**; see text for additional conditions and evidence. [d]*O*-Acetyl substituents would not survive the conditions of per-*O*-methylation required for positive-ion f.a.b.-m.s. [e]Indicates that ions were not detected.

The identity of the aceric acid-containing heptasaccharide in fractions 34 to 40 was confirmed by negative-ion f.a.b.-m.s. (see Table IV). The negative-ion spectrum of the native material contained signals at *m/z* 1055 and 1097 that correspond to $(M - H)^-$ pseudomolecular ions of the heptasaccharide and the monoacetylated heptasaccharide, respectively. The unacetylated, monoacetylated, and diacetylated heptasaccharides were observed[7] upon similar f.a.b.-m.s. analysis of the sycamore oligosaccharide. The ^{1}H-n.m.r. spectrum of the heptasaccharide in fractions 34 to 40 confirmed the presence of acetyl groups as it contained singlets at δ 2.16 and 2.12 (ref. 22). The signals were integrated, and then compared to those of the methyl groups of aceric acid, fucosyl, and rhamnosyl residues; the signals at δ 2.16 and 2.12 corresponded to less than 0.1 and 0.01 mol, respectively, of acetyl group per mol of heptasaccharide.

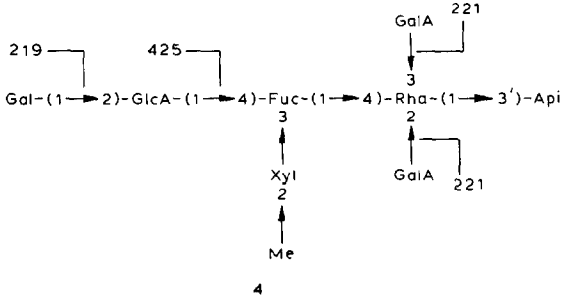
A positive-ion f.a.b.-mass spectrum of the material in fractions 34 to 40 was obtained after deuterium reduction of the reducing-terminal apiose and per-*O*-(trideuteriomethyl)ation of the product. [*Note:* Per-*O*-(trideuteriomethyl)ation was used so that fragment ions containing terminal rhamnosyl residues could be distinguished from those containing terminal 2-*O*-methylfucosyl residues. Per-*O*-(trideuteriomethyl)ated rhamnosyl and per-*O*-(trideuteriomethyl)ated 2-*O*-methylfucosyl residues differ in mass by 3 a.m.u.] The positive-ion spectrum contained signals at *m/z* 1349, 1366, 1371, and 1387, corresponding to the $(M + H)^+$, $(M + NH_4)^+$, $(M + Na)^+$, and $(M + K)^+$ pseudomolecular ions, respectively, of the heptasaccharide derivative. In addition, the positive-ion spectrum contained signals corresponding to A^+-type fragment-ions[23], at *m/z* 195, 198, 364, 754, 948, and 1128, that allowed unambiguous deductions of the sequence of the glycosyl residues of the heptasaccharide (see Formula **3**). The results of positive-ion f.a.b.-m.s. analysis of the aceric acid-containing heptasaccharide from rice RG-II were identical to those obtained by Spellman *et al.*[7] on analyzing the sycamore heptasaccharide.

F.a.b.-m.s. evidence was also obtained of a hexasaccharide in fractions 34 to 40 (see Table IV). The hexasaccharide was probably derived from aceric acid-containing heptasaccharide (see Formula **3**) by loss of the 2-*O*-methylfucosyl residues during partial acid hydrolysis of the polysaccharide. The negative-ion spectrum of the native material contained a signal at *m/z* 911 that corresponds to the $(M - H)^-$ pseudomolecular ion of the proposed hexasaccharide. The positive-ion spectrum of per-*O*-(trideuteriomethyl)ated oligosaccharide-alditols contained signals at *m/z* 1172, 1189, 1194, and 1210, corresponding to the $(M + H)^+$, $(M + NH_4)^+$, $(M + Na)^+$, and $(M + K)^+$ pseudomolecular ions, respectively, of the hexasaccharide derivative. In addition, signals corresponding to A^+-type fragment-ions[23] at *m/z* 198, 364, 771, and 951 were observed, consistent with the postulated structure Rha→Ara→Gal→AceA→Rha→Apiitol. The A^+-type fragment-ion at *m/z* 577, corresponding to Rha→Ara→Gal$^+$, was not observed. Presence of the hexasaccharide in fractions 34 to 40 is consistent with the presence in these fractions of 4-linked galactosyl residues (see Table III), because removal of 2-*O*-methylfucosyl residues from the aceric acid-containing heptasaccharide would convert 2,4-linked galactosyl into 4-linked galactosyl residues (see Formula **3**).

