The location of the 1:2 borate-diol ester cross-link in the dimer of the plant cell wall polysaccharide rhamnogalacturonan II (RG-II) has been determined. The ester cross-links the apiofuranosyl residue of the 2-O-methyl-D-xylose-containing side chains in each of the subunits of the dimer. The apiofuranosyl residue in each of the two aceric acid-containing side chains is not esterified. The site of borate esterification is identical in naturally occurring and in in vitro synthesized dimer. Pb\(^{2+}\), La\(^{3+}\), and Ca\(^{2+}\) increase dimer formation in vitro in a concentration- and pH-dependent manner. Pb\(^{2+}\) is the most effective cation. The dimer accounts for 55\% of the RG-II when the monomer (0.5 mM) is treated for 5 min at pH 3.5 with boric acid (1 mM) and Pb\(^{2+}\) (0.5 mM); at pH 5 the rate of conversion is somewhat slower. Hg\(^{2+}\) does not increase the rate of dimer formation. A cation's charge density and its ability to form a coordination complex with RG-II, in addition to steric factors, may regulate the rate and stability of dimer formation in vitro. Our data provide evidence that the structure of RG-II itself determines which apiofuranosyl residues are esterified with borate and that in the presence of boronic acid and certain cations, two RG-II monomers self-assemble to form a dimer.

Rhamnogalacturonan II (RG-II)\(^3\) is a low molecular weight, structurally complex pectic polysaccharide released from the primary walls of plants by treatment with endo-\(\alpha\)-1,4-polygalacturonase (1). The results of recent studies have established that RG-II exists predominantly in the cell walls of all higher plants as a dimer (dRG-II-B) that is cross-linked by a 1:2 borate-diol ester (2–5). Borate ester cross-linking of pectin may be required for the normal growth and development of plants (4–6) because boron deficiency results in the inhibition of plant growth and in the formation of cell walls with markedly altered physical properties (7–11).

A single 1:2 borate-di-ol ester is believed to cross-link two RG-II molecules. 1 mol of the dimer contains 1 mol of boron (3, 4, 12–14). We have proposed that two of the four 3'-linked apiofuranosyl (Api\(^f\)) residues present in dRG-II-B are cross-linked by borate because the dimer contains approximately equimolar amounts of 3'- and 2,3,3'-linked Api\(^f\) residues, whereas monomeric RG-II (mRG-II) contains only 3'-linked Api\(^f\) residues (4, 12). However, two different Api\(^f\)-containing side chains are attached to the backbone of each RG-II monomer (chains A and B in Fig. 1). It is not known whether the borate cross-link involves the Api\(^f\) residue in one of each of the two types of Api\(^f\)-containing side chains or if the borate is attached to the same type of Api\(^f\)-containing side chains in each monomer.

The ability to form dRG-II-B from mRG-II and boric acid in vitro provides a convenient model system to examine the mechanism of dimer formation (4). In addition, such studies are likely to provide information on the ability of dRG-II-B, which is present in fermented beverages such as wine, to form complexes with heavy metals (15). We have suggested that steric factors regulate dimer formation because only divalent cations with an ionic radius >1.1 Å (e.g. Pb\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\)) increase the rate of dRG-II-B formation significantly (4). However, the function of cations in dimer formation has not been established, nor is it known if the 1:2 borate-di-ol ester is located on the same glycosyl residue(s) in naturally occurring and in vitro synthesized dRG-II-B. We now report that the same two Api\(^f\) residues are the sites of borate esterification in naturally occurring and in vitro synthesized dRG-II-B. The effects of selected mono-, di-, tri-, and tetravalent cations on dRG-II-B formation are described as are the abilities of Pb\(^{2+}\), Cu\(^{2+}\), and La\(^{3+}\) to increase the rate of dimer formation from mRG-II and boric acid.

