# Occurrence of the Primary Cell Wall Polysaccharide Rhamnogalacturonan II in Pteridophytes, Lycophytes, and Bryophytes. Implications for the Evolution of Vascular Plants[1]

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Borate ester cross-linking of the cell wall pectic polysaccharide rhamnogalacturonan II (RG-II) is required for the growth and development of angiosperms and gymnosperms. Here, we report that the amounts of borate cross-linked RG-II present in the sporophyte primary walls of members of the most primitive extant vascular plant groups (Lycopsida, Filicopsida, Equisetopsida, and Psilopsida) are comparable with the amounts of RG-II in the primary walls of angiosperms. By contrast, the gametophyte generation of members of the avascular bryophytes (Bryopsida, Hepaticopsida, and Anthocerotopsida) have primary walls that contain small amounts (approximately 1% of the amounts of RG-II present in angiosperm walls) of an RG-II-like polysaccharide. The glycosyl sequence of RG-II is conserved in vascular plants, but these RG-IIs are not identical because the non-reducing L-rhamnosyl residue present on the aceric acid-containing side chain of RG-II of all previously studied plants is replaced by a 3-*O*-methyl rhamnosyl residue in the RG-IIs isolated from *Lycopodium tristachyum*, *Ceratopteris thalictroides*, *Platycerium bifurcatum*, and *Psilotum nudum*. Our data indicate that the amount of RG-II incorporated into the walls of plants increased during the evolution of vascular plants from their bryophyte-like ancestors. Thus, the acquisition of a boron-dependent growth habit may be correlated with the ability of vascular plants to maintain upright growth and to form lignified secondary walls. The conserved structures of pteridophyte, lycophyte, and angiosperm RG-IIs suggests that the genes and proteins responsible for the biosynthesis of this polysaccharide appeared early in land plant evolution and that RG-II has a fundamental role in wall structure.

Plants first colonized the land approximately 480 million years ago. These early land plants, which are believed to be related to extant bryophytes (Kenrick and Crane, 1997; Qiu and Palmer, 1999), subsequently gave rise to the tracheophytes that now dominate the terrestrial environment. Many of the morphological and biochemical changes that allowed plants to adapt to life on land have been documented (Graham, 1993; Niklas, 1997). However, the limited amount of information available on the composition and architecture of non-flowering plant cell walls (Ligrone et al., 2002; Popper and Fry, 2003) is an impediment to understanding the evolutionary origins of cell walls and the changes in wall structure that occurred during the evolution of land plants.

All growing plant cells are surrounded by a polysaccharide-rich primary wall. Primary walls regulate cell expansion and also have important roles in plant growth and development, in the defense against micro-organisms (Carpita and Gibeaut, 1993), and in intercellular signaling (Ridley et al., 2001). The primary walls of seed-bearing tracheophytes (gymnosperms and angiosperms) are composed predominantly of cellulose, hemicellulose, and pectin together with lesser amounts of structural glycoproteins, minerals, enzymes, and phenolic esters (Carpita and Gibeaut, 1993). Much less is known about the composition of the primary walls of bryophytes (Bryopsida [mosses], Hepaticopsida [liverworts], and Anthocerotopsida [hornworts]) and the spore-bearing tracheophytes that include the lycophytes (Lycopodiales [club mosses] and Selaginellales [spike mosses]) and pteridophytes (Filicopsida [ferns], Equisetopsida [horsetails], and Psilopsida [whisk ferns]).

Recent studies have shown that boron has a major role in maintaining wall structure and that the boron requirements of flowering plants are correlated with the amounts of pectin in their primary walls (Loomis and Durst, 1992; Hu et al., 1996; Brown et al., 2002). Boron exists in primary walls predominantly as a 1:2

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borate-diol ester that covalently cross-links two chains of the pectic polysaccharide rhamnogalacturonan II (RG-II; Kobayashi et al., 1996; Ishii and Matsunaga, 1996; O'Neill et al., 1996), which has the added effect of covalently cross-linking the two homogalacturonan chains upon which the RG-II molecules were constructed (Ishii and Matsunaga, 2001).

Cross-linking of RG-II is believed to result in the formation of a macromolecular pectic network that regulates the size exclusion limit (Fleischer et al., 1999) and the mechanical properties of the primary wall (Ishii et al., 2001). Plants carrying mutations that result in abnormally low amounts of this cross-link are dwarfed (O'Neill et al., 2001) or exhibit reduced intercellular attachment (Iwai et al., 2002). Thus, the structural organization of primary wall pectic polysaccharides is likely to be more important for the growth of flowering plants than had previously been believed (Ishii et al., 2001; O'Neill et al., 2001).

The RG-II synthesized by flowering plants is the most structurally complex naturally occurring polysaccharide yet identified (Ridley et al., 2001). The structure of this polysaccharide (see Fig. 1) is fully conserved in all of the flowering plants analyzed to date (O'Neill et al., 1990; Ridley et al., 2001). RG-II contains 12 different glycosyl residues including the seldom observed sugars D-apiose (Api), L-aceric acid (3-*C*-carboxy-5-deoxy-L-Xyl; AceA), 2-*O*-methyl L-Fuc (2Me-Fuc), 2-*O*-methyl D-Xyl (2Me-Xyl), L-Gal, 2-keto-3-deoxy-D-*lyxo*-heptulosaric acid (Dha), and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo; O'Neill et al., 1990; Ridley et al., 2001). These and other residues (D-GalUA [GalA], D-GlcUA [GlcA], L-rhamnose [Rha], L-Gal [Gal], L-Ara [Ara], and L-Fuc [Fuc]) are interconnected by more than 20 different glycosidic linkages (see Fig. 1). The RG-II backbone contains at least eight 1,4-linked α-D-GalA residues (Whitcombe et al., 1995). Two structurally complex oligosaccharides (Fig. 1, side chains A and B, respectively) are attached by a β-D-Api residue to *O*-2 of the backbone (Ridley et al., 2001). Separate Dha- and Kdo-containing disaccharides (Fig. 1, side chains C and D, respectively) are attached to *O*-3 of the backbone (Ridley et al., 2001). The borate ester that links two RG-II molecules together is formed between the apiosyl residue in side chain A of each monomer (Ishii et al., 1999).

The cumulative results of numerous studies provide compelling evidence that borate cross-linked RG-II is present in the walls of all flowering plants (O'Neill et al., 1990; Matoh et al., 1996). A RG-II-like polysaccharide has also been reported to be present in the walls of the fern *Adiantum* sp., although this putative RG-II was not structurally characterized (Matoh and Kobayashi, 1998). There are no reports describing the presence of RG-II in the other members of the pteridophytes (Equisetopsida and Psilopsida) or in lycophytes and bryophytes. RG-II has not hitherto been isolated from any of these nonflowering plants and structurally characterized.