The glycosyl and glycosyl-linkage compositions of the material in QAE-Sephadex fractions 41 to 48 were very similar to those of fractions 34 to 40 (see Tables II and III). Upon positive-ion f.a.b.-m.s. analysis of the per-*O*-(trideuteriomethyl)ated material, the same pseudomolecular and A^+-type fragment-ions were observed as in the mass spectrum of the material in fractions 34 to 40. Thus, QAE-Sephadex fractions 34 to 40 and 41 to 48 undoubtedly contained the same aceric acid-containing hexa- and hepta-saccharides. However, the levels of rhamnosyl (see Table II) and terminal rhamnosyl (see Table III) residues were higher in fractions 41 to 48 than in 34 to 40, and higher than would be expected were they present only in the hexa- and hepta-saccharides. By forming the alditol acetate derivative and analyzing by g.l.c. and g.l.c.–m.s.[5] in a separate experiment, KDO was detected in fractions 41 to 48. Therefore, the high level of terminal rhamnosyl residues in those fractions can be accounted for by coelution with the aceric acid-containing hexa- and hepta-saccharides from the QAE-Sephadex column of the α-Rha-(1→5)-KDO disaccharide already described. The disaccharide was not observed in the positive-ion f.a.b.-mass spectrum of the per-*O*-(trideuteriomethyl)ated material, because KDO would not have survived the alkylation conditions.

In summary, aceric acid-containing heptasaccharide (see Formula **3**) was present in QAE-Sephadex fractions 34 to 40. The glycosyl and glycosyl-linkage compositions of the rice heptasaccharide were identical to those of the corresponding sycamore heptasaccharide. The glycosyl-residue sequences of the rice and sycamore heptasaccharides were shown to be identical by f.a.b.-m.s. In addition, f.a.b.-m.s. analysis indicated the presence of a hexasaccharide derived from the heptasaccharide by loss of a 2-*O*-methylfucosyl residue. Fractions 41 to 48 contained the same hexa- and hepta-saccharides as fractions 34 to 40, and, probably, the disaccharide α-Rha-(1→5)-KDO.

2-O-Methylxylosyl-containing octasaccharide. — The glycosyl and glycosyl-linkage compositions of the material in QAE-Sephadex fractions 62 to 70 (see Tables II and III) are consistent with those of the octasaccharide (Formula **4**) that



was postulated[1] to be a component of RG-II. The proposed octasaccharide consists of the glucuronic acid-containing heptasaccharide isolated from sycamore RG-II by Melton *et al.*[6], with an additional 2-*O*-methylxylose glycosidically linked through

O-3 of the fucosyl residue. The low yields of rhamnose and fucose relative to those of the other monosaccharides (see Table II) can be explained by the resistance to acid hydrolysis of the glycosidic bonds linking glycosyluronic acids to these residues in the octasaccharide. The low yield of the highly volatile, permethylated alditol acetate derived from pre-reduced, 3'-linked apiitol prevented its quantitation. Furthermore, the ratio of 2,3,4-linked rhamnosyl to 2-linked glucuronic acid residues could only be assumed to be 1:1, because the corresponding partially *O*-methylated alditol acetates were not separated under the g.l.c. conditions used.

The results of f.a.b.-m.s. analysis of fractions 62 to 70 (see Table IV) provided strong evidence for the presence of the 2-*O*-methylxylose-containing octasaccharide. The positive-ion spectrum of the material in fractions 62 to 70 was obtained after deuterium reduction of the reducing-terminal apiose, per-*O*-methylation, carboxyl-reduction, and remethylation (see the Experimental section). Signals at *m/z* 1554, 1571, and 1576 corresponded to the $(M + H)^+$, $(M + NH_4)^+$, and $(M + Na)^+$ pseudomolecular ions, respectively, of the octasaccharide derivative. In addition, the positive-ion spectrum contained weak signals at *m/z* 219, 221, and 425, corresponding to A^+-type fragment-ions[23], arising as suggested in Formula **4**. The complete glycosyl-residue sequence of the octasaccharide isolated from rice RG-II could not be determined from this experiment, because higher-mass fragment-ions were not observed. Nevertheless, previous studies[1] of sycamore RG-II support the proposed structure.