**EXPERIMENTAL PROCEDURES**

Isolation and Purification of RG-II—dRG-II-B was isolated from the cell walls of sugar beet tubers (3), potato tubers (16), bamboo shoots (17), and red wine (12) as described previously. Formation of mRG-II and dRG-II-B—mRG-II was generated by treating dRG-II-B (50 mg) for 30 min at room temperature with 0.1 M HCl (10 ml). The solution was dialyzed (1,000 molecular weight cutoff) at 4 °C against deionized water, and the RG-II was then converted to its sodium form by elution through a column (1 × 5 cm) containing Chelex-100 (Na\(^{+}\) form, Bio-Rad). The eluant was then freeze dried. dRG-II-B was generated in vitro by treating mRG-II (10 mg) for 4 days at room temperature with 50 mM potassium phthalate (10 ml), pH 3.5, containing 15 mM boric acid. dRG-II-B/Pb\(^{2+}\) was generated by

---

Tadashi Ishii, Toshiro Matsunaga, Patrice Pellerin, Malcolm A. O’Neill, Alan Darvill, and Peter Abersheim

*This work was supported in part by United States Department of Energy Grants DE-FG05-93ER20097 and DE-FG02-96ER20220, by Hercules Inc., Wilmington, Delaware, and by Japanese Ministry of Agriculture, Forestry, and Fisheries Glycoltechnology and Biodesign Program Grant BDF-98-II-1-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be sent: Complex Carbohydrate Research Center, the University of Georgia, 220 Riverbend Rd., Athens GA 30602. Tel.: 706-542-4419; Fax: 706-542-4412; E-mail: mao@ccrc.uga.edu.

*The abbreviations used are: RG-II, rhamnogalacturonan II; Api\(^f\), apiofuranosyl; dRG-II-B, dimeric rhamnogalacturonan-II-boron; GLCMS, gas-liquid chromatography with mass spectrometry; GLC-Cl-MS, gas-liquid chromatography with chemical ionization mass spectrometry; GLC-EI-MS, gas liquid chromatography with electron impact ionization mass spectrometry; Kdo, 3-deoxy-D-manno-octulopyranosyluronic acid; Kdo'ol, 3-deoxy-o-manno-octulopyranosyluronic acid; MeXyl, O-methyl-xylose; Api, apiofuranosyl; SEC, size-exclusion chromatography.
treating mRG-II (10 mg) for 24 h at room temperature with 50 mM potassium phthalate (5 ml), pH 3.5, containing 1 mM borate acid and 0.5 mM Pb(OAc)₂. The solutions were dialyzed separately (1,000 molecular weight cutoff) against deionized water and then freeze dried. The content of mRG-II, dRG-II-B, and dRG-II-B/Pb was determined by inductively coupled plasma atomic emission spectroscopy (4) and by inductively coupled plasma mass spectrometry (3).

**Methylation and Carboxyl Reduction of Methylated RG-II—** Separate solutions of mRG-II and dRG-II-B (~5 mg) in water (5 ml) were mixed with dimethyl sulfoxide (1 ml) and the mixtures purged with argon for 2 h at room temperature to remove air (18). Potassium methylsulfonyl-methanide (200 μl, 2.5 M in dimethyl sulfoxide) was then added, and the solutions were kept at room temperature for 2 h. The mixtures were then diluted with an equal volume of water and flushed with argon to remove the excess methyl iodide. The solutions were then dialyzed (1,000 molecular weight cutoff) for 24 h against deionized water and then freeze dried. The methylated, carboxyl-reduced borate ester was described (19). The methylated, carboxyl-reduced polysaccharide was desalted by dialysis and then freeze dried.

**Generation of the Rha-apitido Derivatives by Partial Fragmentation of Methylated and Carboxyl-reduced RG-II—** The methylated, carboxyl-reduced polysaccharide (~1 mg) was partially fragmented by treatment with 2 h at room temperature to generate the methylated polysaccharide (prolonged exposure (~3 h) of the dimer to the methylating reagents resulted in partial cleavage of the borate ester). The solutions were then diluted with an equal volume of water and flushed with argon to remove the excess methyl iodide. The solutions were then dialyzed (1,000 molecular weight cutoff) for 24 h against deionized water and then freeze dried.

**RESULTS**

A chemical procedure to locate the borate-esterified apiosyl residues in dRG-II-B—Two of the four Apif residues in dRG-II-B have been shown, by glycosyl linkage composition analysis, to be the probable sites of borate esterification (4, 12, 13). There are three ways the borate ester can cross-link two RG-II monomers. The borate could cross-link an Apif residue of side chain A to B or to another A, or two B side chains may be cross-linked. We describe in the following paragraphs experiments designed to determine which apiosyl residues are cross-linked by the borate ester using a chemical method (see Fig. 2).
originally developed to sequence complex carbohydrates (19).