Here, we report that comparable amounts of borate cross-linked RG-II are present in the walls of angiosperms and plants selected from the lycophytes and pteridophytes, which are among the most primitive

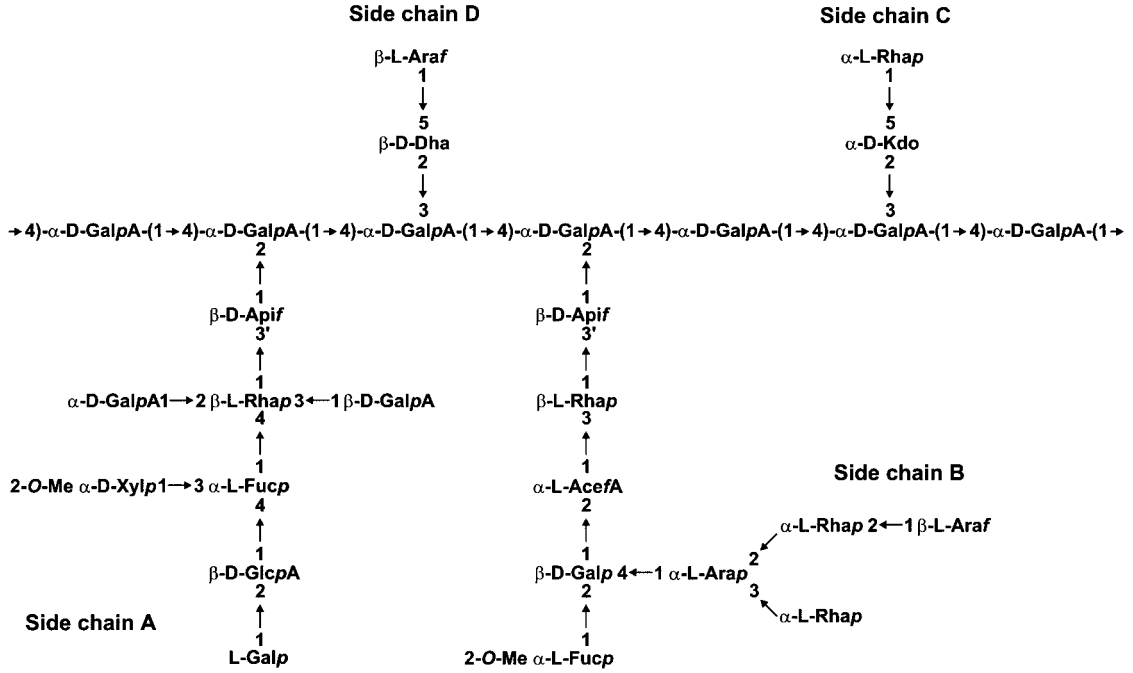



**Figure 1.** The glycosyl sequence of RG-II isolated from angiosperm cell walls. The locations on the backbone of the four side chains (A–D) relative to one another have not been determined with certainty. The anomeric configuration of the terminal L-Gal residue in side chain A is not known.

plants that have evolved lignified vascular tissues. By contrast, the gametotphyte generation of members of the Bryopsida, Hepaticopsida, and Anthocerotopsida, which contain those land plants that do not form lignified vascular tissue, have primary walls that contain small amounts (approximately 1% of the amounts of RG-II present in angiosperm walls) of an RG-II-like polysaccharide. We provide evidence that the structural features of RG-II in angiopsperms are conserved in pteridophytes and lycophytes.

## RESULTS

### Lycophyte and Pteridophyte Walls Contain Glycosyl Residues That Are Diagnostic of RG-II

Cell walls were isolated from actively growing regions of the aerial portions of the gametophyte generation of 13 different species of bryophytes, and the sporophyte generation of six different species of lycophytes and 12 different species of pteridophytes (see Table I).

In preliminary experiments we established that four of the characteristic RG-II glycoses (Api, AceA, 2Me-Fuc, and 2Me-Xyl; see Fig. 1) are reliably detected by gas chromatography in combination with electron impact mass opectrometric analysis of the alditol acetates derived from samples that contained 0.5% (w/w) RG-II. This value was obtained by analyzing material that contained known amounts of wine RG-II (0.1–5.0 mg) mixed with cellulose (100 mg). A mixture that contained 0.1% (w/w) RG-II gave total ion current peaks corresponding to Fuc and 2Me-Xyl, but not Api and AceA. This was expected because the GC/EI-MS procedure (York et al., 1985) underestimates by at least 50% the amounts of Api and AceA present in RG-II.

Peaks corresponding to Api, AceA, 2Me-Fuc, and 2Me-Xyl were detected by GC/EI-MS analysis of the alditol acetate derivatives from all of the pteridophyte and lycophyte walls (1–2 mg; see Table I for the plants used in this study). These peaks gave electron impact mass spectra that were indistinguishable from the corresponding derivatives generated from red wine RG-II. This result by itself made it highly likely that pteridophyte and lycophyte primary walls contain RG-II. The presence of RG-II in bryophyte walls (1–2 mg; see Table I for the plants used in this study) could not be established with certainty because only trace amounts of 2Me-Fuc and 2Me-Xyl, but no Api or AceA, were detected by GC/EI-MS analysis.

### The Contents of Boron and of Borate Cross-Linked RG-II in the Cell Walls of Pteridophytes, Lycophytes, and Bryophytes

Approximately 95% of the RG-II present in angiosperm walls is solubilized by Driselase, a commercially available mixture of glycanses that include pectinase, hemicellulase, and cellulase (Ishii and Matsunaga, 1996). The solubilized RG-II can then be quantified as borate cross-linked RG-II by size-exclusion chromatography in combination with inductively coupled plasma mass spectrometry (SEC/ICP-MS; Matsunaga et al., 1997).