The f.a.b.-mass spectrum of the per-*O*-methylated material in fractions 62 to 70 contained additional, less intense pseudomolecular ions corresponding to oligosaccharide-alditols other than that derived from the 2-*O*-methylxylose-containing octasaccharide already discussed. These pseudomolecular ions (see Table IV) correspond to oligosaccharide-alditols having the compositions of Hex_1HexA_3-$Deoxyhex_2Pent_1$-itol [*m/z* 1394, 1411, and 1416 for the $(M + H)^+$, $(M + NH_4)^+$, and $(M + Na)^+$ ions, respectively], $Hex_1HexA_2Deoxyhex_2Pent_2$-itol [*m/z* 1348, 1365, and 1370 for the $(M + H)^+$, $(M + NH_4)^+$, and $(M + Na)^+$ ions, respectively], $Hex_1HexA_2Deoxyhex_2Pent_1$-itol [*m/z* 1188, 1205, and 1210 for the $(M + H)^+$, $(M + NH_4)^+$, and $(M + Na)^+$ ions, respectively], and finally $HexA_2Deoxyhex_2Pent_2$-itol [*m/z* 1144, 1161, and 1166 for the $(M + H)^+$, $(M + NH_4)^+$, and $(M + Na)^+$ ions, respectively]. The compositions are consistent with the following structures, which are all related to octasaccharide **4** by loss of one, or two, residue(s): Gal→GlcA→Fuc→Rha(GalA)(GalA)→Apiitol (octasaccharide minus methyl-Xyl), Gal→GlcA→Fuc(2-*O*-methyl-Xyl)→Rha(GalA)→Apiitol (octasaccharide minus GalA), Gal→GlcA→Fuc→Rha(GalA)→Apiitol, (octasaccharide minus methyl-Xyl and GalA), and GlcA→Fuc(2-*O*-methyl-Xyl)→Rha(GalA)→Apiitol or 2-*O*-methyl-Xyl→Fuc→Rha(GalA)(GalA)→Apiitol (octasaccharide minus Gal and either GalA or GlcA). These compounds were either generated during chemical modification(s) required for f.a.b.-m.s. analysis of the per-*O*-methylated 2-*O*-methylxylose-containing octasaccharide, or present in the intact polysaccharide in exceedingly low proportions; glycosyl linkages diagnostic of their proposed

structures were not detected in the intact polysaccharide (see Table I) or among the products of partial acid hydrolysis (see Table III).

Evidence of terminal arabinosyl residues linked to galactosyluronic acids. — The material eluted from the QAE-Sephadex column in fractions 111 to 117 (see Fig. 3) contained a high level of 4-linked galacturonic acid, and lower levels of glucuronic acid and neutral glycosyl residues (see Tables II and III). Melton *et al.*[6] obtained f.a.b.-m.s. evidence for oligosaccharides containing up to eight consecutive galactosyluronic acid residues, following partial acid hydrolysis of per-*O*-methylated sycamore RG-II. The galacturonic acid in fractions 111 to 117 was probably present as linear segments of α-(1→4)-linked galactosyluronic acid residues. Melton *et al.*[6] also reported the linkage of terminal arabinofuranosyl residues to galactosyluronic acid residues through O-3 in sycamore RG-II. Both terminal arabinofuranosyl and 3,4-linked galacturonic acid residues were present in fractions 111 to 117 (see Table III). Thus, some of the arabinofuranosyl residues in rice RG-II may be linked to galacturonic acid through O-3, as they are in[6] sycamore RG-II.

Evidence of the attachment of the 2-O-methylxylose-containing octasaccharide to oligogalactosyluronic acids. — The presence of 3′-linked apiosyl residues in fractions 111 to 117 (see Table III) indicated that not all apiosyl glycosidic bonds in rice RG-II were cleaved by the partial acid treatment used. [*Note:* Cleavage of apiosyl glycosidic bonds would have produced reducing-terminal apiose that would have been detected as 3′-linked apiitol, as in fractions 34 to 40, 41 to 48, and 62 to 70.] The 3′-linked apiosyl residues, along with other variously linked glycosyl residues [3,4-linked fucosyl, terminal galactosyl, 2-linked glucuronic acid, 2,3,4-linked rhamnosyl, and terminal (2-*O*-methyl) xylosyl residues], were present in fractions 111 to 117 (see Table III) in molar ratios characteristic of 2-*O*-methylxylose-containing octasaccharide **4** already discussed. These observations suggested that one or more 2-*O*-methylxylose-containing octasaccharides remained linked to other RG-II glycosyl residues in fractions 111 to 117. The 2-*O*-methylxylose-containing octasaccharide(s) may have been linked to galacturonic acid, because the only other non-terminal residues detected in the fraction were galactosyluronic acid residues.