The $^{13}$B NMR spectrum of methylated dRG-II-B in dimethyl sulfoxide contains a signal at $\delta$ 8.8 which corresponds to a 1:2 borate-diol ester (2–4) and establishes that the cross-link had not been hydrolyzed during the methylation reaction. Borate-esterified Api residues are not methylated, whereas unesterified Api residues are methylated at O-2 and O-3 (Fig. 2, step i). The methylated, carboxyl-reduced RG-II dimer was then partially fragmented by treatment with formic acid (Fig. 2, step ii). This treatment hydrolyzes the 1:2 borate-diol ester thereby exposing the hydroxyls to which it was attached and also generates a mixture of partially methylated oligoglycoses including the partially methylated disaccharide Rhap-(1–3′)-Api. The Rhap residue linked to the Api residue from side chain A has no O-methyl groups (Fig. 2, step i) whereas the Rhap residue linked to Api residue from side chain B has O-methyl groups at O-2 and O-4 (Fig. 2, step i). This provides a way to identify the side chain from which each Rhap-(1–3′)-Api originated. The methylated oligosaccharides were converted, by reduction with NaBD$_4$, to their corresponding partially methylated oligoglycosyl alditols (Fig. 2, step iii) and the free hydroxyl groups then O-acetylated (Fig. 2, step iv).

The partially O-methylated alditol from a borate esterified 3i-linked Api residue is acetylated at O-1, O-2, O-3, and O-4 (see 1 in Fig. 2), 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 (see 2 and 3 in Fig. 2). The Rhap residue originating from side chain A would be acetylated at O-2, O-3, and O-4 (see 1 and 2 in Fig. 2). The Rhap residue originating from side chain B is methylated at O-2 and O-4 and acetylated at O-3 (see 3 in Fig. 2). These Rhap-(1–3′)-apiitol derivatives differ in the number and positions of O-methyl and O-acetyl groups and are distinguished by using GLC-CI-MS to monitor their $[M + NH_4]^+$ ions ($m/z$ 611 (1), 555 (2), and 499 (3)). The locations 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 derivative.

The Api Residue of 2-O-MeXyl-containing Side Chain A is the Site of Borate Esterification—Naturally occurring dRG-II-B and mRG-II formed in vitro were methylated and carboxyl reduced. The Rhap-(1–3′)-apiitol derivatives were then generated and characterized by GLC-MS.

Two Rhap-(1–3′)-apiitol derivatives were generated from methylated, carboxyl-reduced dRG-II-B that were shown, by GLC-CI-MS, to have $[M + NH_4]^+$ ions at $m/z$ 611 (1) and 499 (3).
insets mass spectra were obtained at an ionization potential of 70 eV. The fragment ions of each derivative define the locations of the mRG-II. 

reduced dRG-II-B in which 50% of the Api derivatives that are generated from methylated and carboxyl-reduced mRG-II. The EI mass spectrum of derivative (1) has a molecular mass of 593 (611–18) and thus is fully methylated. The fragment ions at m/z 273 and 304 in the EI mass spectrum of (1) (Fig. 3A) confirm that the apiitol and the Rha residue do not contain O-methyl groups. Per-O-acetylated apiitol is the expected derivative of a borate-esterified Apif residue. The Rha residue must have been substituted at O-2, O-3, and O-4 because it contains no O-methyl groups and thus is the expected product of the Rha-(1→3')-apiitol derivatives of side chain A (see Fig. 1). Taken together these data establish that the partially methylated and O-acetylated monoglycosyl 3-deoxyoctitol originating from borate-esterified mRG-II contains no borate. Indeed, mRG-II contains no borate. It is important that these results demonstrate that our chemical procedure does distinguish between borate-esterified and unesterified apiitol derivatives.

The disaccharide α-L-Rhap-(1→5)-KdoαL is attached to C-3 of one of the RG-II backbone GalpA residues (20). The Kdo residue has cis hydroxyl groups at C-7 and C-8 which are a potential site for borate esterification. The following experiments were performed to determine if the Kdo is borate-esterified. The partially methylated and O-acetylated monoglycosyl alditol derivative of Rha-(1→5)-Kdo was generated by partial acid hydrolysis of methylated, carboxyl-reduced mRG-II and from naturally occurring dRG-II-B. The GLC-CI mass spectrum of the Rha-(1→5)-Kdo′ol derivative contained a [M + NH₄⁺]⁺ ion at m/z 659 (data not shown) irrespective of whether it was generated from mRG-II or dRG-II-B. The mass of this ion establishes that Rha-(1→5)-Kdo′ol did not originate from borate-esterified Rha-(1→5)-Kdo. No evidence was obtained by GLC-CI-MS for a Rha-(1→5)-Kdo′ol derivative with a [M + NH₄⁺]⁺ ion at m/z 659, the mass of the ion expected for a monoglycosyl 3-deoxyoctitol originating from borate-esterified Rha-(1→5)-Kdo. Thus, we conclude that the Kdo residue in RG-II is unlikely to be cross-linked by a borate ester.