Similar amounts of boron are present in pteridophyte ($21.0 \pm 8.9$ $\mu$g boron $g^{-1}$ dry weight wall, $n = 12$), lycophyte ($15.1 \pm 11.5$ $\mu$g boron $g^{-1}$ dry weight wall, $n = 7$), and bryophyte ($12.4 \pm 9.9$ $\mu$g boron $g^{-1}$ dry weight wall, $n = 13$) walls. However, these amounts do not necessarily reflect the amount of RG-II dimer present in the walls because boron may be bound non-specifically to polysaccharides. The

**Table I.** *The plants used in this study*

| Bryophytes | Lycophytes | Pteridophytes |
|---|---|---|
| Bryopsida | Lycopodiales | Psilopsida |
| *Dicranodontium denudatum*[a] | *Huperzia lucidula*[b] | *Psilotum nudum*[c,d] |
| *Hypnum oldhamii*[a] | *Lycopodium nummularifolium*[d] | Equisetopsida |
| *Loeskeobryum cavifolium*[a] | *Lycopodium scariosum*[c] | *Equisetum hyemale*[c] |
| *Platyhypnidium riparioides*[a] | *Lycopodium tristachyum*[b] | *Equisetum variegatum*[d] |
| *Pogonatum japonicum*[a] | Selaginellales | Filicopsida |
| *Polytrichum juniperinum*[a] | *Selaginella kraussiana*[c] | *Angiopteris lygodiifolia*[d] |
| *Sphagnum palustre*[a] | *Selaginella tamariscina*[d] | *Ceratopteris thalictroides*[d] |
| *Takakia ceratophylla*[e] | | *Ceratopteris richardii*[b] |
| Hepaticopsida | | *Cyrtomium falcatum*[d] |
| *Bazzania pompeana*[a] | | *Lacosteopsis orientalis*[d] |
| *Conocephalum conicum*[a] | | *Matteuccia struthiopteris*[d] |
| *Marchantia polymorpha*[c] | | *Platycerium bifurcatum*[c] |
| *Pallavicinia subciliata*[a] | | *Sceptridium* sp.[d] |
| Anthocerotopsida | | *Salvinia molesta*[d] |
| *Phaeoceros carolinianus*[a] | | |

[a]Plants collected from fields in Japan. [b]Purchased from The Carolina Biological Supply Company (Burlington, NC). [c]Obtained from the greenhouses of The Department of Plant Biology, The University of Georgia. [d]Obtained from the greenhouses of National Science Museum (Tsukuba, Japan). [e]Plants collected from fields in China.

amount of boron solubilized by Driselase treatment of the these walls varied form species to species. Between 12% and 73% of the boron solubilized by Driselase from pteridophyte and lycophyte walls eluted in the region for borate cross-linked RG-II (Fig. 2, A and B), whereas boric acid accounted for at least 95% of the boron in the bryophyte digests (Fig. 3, A and B). Angiosperm borate cross-linked RG-II contains discernible amounts of $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, and $Pb^{2+}$ (O'Neill et al., 1996; Ishii et al., 1999). Signals corresponding to these elements co-eluted with $^{11}B$ in the Driselase digests of all of the pteridophyte, lycophyte, and bryophyte walls (data not shown), which provides additional evidence that all of these walls contain RG-II.

The RG-II dimer content of the walls was calculated from the amount of bound borate that eluted in the region containing the RG-II dimer and by assuming that the boron content of the RG-II dimer is 1 mg $g^{-1}$ (Ishii and Matsunaga, 1996; Kaneko et al., 1997). These data were then used to calculate the average amounts of RG-II in the walls of each plant group (see Table II). The differences in the amounts of borate cross-linked RG-II in pteridophyte (9.3 ± 4.9 mg RG-II $g^{-1}$ dry weight) and lycophyte (7.4 ± 6.0 mg RG-II $g^{-1}$ dry weight) walls are not statistically significant. Moreover, the amounts of RG-II in the pteridophyte and lycophyte cell walls are roughly of the same order of magnitude as dicots and nongraminaceous monocots and the Gramineae (Table II). In contrast, RG-II accounts for only 0.10 ± 0.06 mg $g^{-1}$ dry weight bryophyte cell walls. This amount is significantly different ($P < 0.0001$) from the amounts of RG-II in vascular plant walls. Thus, it is likely that bryophyte gametophyte walls contain much less RG-II than vascular plant cell walls.

### Structural Characterization of RG-II from the Walls of Lycophytes and Pteridophytes

A mixture of rhamnogalacturonan I (RG-I), RG-II, and partially methyl-esterified oligogalacturonides are generated by treating angiosperm walls with homogeneous endopolygalaturonase (EPG; O'Neill et al., 1990). Thus, the walls of selected lycophytes and pteridophytes (see Table III) were treated with EPG, and the products were fractionated by SEC with refractive index detection (SEC/RI).

Peaks that eluted in the region expected for monomeric and dimeric RG-II were detected by SEC/RI of the EPG-soluble material obtained from the walls of *C. thalictroides*, *S. molesta*, *P. bifurctatum*, *P. nudum*, *E. hyemale*, *L. tristachyum*, and *S. kraussiana*. At least 80% of each of the RG-IIs in these extracts eluted as the dimer (see Fig. 4, A and B) and had glycosyl residue (Table III) and glycosyl-linkage (Table IV) compositions that are, with the exception of the presence of 3-*O*-methyl rhamnose (3Me-Rha), indistinguishable from those of angiopserm RG-II. The boron contents of the RG-II from *C. thalictroides* (1.03 mg boron $g^{-1}$ RG-II) and *S. molesta* (0.55 mg boron $g^{-1}$ RG-II) are comparable with the boron contents of angiosperm RG-II (Ishii and Matsunaga, 1996; O'Neill et al., 1996; Kaneko et al., 1997). The $^{11}B$-NMR spectrum of *C. thalictroides* RG-II (Fig. 4C) contains a signal at δ-9.6 that originates from a 1:2 borate:diol diester (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996). To confirm that the pteridophyte RG-II