DISCUSSION

The results presented here demonstrate that RG-II is a component of the primary cell-walls of a monocotyledonous plant. Rice RG-II was found to contain the same monosaccharide constituents[2–5] and points of attachment of glycosidic linkages[1,2] that are known to be present in sycamore RG-II. However, the absolute configurations of the monosaccharide components were not determined. Previously, no polysaccharide isolated from the cell walls of a monocot had been found to have the features of RG-II. The primary cell-walls of suspension-cultured Douglas fir, a gymnosperm, also contain[24] a polysaccharide having both the glycosyl

composition and glycosyl linkages of RG-II. Thus, RG-II appears to be a widespread feature of the primary cell-walls of higher plants.

We have also shown that rhamnogalacturonan I (RG-I), a polysaccharide structurally distinct from RG-II, is a component of the primary walls of suspension-cultured rice-cells. RG-I had been isolated from two other monocot sources: cell walls of rice endosperm[21] and primary walls of suspension-cultured maize *(Zea mays)* cells[11]. The cell walls of rice endosperm have been studied in detail (see Shibuya and Nakane[21] and references cited therein), but it was not shown that they contain RG-II. The primary walls used in the present study were isolated from suspension-cultured embryonic cells and may differ from the cell walls of endosperm, which is a triploid storage-tissue.

The presence of a 2-*O*-methylxylose-containing octasaccharide as a component of RG-II has now been established. The results of f.a.b.-m.s. clearly showed the octasaccharide to be among the products of partial-acid-hydrolysis of rice RG-II. The existence of such an octasaccharide in sycamore RG-II was suggested by Stevenson *et al.*[1]. They isolated a tetrasaccharide [2-*O*-methyl-Xyl-(1→4)-Fuc-(1→3)-Rha-(1→3′)-Apiitol] and identified 2-*O*-methylxylose as the glycosyl residue linked through O-3 of the 3,4-linked fucose in RG-II. It was concluded that the tetrasaccharide overlaps the structure of the glucuronic acid-containing heptasaccharide isolated by Melton *et al.*[6]; that heptasaccharide contains 4-linked fucosyl and no 2-*O*-methylxylose. RG-II contains 3,4-linked, but no 4-linked, fucose[1,2], indicating loss of one or more glycosyl residues from O-3 of fucose during the partial acid hydrolysis used to isolate the heptasaccharide. More direct evidence[1] of the presence of the octasaccharide in RG-II isolated from Pectinol AC has been obtained by using conditions of partial acid hydrolysis and ion-exchange chromatography similar to those described herein. The sequences of the octasaccharides derived from Pectinol AC and rice RG-II were not established; the sequence proposed here and elsewhere[1] is based on that of the heptasaccharide isolated by Melton *et al.*[6].

The complete residue-sequence of the aceric acid-containing heptasaccharide isolated from rice RG-II was deduced from the fragment-ions observed upon f.a.b.-m.s. analysis. The overall structure was then assigned by combining this sequence information with the glycosyl and glycosyl-linkage compositions. The complete structure (including anomeric configurations of the glycosidic linkages and absolute configurations of the glycosyl residues) of the aceric acid-containing heptasaccharide isolated from sycamore RG-II had previously been determined[7]; the linkage of aceric acid and the anomeric configurations of glycosidic bonds in the heptasaccharide isolated from rice RG-II were not determined, but were assumed to be the same as those in sycamore RG-II. However, all of the structural features of the aceric acid-containing heptasaccharide from rice RG-II that were determined are identical to those of the corresponding heptasaccharide from sycamore RG-II.

The structural features of RG-II isolated from the primary walls of suspension-cultured rice and sycamore cells are remarkably alike. Their glycosyl-

linkage compositions are virtually identical. Furthermore, the same oligosaccharides are released from the polysaccharide by partial acid hydrolysis, including two unusual disaccharides [β-Ara-(1→5)-DHA and α-Rha-(1→5)-KDO], an aceric acid-containing heptasaccharide, and a 2-*O*-methylxylose-containing octasaccharide.

Rhamnogalacturonan II is a minor component of rice cell-walls, perhaps accounting for as little as 0.1% of the wall by weight. Even in dicot primary cell-walls, where the pectic polysaccharides account for ~30% of the walls (ref. 12 and refs. cited therein), RG-II accounts for only 3 to 4% of the walls[2]. Considering the low levels of RG-II in the primary cell-walls of monocots and dicots, any structural role that RG-II may serve is unlikely to constitute its primary function.

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