The Location of the Borate Ester Is Identical in Naturally Occurring and in Vitro Synthesized dRG-II-B—The mechanism of dRG-II-B formation in plants is not known (4, 5). Thus the location of the 1:2 borate-diol ester may differ in naturally occurring and in vitro synthesized dRG-II-B. We performed an experiment to determine whether the location of the borate ester is the same in naturally occurring and in vitro synthesized dRG-II-Bs.

Dimeric RG-II-B isolated from sugar beet, potato, bamboo shoots, and red wine, dRG-II-B/Pb synthesized from mRG-II, boric acid and Pb²⁺, and dRG-II-B synthesized from mRG-II and boric acid were methylated and carboxyl reduced. The Rha-(1→3')-apiitol derivatives were generated (see Fig. 2) and then characterized by GLC-CI-MS and GLC-EL-MS. The Apif residue of the 2-O-MeXyl-containing side chains but not the Apif residue of the aceric acid-containing side chains was es-

![Figure 3](image-url)  
**FIG. 3.** GLC-EI-MS fragmentation patterns of the Rha-apiitol derivatives that are generated from methylated and carboxyl-reduced dRG-II-B in which 50% of the Apif residues are esterified with borate and from methylated and carboxyl-reduced mRG-II. Panels A, B, and C show the GLC-EI mass spectra of Rha-(1→3')-apiitol derivatives 1, 2, and 3, respectively. The EI mass spectra were obtained at an ionization potential of 70 eV. The insets in each panel show the structure of each derivative and the expected primary fragment ions from each derivative. The primary fragment ions of each derivative define the locations of the O-methyl and O-acetyl groups. For details, see "Results."
Borate Ester Cross-linked Apiosyl Residues of RG-II Dimers

**Location of the borate ester in naturally occurring and in vitro synthesized dRG-II-B**

Methylated, carboxyl-reduced dRG-II-B was partially fragmented by treatment with formic acid. The methylated Rha-apiose derivatives generated were converted to their Rha-apiitol derivatives, and the free hydroxyl groups were O-acetylated. The positions of the O-methyl and O-acetyl groups in Rha-apioitol derivatives 1–3 were determined by GLC-MS and define which side chain (A or B, see Fig. 1) is esterified with borate.

| Source of RG-II | % RG-II-B | 2-O-MeXyl-containing side chain A | Aeric acid-containing side chain B |
|----------------|-----------|----------------------------------|----------------------------------|
|                |           | Relative proportion | Relative proportion | Relative proportion |
| Sugar beet<sup>a</sup> | 95        | 100                 | 0                   | 0                   | 100                  |
| Potato<sup>b</sup> | 95        | 100                 | 0                   | 0                   | 100                  |
| Bamboo<sup>c</sup> | 90        | 100                 | 0                   | 0                   | 100                  |
| Red wine<sup>d</sup> | 80        | 100                 | 0                   | 0                   | 100                  |
| mRG-II<sup>e</sup> | 0         | 0                   | 100                 | 0                   | 100                  |
| dRG-II-B/Pb<sup>f</sup> | 20        | 30                  | 70                  | 0                   | 100                  |

**In vitro synthesized**

| dRG-II-B<sup>g</sup> | 70        | 88                  | 12                  | 0                   | 100                  |
| mRG-II<sup>h</sup> | 100       | 81                  | 19                  | 0                   | 100                  |
| dRG-II-B/Pb<sup>i</sup> | 70        | 57                  | 43                  | 0                   | 100                  |
| dRG-II-B | 95        | 100                 | 0                   | 0                   | 100                  |
| dRG-II-B | 100      | 93                  | 7                   | 0                   | 100                  |

<sup>a</sup>The percent of dRG-II-B and mRG-II was determined by SEC.