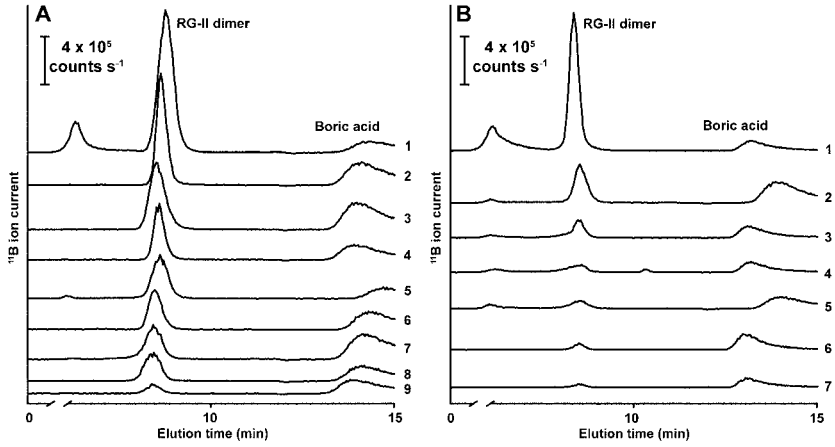



**Figure 2.** SEC/ICP-MS $^{11}B$ profiles of the material released by Driselase treatment of pteridophyte and lycophyte walls. A, The $^{11}B$ profiles of the material solubilized from pteridophyte walls with Driselase. 1, *A. lygodiifolia*; 2, *C. thalictroides*; 3, *E. variegatum*; 4, *P. nudum*; 5, *C. falcatum* (gametophyte); 6, *C. falcatum* (sporophyte); 7, *M. struthiopteris*; 8, *S. molesta*; and 9, *L. orientalis*. B, The $^{11}B$ profiles of the material solubilized from lycophyte walls with Driselase. 1, *S. kraussiana*; 2, *S. tamariscina*; 3, *L. scariosum*; 4, *H. lucidula*; 5, *L. nummularifolium*; 6, *L. tristachyum* (leaf); and 7, *L. tristachyum* (rhizome). Cell walls (5–10 mg) were suspended in 15 mM ammonium acetate (1 mL), pH 5.0, containing Driselase (5 mg). The mixture was shaken for 20 h at 30°C and then centrifuged. The suspension was filtered through a 0.45-μm nylon membrane, and a portion (100 μL) of the soluble material was analyzed by SEC/ICP-MS. The $^{11}B$ ion count scale is shown by the vertical bar ($4 \times 10^5$ counts $s^{-1}$). No borate cross-linked RG-II was detected in a control sample containing Driselase alone.

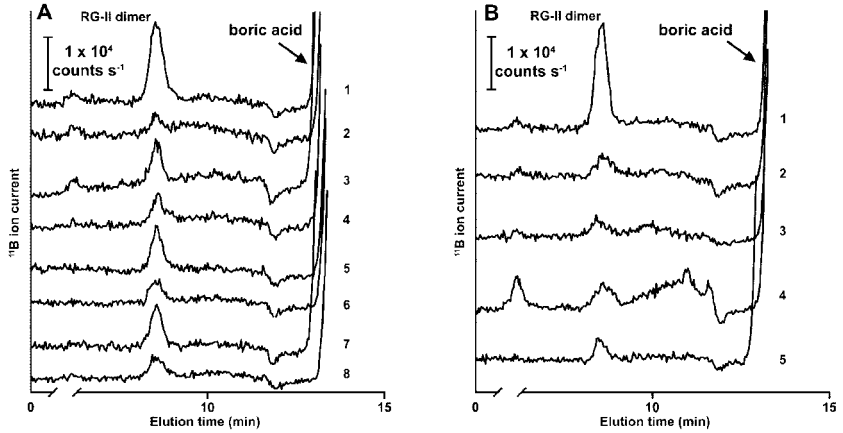



**Figure 3.** SEC/ICP-MS $^{11}B$ profiles of the material released by Driselase treatment of bryophyte walls. A, The $^{11}B$ profiles of the material solubilized from Bryopsida walls with Driselase. 1, *H. oldhamii*; 2, *D. denudatum*; 3, *L. cavifolium*; 4, *P. riparioides*; 5, *P. japonicum*; 6, *P. juniperum*; 7, *S. palustre*; and 8, *T. ceratophylla*. B, The $^{11}B$ profiles of the material solubilized from Hepaticopsida and Anthocerotopsida walls with Driselase. 1, *B. pompeana*; 2, *C. conicum*; 3, *M. polymorpha*; 4, *P. subciliata*; and 5, *P. carolinianus*. Cell walls (5–10 mg) were suspended in 15 mM ammonium acetate (1 mL), pH 5.0, containing Driselase (5 mg). The mixture was shaken for 20 h at 30°C and then centrifuged. The suspension was filtered through a 0.45-$\mu$m nylon membrane, and a portion (200 $\mu$L) of the soluble material was analyzed by SEC/ICP-MS. The $^{11}B$ ion count scale is shown by the vertical bar ($1 \times 10^4$ counts $s^{-1}$). This scale is $\times 40$ more sensitive than the corresponding scale in Figure 2. The $^{11}B$ signal for boric acid corresponds to the large peak beginning at approximately 13 min. The maximum intensity of this signal was between 1.6 and $7.0 \times 10^5$ counts $s^{-1}$. No borate cross-linked RG-II was detected in a control sample containing Driselase alone.

exists as a borate, cross-linked dimer solutions of the EPG-soluble material from *P. nudum* and *E. hyemale* were treated for 45 min at 22°C with 0.1 M HCl. These conditions, which are known to hydrolyze the borate diester and generate monomeric RG-II and boric acid (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996), convert the pteridophyte RG-II dimer into monomer (Fig. 4, A and B, middle profiles). The dimer reformed (Fig. 4, A and B, bottom profiles) when the monomer was reacted with boric acid for 3 h at pH 3.5 in the presence of $Pb(OAc)_2$. These results establish that lycophyte and pteridophyte cell walls contain borate cross-linked RG-II and that in vitro, the cross-link has chemical properties that are indistinguishable from those of angiosperm RG-II.

### Side Chain B of Lycophyte and Pteridophyte RG-II Contains 3-*O*-Methyl Rhamnosyl Residues

Somewhat unexpectedly, 3Me-Rha was detected in the RG-IIs isolated from *C. thalictroides*, *C. richardii*, *S. molesta*, *P. bifurctatum*, *P. nudum*, and *L. tristachyum* (Table III). This glycosyl residue is not present in

**Table II.** *The amounts of borate cross-linked RG-II in the cell walls of bryophytes, lycophytes, pteridophytes, dicotyledenous and non-graminaceous monocots, and the gramineae*

The amounts of RG-II in the non-flowering plant walls were determined by SEC/ICP-MS analyses of the material solubilized from bryophytes, lycophytes, and pteridophytes by Driselase.

| Plant Group | Average Amount of Borate Cross-Linked RG-II | Range of the Amount of Borate Cross-Linked RG-II |
|---|---|---|
| | *mg $g^{-1}$ dry wt cell wall ± SD* | *mg $g^{-1}$ dry wt cell wall* |
| Bryophytes<br>*n* = 13[a] | 0.1 ± 0.06 | 0.04–0.25 |
| Lycophytes<br>*n* = 7 | 7.4 ± 6.2 | 2.0–19.1 |
| Pteridophytes<br>*n* = 12 | 9.3 ± 4.9 | 1.9–19.9 |
| Dicotyledons and non-graminaceous monocotyledons[b]<br>*n* = 18 | 22.8 ± 8.5 | 5.0–36.0 |
| Gramineae[c]<br>*n* = 4 | 3.8 ± 1.0 | 3.0–5.0 |

[a]*n*, Number of different plant species analyzed. [b]Amounts calculated from Matoh et al. (1996). [c]Amounts calculated from Matoh et al. (1996) and Kaneko et al. (1997).