<sup>b</sup>The numbers in parentheses correspond to the methylated and acetylated Rha-apiitol derivatives shown in Figs. 2 and 3.

<sup>c</sup>The relative proportions of the esterified and nonesterified Api residues were estimated from the GC-EL-MS peak areas of 1–3.

<sup>d</sup>Naturally occurring dRG-II-B.

<sup>e</sup>mRG-II was generated by treating dRG-II-B for 30 min at 20 °C with 0.1 M HCl.

<sup>f</sup>dRG-II-B/Pb was synthesized in vitro by treating mRG-II with boric acid at pH 3.5 for 4 days at room temperature.

<sup>g</sup>dRG-II-B/Pb was synthesized in vitro by treating mRG-II with boric acid and Pb(NO<sub>3</sub>)<sub>2</sub> at pH 3.5 for 24 h at room temperature. For further details, see "Results."

**Di- and Trivalent Cations Increase the Rate of Formation of dRG-II-B**—We have provided evidence that the location of the 1:2 borate-diol ester is identical in naturally occurring and in vitro synthesized dRG-II-Bs. Thus, the mechanism of dimer formation can be analyzed using in vitro synthesis of dRG-II-B. In a previous study we showed that only divalent cations with an ionic radius of >1.1 Å increased dimer formation in vitro.<sup>4</sup> We now provide evidence that the ionic radius of the cation is only one of several factors that regulate dimer formation in vitro.

Divalent cations (Sr<sup>2+</sup>, Pb<sup>2+</sup>, and Ba<sup>2+</sup>) with an ionic radius >1.10 Å and trivalent cations (Eu<sup>3+</sup>, Pr<sup>3+</sup>, La<sup>3+</sup>, and Ce<sup>3+</sup>) with an ionic radius >0.90 Å significantly increased the amount of dimer formed in 24 h, whereas Ca<sup>2+</sup> and Cd<sup>2+</sup> (ionic radius of 0.99 and 0.95 Å, respectively) caused a small increase in the amount of dimer formed (Table II). Somewhat unexpectedly, Hg<sup>2+</sup>, which has an ionic radius of 1.10 Å, has no discernible effect on the amount of dimer that formed (Table II). This may result from the fact that Hg<sup>2+</sup> typically does not form stable coordination complexes with oxygen-donor ligands such as RG-II (21). Those cations that do increase the amount of dimer formed all have an affinity for oxygen-donor ligands (21). These results provide additional evidence that the cation-dependent increase in the rate of dimer formation in vitro most likely involves the formation of an RG-II-cation coordination complex. Thus, charge, ionic radius, and ligand-donor-atom selection are all factors that determine whether a particular cation will increase the amount of dRG-II-B formed in vitro.

**Effect of mono-, di-, tri-, and tetravalent cations on the amount of dRG-II-B formed from mRG-II and boric acid in 24 h at pH 3.7**

| Cation | Ionic radius<sup>a</sup> | dRG-II-B formed in 24 h<sup>b</sup> |
|--------|---------------------------|----------------------------------|
| None   | 0.00                      | 25                               |
| Na<sup>+</sup> | 0.97                     | 25                               |
| K<sup>+</sup> | 1.33                     | 25                               |
| Mg<sup>2+</sup> | 0.66                     | 20                               |
| Ni<sup>2+</sup> | 0.69                     | 25                               |
| Cu<sup>2+</sup> | 0.72                     | 20                               |
| Zn<sup>2+</sup> | 0.74                     | 30                               |
| Sn<sup>2+</sup> | 0.93                     | 15                               |
| Cd<sup>2+</sup> | 0.97                     | 30                               |
| Ca<sup>2+</sup> | 0.99                     | 35                               |
| Hg<sup>2+</sup> | 1.10                     | 25                               |
| Sr<sup>2+</sup> | 1.12                     | 90                               |
| Pb<sup>2+</sup> | 1.20                     | 100                              |
| Ba<sup>2+</sup> | 1.34                     | 95                               |
| Al<sup>3+</sup> | 0.55                     | 20                               |
| Sc<sup>3+</sup> | 0.76                     | 20                               |
| Eu<sup>3+</sup> | 0.95                     | 85                               |
| Pr<sup>3+</sup> | 0.99                     | 90                               |
| Ce<sup>3+</sup> | 1.01                     | 95                               |
| La<sup>3+</sup> | 1.03                     | 90                               |
| Ce<sup>4+</sup> | 0.87                     | 20                               |

<sup>a</sup>The crystal ionic radii of the cation (32).

<sup>b</sup>The values are nonequilibrium amounts of dRG-II-B.

**dRG-II-B Formation from mRG-II and Boric Acid Is Rapid in the Presence of Selected Di- and Trivalent Cations**—We showed previously that certain divalent cations increase the rate of dRG-II-B formation in vitro, although the rate achieved was relatively slow (4). We now show that dRG-II-B is formed within minutes from mRG-II and boric acid at pH 3.5 when in the presence of an appropriate divalent or trivalent cation.

Pb<sup>2+</sup> and La<sup>3+</sup> both induce a rapid, concentration-dependent increase in the rate of dRG-II-B formation at pH 3.5 (Fig. 4, A and B). Within 5 min in the presence of 0.5 mM Pb<sup>2+</sup>, ~55% of...
the monomer is converted to the dimer, and >90% conversion occurs within 1 h (Fig. 4A). La$^{3+}$ is less effective than Pb$^{2+}$ because only 60% of the monomer is converted to the dimer in 1 h, and the conversion is ~80% after 6 h (Fig. 4B). Ca$^{2+}$ is considerably less effective than both Pb$^{2+}$ and La$^{3+}$ (Fig. 4C). The rate of dimer formation in the presence of Pb$^{2+}$ and La$^{3+}$ is somewhat slower at pH 5 (Fig. 4, D and E). Ca$^{2+}$, even at high concentration (50 mM), is again considerably less effective than both Pb$^{2+}$ and La$^{3+}$ at pH 5 (Fig. 4F). Dimer formation is barely detectable within 6 h at pH 3.5 or 5 in the absence of added cations under the conditions of our experiments (Fig. 4, A and D), confirming that dimer formation in vitro is pH- and cation-dependent (4).

**DISCUSSION**

The Location of the Borate Ester Cross-link in dRG-II-B—We have provided evidence that a single 1:2 borate-diol ester in dRG-II-B cross-links the 3'-linked Api$f$ residues of the 2-O-MeXyl-containing side chains of the two mRG-II subunits but does not cross-link the 3'-linked Api$f$ residues of the aceric acid-containing side chains. The location of the B ester is the same in dRG-II-B isolated from natural sources and synthesized in vitro. However, 1:2 borate-apioside esters can exist in either of two diastereomeric forms (bis(β-D-api)-1,2:3,2,3-borate). Indeed, both diastereoisomers are formed when methyl-β-D-apioside is reacted with borate. It is not known if naturally occurring and in vitro synthesized dRG-II-B contain the same diastereoisomer. Nevertheless, we conclude that, irrespective of the diastereoisomer formed, the structure of RG-II itself determines which Api$f$ residues are esterified with borate.

We have reported previously that the maximum rate of dRG-II-B formation in vitro occurs between pH 3 and 4 and in the presence of selected divalent cations (4). In contrast, the 1:2 borate-diol esters of methyl-β-D-apioside form only above pH 5.2 even in the presence of cations. Thus, the pH- and cation-dependent formation of Api$f$ 1:2 borate-diol esters in dRG-II-B is determined by the structural characteristics of RG-II. We propose that the charge density of side chain A, which contains three uronosyl residues (see Fig. 1), may explain, in part, the requirement for divalent cations in dimer formation. Additional factors, including the conformations of both the 2-O-MeXyl- and aceric acid-containing side chains, are likely to contribute to the specific location of the borate ester.

**dRG-II-B Formation in Vitro and in Muro May Be Promoted by Different Divalent Cations**—The rate of cation-dependent dimer formation in vitro and the rate of dimer formation in suspension-cultured *Chenopodium album* cells are comparable. However, those cations that promote dimer formation in vitro (see Table II) are unlikely to be present at concentrations sufficiently high to promote dimer formation in muro (22, 23). In contrast, calcium is present in plant cell walls at mM concentrations (24), although most (>95%) of this calcium is bound, and the “free” calcium content of the wall is typically <5 mM. Furthermore, calcium ions have been reported to stabilize the borate ester cross-link in muro. Nevertheless, low concen-

---

2 T. Ishii and H. Ono, unpublished data.

3 A. Fleischer, M. A. O’Neill, and R. Ehwald, submitted for publication.
tations of Ca^{2+} (0.5 mM) do not promote rapid dimer formation in vitro under the conditions of our experiments, although higher concentrations (>5 mM) are somewhat effective (see Fig. 4, C and F). We suggest that the roles of divalent cations in regulating borate ester cross-linking of RG-II in vitro and in muro are not the same because soluble and wall-bound mRG-II differ.