**Table III.** *Glycosyl residue compositions of the RG-II isolated from lycophyte, pteridophyte, gymnosperm, and angiopserm cell walls*

RG-II was isolated by SEC from the material solubilized from the walls of lycophytes and pteridophytes by treatment with EPG. The glycosyl residue compositions were determined by GC/EI-MS analysis of the alditol acetates.

| Plant | Glycosyl Residue | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MeFuc | MeRha | Rha | Fuc | MeXyl | Ara | Ace | Api | Man | Gal | Glc | GlcA | GalA | Dha | Kdo |
| | *mol%* | | | | | | | | | | | | | | |
| *C. thalictroides* | 2 | 3 | 15 | 2 | 4 | 13 | 2 | 6 | nd[a] | 9 | 7 | 5 | 37 | d[b] | d |
| *S. molesta* | 4 | 3 | 15 | 3 | 4 | 10 | 2 | 7 | 1 | 11 | 7 | 3 | 34 | d | d |
| *C. richardii*[c] | 2 | 2 | 10 | 2 | 3 | 12 | 1 | 5 | 1 | 8 | 3 | 3 | 42 | 2 | 3 |
| *P. bifurcatum* | 3 | 6 | 8 | 2 | 3 | 11 | 1 | 4 | 5 | 13 | 5 | 2 | 33 | 2 | 2 |
| *E. hyemale* | 3 | nd | 8 | 2 | 3 | 18 | 2 | 5 | 1 | 22 | nd | 2 | 30 | 2 | 2 |
| *P. nudum* | 3 | 7 | 9 | 2 | 3 | 14 | 1 | 5 | 6 | 15 | 4 | 2 | 26 | 1 | 2 |
| *S. kraussiana* | 3 | nd | 12 | 2 | 4 | 15 | 1 | 5 | 1 | 11 | 1 | 2 | 39 | 2 | 2 |
| *L. tristachyum* | 3 | 2 | 10 | 3 | 3 | 12 | 1 | 6 | 3 | 14 | 3 | 2 | 34 | 2 | 2 |
| Arabidopsis | 3 | nd | 11 | 2 | 3 | 16 | 1 | 5 | 1 | 19 | 1 | 3 | 30 | 1 | 2 |
| Sugar beet (*Beta vulgaris*)[d] | 3 | nd | 11 | 2 | 5 | 11 | d | 5 | nd | 12 | nd | 7 | 38 | 2 | 3 |
| *Pinus densiflora*[e] | 6 | nd | 13 | 2 | 5 | 12 | d | 7 | nd | 6 | nd | 2 | 38 | 3 | 3 |
| Grape (red wine) | 3 | nd | 12 | 2 | 4 | 13 | 2 | 5 | 4 | 13 | 1 | 3 | 34 | 2 | 2 |

[a]nd, Not detected. [b]d, Detected but not quantified. [c]Gametophyte. [d]Ishii and Matsunaga (1996). [e]Shimokawa et al. (1999).

angiosperm RG-II (O'Neill et al., 1990; Ridley et al., 2001). To determine the location of the 3Me-Rha residues, the glycosidic bonds of the apiosyl residues were selectively cleaved with 0.1 M trifluoroacetic acid (TFA; Spellman et al., 1983; Whitcombe et al., 1995). This treatment generates low-$M_r$ material (200–1,400 D) that is enriched with side chains B and D and a high-$M_r$ material (approximately 4 kD) that is enriched with backbone that is substituted with side chains A and C. The side chain B-containing fractions were isolated by SEC, and their glycosyl residue and glycosyl-linkage compositions were determined.

The side chain B material from *C. thalictroides*, *P. bifurctatum*, *P. nudum*, and *L. tristachyum* all contain 2Me-Fuc, Rha, Api, AceA, Ara, and Gal in addition to 3Me-Rha, which itself accounted for between 7 mol% and 15 mol% of the glycoses. Glycosyl linkage composition analyses of the per-*O*-deuteriomethylated oligosaccharides established that the 3Me-Rha was present as a terminal non-reducing residue (data not shown). This result together with the presence of a 2,3-linked Ara*p* residue suggested that in side chain B, the 3Me-Rha is linked to *O*-2 and/or *O*-3 of the Ara*p* residue.

The presence of 3Me-Rha residues in side chain B was confirmed by electrospray ionization MS (ESI-MS) and electrospray ionization product-ion MS (ESI/MS-MS). Somewhat unexpectedly, positive ion ESI-MS gave protonated molecular ions ($[M+H]^+$) that had even masses (Table V). Side chain B oligoglycoses have nominal masses with even numbers and were anticipated to give odd numbered $[M+H]^+$ ions. It is likely that the reducing apiose is converted to its apiosylamine derivative during the removal of ammonium formate by repeated freeze-drying. A glycosylamine is readily formed when a reducing glycose is reacted with a aqueous ammonium salt (Likhosherstov et al., 1986). A glycosylamine-terminated oligosaccharide gives an even numbered $[M+H]^+$ ion because its mass is 1 atomic mass unit lower than the corresponding reducing oligoglycose. Moreover, Y-type fragment ions (Gillece-Castro and Burlingame, 1990) with even masses dominate the product ion spectra generated from side chain B $[M+H]^+$ ions (see Fig. 5), which suggests that the positive charge was localized at the reducing terminus of the fragments, a characteristic of oligoglycosylamines. Additional evidence that the glycosylamine had formed was obtained by ESI-MS analyses of maltopentaose (nominal mass 828 D) that had been treated with ammonium formate or with sodium acetate. Ammonium formate-treated maltopentaose gave ions at *m/z* 828 and 829 that correspond to $[M+H]^+$ from the oligoglycosylamine derivative and the reducing oligoglycose, respectively. The ion at *m/z* 828 was not present in the ESI mass spectrum of sodium acetate-treated maltopentaose (K. Kolli and M.A. O'Neill, unpublished data).