The soluble mRG-II used to form the dimer in vitro and wall-bound RG-II are not identical. Soluble mRG-II has a backbone that contains between 7 and 15 1,4-linked α-D-galacturonosyl residues (12, 25), whereas the wall-bound RG-II backbone is believed to be covalently inserted within a homogalacturonan chain (1). Calcium ions may interact with both homogalacturonan and RG-II and thereby promote cross-link formation in muro. Moreover, wall-bound mRG-II molecules may be structurally constrained in a manner that favors borate ester cross-link formation. In contrast, borate ester formation in vitro is dependent on the direct interaction of di- and trivalent cations with RG-II itself. This interaction is determined, in part, by steric factors, because only divalent cations with ionic radii <1.10 Å and trivalent cations with ionic radii >0.95 Å promote dimer formation in vitro (Table II). Di- and trivalent cations may also stabilize the borate ester cross-link because treating naturally occurring dRG-II-B with EDTA results in the slow but discernible formation of mRG-II. Such results are consistent with a previous report showing that calcium ions form coordination complexes with and stabilize the 1:2 borate-diol esters of glucaric acid (26).

**dRG-II-B Is Formed by the Self-assembly of Two mRG-II Molecules**—dRG-II-B formation in muro must result from either a spontaneous self-assembly or an enzymatically catalyzed process. Our results do not preclude that borate ester formation is enzymically catalyzed in muro. However, we have shown that in the presence of boric acid and certain cations, two RG-II monomers rapidly self-assemble to form a dimer and that the structure of RG-II itself may determine the location of the borate ester.

The ability of plant cell wall polysaccharides to self-assemble into ordered structures has become the subject of considerable debate (27). For example, the parallel arrangement of 1,4-linked β-D-glucan chains in naturally occurring cellulose is believed to result in large part from the organization of the membrane-bound cellulose synthases because the spontaneous assembly of parallel glucan chains is entropically unfavorable (27). The formation of ordered structures is a characteristic of homogalacturonan because this polysaccharide spontaneously forms gels in the presence of calcium. Cellulose formation and the calcium-dependent gelation of homogalacturonan result from noncovalent interchain bonding (27). Moreover, a glycosyl residue in one glucan or galacturonan chain may interact with any of the glycosyl residues in a second chain. In contrast, RG-II dimer self-assembly requires the formation of a covalent borate ester cross-link between the Api’f residue of the same side chain (Fig. 1, side chain A) in each mRG-II subunit. The specificity and cation dependence of this cross-linking suggest that there are precise structural requirements for dRG-II-B formation, and this may explain why the structure of RG-II is highly conserved in higher plants (1).

**Borate Ester Cross-linking of RG-II May Alter the Mechanical Properties of the Plant Cell Wall**—We have provided evidence that a single borate ester cross-links two mRG-II molecules and that the location of the ester is the same in dRG-II-B isolated from different plants. The results of numerous studies suggest that the boron requirement and wall pectin content are correlated in many plants (28, 29) and that boron is required to maintain the mechanical properties of the primary wall (6, 8–11, 30, 31). For example, borate ester cross-linking of RG-II results in a rapid decrease in the wall pore size of suspension-cultured plant cells. Moreover, borate cross-link formation is required to prevent the walls from rupturing when the cells enter the stationary phase of their growth. Thus, the mechanical properties of the cell wall may be determined in large part by the macromolecular pectin network that most likely forms when RG-II is cross-linked by a borate ester. Nevertheless, additional roles for borate ester cross-linking of RG-II cannot be excluded.

In summary, we have shown that a single borate ester cross-links the Api’f residue of the 2-O-MeXyl-containing side chain of each mRG-II subunit in naturally occurring and in vitro synthesized dRG-II-B. Dimer formation in vitro is pH-dependent and occurs within minutes in the presence of certain di- and trivalent cations. RG-II is, to the best of our knowledge, the first example of a plant cell wall pectic polysaccharide that self-assembles to form structurally identical dimers.

**Acknowledgments**—We acknowledge Yuko Takeda of the Forestry and Forest Products Research Institute for technical assistance. We thank Dr. Joelyn Rose and Karen Howard of the CCRC for comments concerning drafts of this manuscript.

**REFERENCES**

1. O’Neill, M. A., Darvill, A. G., and Albersheim, P. (1990) in *Methods of Plant Biochemistry* (Dey, P. M., ed) Vol. 2, pp. 415–441, Academic Press, London.
2. Kobayashi, M., Matoh, T., and Azuma, J.-L. (1996) *Plant Physiol.* 110, 1017–1020.
3. Ishii, T., and Matsunaga, T. (1996) *Carbohydr. Res.* 284, 1–9.
4. O’Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G., and Albersheim, P. (1996) *J. Biol. Chem.* 271, 22925–22930.
5. Matoh, T. (1997) *Plant Soil* 193, 59–70.
6. Brown, P. H., and Hu, H. (1997) *Plant Soil* 196, 211–215.
7. Kouchi, H., and Kumazawa, K. (1975) *Soil Sci. Plant Nutr.* 21, 137–150.
8. Hirsch, A. M., and Torrey, J. G. (1980) *Can. J. Bot.* 58, 856–866.
9. Loomis, W. D., and Durst, R. W. (1992) *BioFactors* 3, 229–239.
10. Hu, H., and Brown, P. H. (1994) *Plant Physiol.* 105, 681–689.
11. Dell, B., and Huang, L. (1997) *Plant Soil* 193, 103–120.
12. Pellerin, P., Doco, T., Vidal, S., Williams, P., Brilouet, J.-M., and O’Neill, M. A. *Carbohydr. Res.* 290, 183–197.
13. Ishii, T., and Kaneko, S. (1998) *Phytochemistry* 49, 1189–1202.
14. Shin, K.-S., Kyohara, H., Matsumoto, T., and Yamada, H. (1998) *Carbohydr. Res.* 307, 97–106.
15. Pellerin, P., and O’Neill, M. A. (1998) *Analysis* 26, M32–M35.
16. Ishii, T. (1997) *Plant Physiol.* 115, 1285–1272.
17. Kaneko, S., Ishii, T., and Matsunaga, T. (1997) *Phytochemistry* 44, 243–248.
18. Stevenson, T. T., Darvill, A. G., and Albersheim, P. (1988) *Carbohydr. Res.* 179, 269–288.
19. McNeil, M., Darvill, A. G., Áman, P., Franzené, L.-E., and Albersheim, P. (1982) *Methods Enzymol.* 83, 3–45.
20. York, W. S., Darvill, A. G., McNeil, M., and Albersheim, P. (1985) *Carbohydr. Res.* 138, 109–126.
21. Frausto da Silva, J. J. R., and Williams, R. J. P. (1991) *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*, Clarendon Press, Oxford.
22. Kobayashi, M., Ohno, K., and Matoh, T. (1997) *Plant Cell Physiol.* 38, 676–683.
23. Matsunaga, T., Ishii, T., and Watanabe-Oda, H. (1997) in *Plant Nutrition for Sustainable Food Production and Environment* (Ando, T., ed) pp. 81–82, Kluwer Academic Publishers, London.
24. MacDougall, A. J., Parker, R., and Selvendran, R. R. (1995) *Plant Physiol.* 106, 1679–1689.
25. Whitcombe, A. J., O’Neill, M. A., Steffan, W., Albersheim, P., and Darvill, A. G. (1995) *Carbohydr. Res.* 271, 15–29.
26. Van Duin, M., Peters, J. A., Kieboom, A. P. G., and Van Bekkum, H. (1987) *Carbohydr. Res.* 162, 65–78.
27. Darvill, A. G. (1992) *Plant Cell Environ.* 15, 1–5.
28. Hu, H., Brown, P. H., and Labavitch, J. M. (1996) *J. Exp. Bot.* 47, 227–232.
29. Matoh, T., Kawaguchi, S., and Kobayashi, M. (1996) *Plant Cell Physiol.* 37, 636–640.
30. Findikli, P., and Golbach, H. E. (1996) *Bot Acta* 109, 463–465.
31. Fleischer, A., Titel, C., and Ehwdal, R. (1998) *Plant Physiol.* 117, 1401–1410.
32. Weast, R. C. (1983) in *CRC Handbook of Chemistry and Physics* (Weast, R. C., ed) 64th Ed., p F-170, CRC Press, Boca Raton, FL.