The ESI mass spectra of the side chain B fraction from *P. nudum* and *P. bifurcatum* both contain an ion at *m/z* 1,272 that corresponds to $[M+H]^+$ of a glycosylamine-terminated oligosaccharide composed of one Api, one Rha, one AceA, one Gal, one Ara, three 6-deoxy methyl hexoses, and one *O*-acetyl residue (Table V). The corresponding oligosaccharides (*m/z* 1,230) that contain no *O*-acetate or that contain one less 6-deoxy methyl hexose residue with either zero (*m/z* 1,070) or one *O*-acetate (*m/z* 1,112) are also present. The product ion spectra of the ions at *m/z* 1,070 (Fig. 5A) and 1,272 (Fig. 5C) contain fragment ions that are consistent with the presence of one and two terminal non-reducing 3-*O*-Me Rha residues, respectively, and the presence of *O*-acetyl groups on either the AceA or 2Me-Fuc residue (see Fig. 6; oligosaccharides 2, 2a, 2b, 6, 6a, 6b in Table V).

The ESI mass spectrum of side chain B from *P. bifurcatum* and *L. tristachyum* contain an ion at

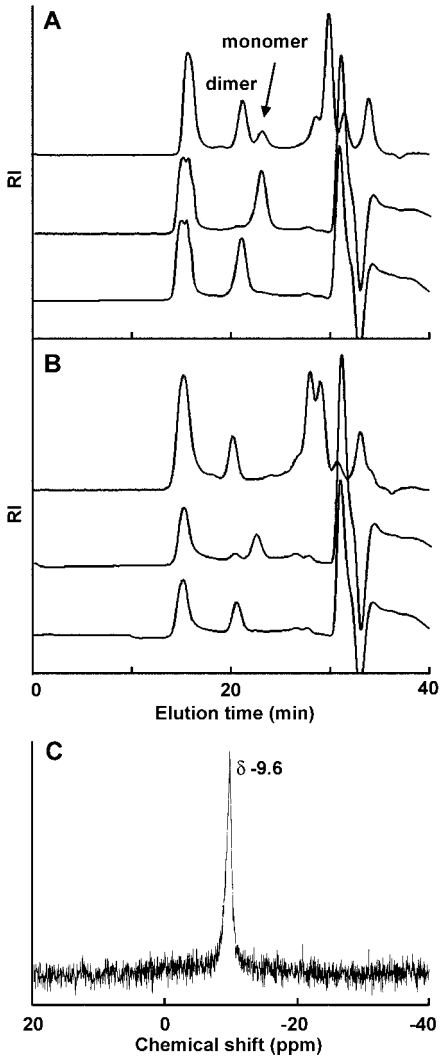



**Figure 4.** Properties of the borate cross-link in pteridophyte RG-II. A, SEC profiles of the material solubilized from *E. hyemale* walls with a homogeneous EPG. Top profile, Control, no treatment. Middle profile, Material in top profile after treatment for 45 min at room temperature with 0.1 M HCl. Bottom profile, 0.1 M HCl-treated material after reaction for 3 h at pH 3.7 with 1 mM boric acid and 1 mM $Pb(OAc)_2$. B, SEC profiles of the material solubilized from *P. nudum* walls with a homogeneous EPG. Top profile, Control, no treatment. Middle profile, Material in top profile after treatment for 45 min at room temperature with 0.1 M HCl. Bottom profile, HCl-treated material (0.1 M) after reaction for 3 h at pH 3.7 with 1 mM boric acid and 1 mM $Pb(OAc)_2$. The elution positions of the RG-II dimer and monomer are shown. C, The $^{11}$B-NMR spectrum of borate cross-linked RG-II isolated from *C. thalictroides* cell walls. The $^{11}$B signal at δ-9.6 originates from a 1:2 borate-diol diester (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996).

*m/z* 1,258 that corresponds to $[M+H]^+$ of a glycosylamine-terminated oligosaccharide composed of one Api, two Rha, one AceA, one Ara, one Gal, two 6-deoxy methyl hexoses, and one *O*-acetyl residue. The product ion spectra of this oligosaccharide (Fig. 5B) contain ions that are consistent with the presence of terminal non-reducing Rha and 3Me-Rha residues.

**Table IV.** *Glycosyl-linkage compositions of RG-II isolated form the walls of C. thalictroides, S. molesta, sugar beet, and bamboo (Phyllostachys edulis)*

RG-II was isolated by SEC from the material solubilized from *C. thalictroides* and *S. molesta* walls by treatment with EPG. The glycosyl-residue compositions were determined by GC/EI-MS analysis of the per-*O*-methylated alditol acetates.

| Linkage | *C. thalictroides* | *S. molesta* | Sugar Beet[a] | Bamboo[b] |
|---|---|---|---|---|
| | | *Mol%* | | |
| T-Rhap[c] | 7[d] | 6[d] | 5 | 5 |
| 2-Rhap | nd[e] | nd | 4 | 2 |
| 3-Rhap | 5 | 5 | 7 | 5 |
| 2,3,4-Rhap | 6 | 5 | 8 | 5 |
| T-Fucp[f] | 5 | 4 | 6 | 7 |
| 3,4-Fucp | 5 | 6 | 3 | 6 |
| T-Arap | nd | nd | 6 | nd |
| T-Araf | 4 | 5 | 5 | 6 |
| 2,3-Arap | 6 | 6 | nd | nd |
| T-Xylp[g] | 3 | 4 | 7 | nd |
| 3′-Apif | 5 | 5 | 7 | 5 |
| 2,3,3′-Apif | 5 | 4 | 3 | nd |
| T-Galp | 6 | 5 | 7 | 11 |
| 2,4-Galp | 9 | 7 | 6 | 8 |
| 2-GlcpA | 4 | 4 | 5 | 3 |
| T-GalpA | 12 | 15 | 12 | 7 |
| 4-GalpA | 10 | 8 | 2 | 6 |
| 2,4-GalpA | nd | nd | 4 | 5 |
| 3,4-GalpA | 5 | 5 | 3 | 11 |
| 2,3,4-GalpA | 3 | 5 | nd | 8 |

[a]Calculated from Ishii and Kaneko (1998). [b]Calculated from Kaneko et al. (1997). [c]T-RhaP, 2,3,4-Tri-*O*-methyl-1,5-di-*O*-acetylrhamnitol. [d]Includes terminal non-reducing 3-*O*-methyl rhamnosyl residues. [e]nd, Not detected. [f]From terminal non-reducing 2-*O*-methyl fucosyl residues. [g]From terminal non-reducing 2-*O*-methyl xylosyl residues.

Again, the *O*-acetate is most likely located on the AceA or 2Me-Fuc residues (see Fig. 6; oligosaccharides 5, 5a, and 5b in Table V). In a separate series of experiments, octasaccharide 5a was also shown by negative ion ESI-MS to be generated by selective acid hydrolysis of *C. thalictroides* RG-II.

The ESI mass spectrum of side chain B from Arabidopsis, *E. hyemale*, and *S. kraussiana* contain ions at *m/z* 1,056, 1,098, and 1,140 that correspond to $[M+H]^+$ from glycosylamine-terminated oligosaccharides composed of one Api, two Rha, one AceA, one Ara, one Gal, and one 6-deoxy methyl hexose with zero, one, or two *O*-acetyl residues (see Table IV). No oligosaccharides larger than a heptasaccharide were detected. In contrast, side chain B from wine RG-II contains a glycosylamine-terminated hepta- (1, 1a, and 1b), octa- (3 and 3a and 4, 4a, and 4b), and nonasaccharide (7, 7a, and 7b) with zero, one, or two *O*-acetyl residues (see Table IV; Fig. 6). It is likely that the hepta- and octasaccharide are generated from the nonasaccharide when wine RG-II is treated with warm 0.1 M TFA (Whitcombe et al., 1995). We conclude that the side chain B heptasac-

**Table V.** *ESI-MS protonated molecular ions of the oligosaccharides present in side chain B fractions generated by selective acid hydrolysis of RG-II from lycophytes, pteridophytes, and angiosperms*

RG-II was isolated by SEC from the material solubilized from the lycophyte and pteridophyte walls by treatment with EPG. The purified RG-II was then treated for 24 h at 40°C with 0.1 M TFA to selectively hydrolyze the glycosidic bond of the apiosyl residue of side chain B. Side chain B fractions were isolated by SEC and analyzed by ESI-MS and ESI/MS-MS in the positive ion mode.

| $[M + H]^+$ | Deduced Number of Glycosyl and *O*-Acetyl Residues[a] | | | | | | | | Fragment[b] | Plant Source[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Api | Rha | Ara | Gal | AceA | 2MeFuc | 3MeRha | *O*-Acetyl | | |
| 1,056 | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | Rw,At,Eh,Sk |
| 1,070 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 2 | Pn, Pb |
| 1,098 | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 1a | Rw,At,Eh,Sk |
| 1,112 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2a | Pn, Pb |
| 1,140 | 1 | 2 | 1 | 1 | 1 | 1 | 0 | 2 | 1b | Rw,At,Eh,Sk |
| 1,154 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2c | Pn, Pb |
| 1,188 | 1 | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 3 | Rw |
| 1,202 | 1 | 3 | 1 | 1 | 1 | 1 | 0 | 0 | 4 | Rw |
| 1,216 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 0 | 5 | Lt |
| 1,230[d] | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 0 | 6 | Pn, Pb |
| 1,230[d] | 1 | 2 | 2 | 1 | 1 | 1 | 0 | 1 | 3a | Rw |
| 1,244 | 1 | 3 | 1 | 1 | 1 | 1 | 0 | 1 | 4a | Rw |
| 1,258 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 5a | Pb, Lt,Ct[e] |
| 1,272 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 6a | Pn, Pb |
| 1,286 | 1 | 3 | 1 | 1 | 1 | 1 | 0 | 2 | 4b | Rw |
| 1,300 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 5b | Lt |
| 1,314 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 6b | Pn, Pb |
| 1,334 | 1 | 3 | 2 | 1 | 1 | 1 | 0 | 0 | 7 | Rw |
| 1,376 | 1 | 3 | 2 | 1 | 1 | 1 | 0 | 1 | 7a | Rw |
| 1,418 | 1 | 3 | 2 | 1 | 1 | 1 | 0 | 2 | 7b | Rw |

[a]The number of glycosyl and *O*-acetyl residues was determined from the $[M + H]^+$ ion. The glycosyl residue compositions of the oligosaccharides were determined by GC/EI-MS analyses of the alditol acetate derivatives. [b]The deduced glycosyl sequences of oligosaccharide fragments 1, 5, 6, and 7 are shown in Figure 6. [c]At, Arabidopsis; Ct, *C. thalictroides*; Eh, *E. hyemale*; Lt, *L. tristachyum*; Pb, *P. bifurcatum*; Pn, *P. nudum*; Rw, red wine; Sk, *S. kraussiana*. [d]3a and 6 have the same molecular mass. Only *P. nudum* and *P. bifurcatum* RG-IIs contain 3-*O*-methyl rhamnosyl residues (see Table III). [e]Fragment 5a generated from *C. thalictroides* RG-II was detected by negative ion ESI-MS.

charides from the RG-IIs of Arabidopsis, *E. hyemale*, and *S. kraussiana* RG-II have nearly identical structures and that in these plants, side chain B is no larger than a heptasaccharide. In contrast, side chains B in the RG-IIs of *C. thalictroides*, *P. bifurcatum*, *P. nudum*, and *L. tristachyum* contain one or more terminal nonreducing 3Me-Rha residues and are probably octasaccharides.

Kdo was present in the side chain B fractions from all of the pteridophyte and lycophyte RG-IIs examined. Selective acid hydrolysis of the RG-II from *C. thalictroides* generated a fraction that contained only Rha and Kdo. This result is consistent with the presence of the disaccharide α-L-Rhap-(1→5)-Kdo (side chain D) that is known to be linked to the backbone of angiosperm RG-II (O'Neill et al., 1990; Ridley et al., 2001).

Glycosyl residues that are characteristic of side chain A (GlcA and 2Me-Xyl) and side chain C (Dha; O'Neill et al., 1990) were present in the high-$M_r$ fraction that remained after treating the pteridophyte and lycophyte RG-IIs with warm 0.1 M TFA. We presume that the Dha originated from the disaccharide β-L-Ara*f*-(1→5)-Dha (side chain C) that is known to be linked to the backbone of angiosperm RG-II (O'Neill et al., 1990; Ridley et al., 2001). L-Gal accounted for between 40% and 80% of the Gal in the high-$M_r$ fractions after 0.1 M TFA treatment of the RG-II isolated from pteridophytes, lycophytes, Arabidopsis, and wine (data not shown). This monosaccharide has been identified as a component of side chain A in angiosperm RG-II (Reuhs et al., 2003). It is likely that TFA treatment does not release all of side chain B from the 1,4-linked Gal*p*A backbone of RG-II, because small amounts of the glycoses characteristic of side chain B were also present in the high-$M_r$ fraction.

Taken together, these results established that the RG-IIs synthesized by lycophytes, pteridophytes, and flowering plants have almost identical glycosyl residue and glycosl-linkage compositions. Moreover, the four specifically arranged side chains (A–D) that are linked to the backbone of angiosperm RG-II are all present in the RG-IIs of lycophytes and pteridophytes.

## DISCUSSION

### Borate Ester Cross-Linked RG-II Is a Component of Lycophyte and Pteridophyte Cell Walls

Our study is the first to provide compelling evidence that lycophyte, pteridophyte, and seed plant

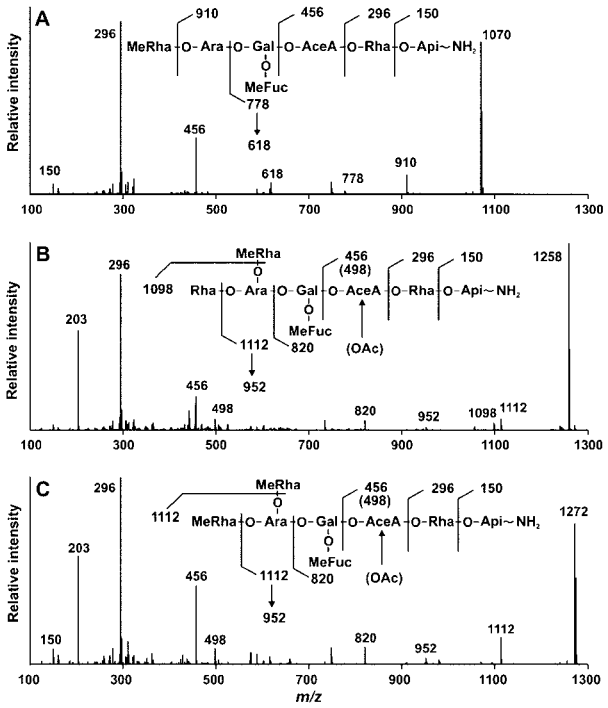


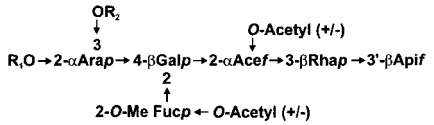

**Figure 5.** ESI-MS-MS product ion mass spectra of selected side chain B oligosaccharides generated from pteridophyte RG-II. A, The product ion spectrum of the glycosylamine-terminated heptasaccharide 2 (*m/z* 1,070) generated from *P. nudum* RG-II. B, The product ion spectrum of the glycosylamine-terminated mono-*O*-acetylated octasaccharide 5a (*m/z* 1,258) generated from *P. bifurcatum* RG-II. C, The product ion spectrum of the glycosylamine-terminated mono-*O*-acetylated octasaccharide 6a (*m/z* 1,272) generated from *P. nudum* RG-II.

cell walls contain similar amounts (2–36 mg $g^{-1}$ wall) of borate ester cross-linked RG-II (see Table II) and that in all of the plants that RG-II has been characterized, the structure of RG-II is conserved. There is so little RG-II (40–250 $\mu$g $g^{-1}$ wall) in bryophyte walls that we have been unable to isolate sufficient amounts of this polysaccharide from any bryophyte for structural characterization. Thus, the possibility cannot be discounted that the material obtained from bryophyte walls that has been identified by SEC/ICP-MS as borate cross-linked RG-II (see Fig. 3) is another boron-containing polysaccharide. However, trace amounts of 2Me-Fuc and 2Me-Xyl are present in bryophyte wall, and these modified glycoses have only been associated with RG-II in all other plants studied. Moreover, the publicly available *Physcomitrella* expressed sequence tag database (http://www.moss.leeds.ac.uk/blast/) contains an expressed sequence tag sequence (ga68F10.y1) with 75% identity to a gene that encodes CMP-Kdo synthase in maize (*Zea mays*; CAB89846; Royo et al., 2000a) and a putative CMP-kdo synthase from Arabidopsis (At1g53000). CMP-Kdo is required for the biosynthesis of Kdo-containing polysaccharides in bacteria (Royo et al., 2000b). RG-II is, as far as we are aware, the only Kdo-containing polysaccharide that is synthesized by land plants.

Some members of the lycophytes and pteridophytes synthesize RG-II that contains terminal nonreducing 3Me-Rha residues (see Fig. 6). This glycosyl residue itself is not diagnostic for lycophyte or pteridophyte RG-II because it was not detected in the RG-II synthesized by *E. hyemale* and *S. kraussiana*. Moreover, 3Me-Rha accounts for between 1% and 3% of the glycoses in bryophyte walls (T. Matsunaga, T. Ishii, S. Matsumoto, M. Higuchi, A. Darvill, P. Albersheim, and M.A. O'Neill, unpublished data), which themselves contain barely detectable amounts of borate cross-linked RG-II.

The 3Me-Rha residue is linked to *O*-2 and/or *O*-3 of the Ara*p* residue in side chain B of lycophyte and pteridophyte RG-IIs (Fig. 6, top). This Ara*p* residue is substituted with $\alpha$-L-Rha*p* residues in angiosperm RG-II (see Fig. 6, top). Replacing Rha with 3Me-Rha has no discernible affect on the ability of pteridophyte RG-II to form a dimer in vitro. The monomers generated from *P. nudum* and *E. hyemale* RG-IIs are fully converted to the dimer under our in vitro conditions (See Fig. 4, A and B), even though the structure of each side chain B differs (see Fig. 6). In contrast, RG-II dimer formation and the stability of the

A




B

| Plant | Side chain $R_1$ | Side chain $R_2$ | Oligosaccharide fragment 0 *O*-Ac | 1 *O*-Ac | 2 *O*-Ac |
|---|---|---|---|---|---|
| *A. thaliana*<br>*E. hyemale*<br>*S. Kraussiana* | αRha*p* | H | 1 | 1a | 1b |
| *C. thalictroides*<br>*L. tristachyum*<br>*P. bifurcatum* | αRha*p* | 3-*O*-Me Rha*p* | 5 | 5a | 5b |
| *P. bifurcatum*<br>*P. nudum* | 3-*O*-Me Rha*p* | 3-*O*-Me Rha*p* | 6 | 6a | 6b |
| Grape<br>(red wine) | βAra*p*→2αRha*p* | αRha*p* | 7 | 7a | 7b |

**Figure 6.** The deduced glycosyl sequences of side chain B of the RG-IIs isolated from the primary walls of lycophytes, pteridophytes, and angisoperms. A, The deduced glycosyl sequences of the largest side chain B oligosaccharide present on each of the RG-IIs. $R_1$ and $R_2$ show the locations of the different glycosyl residues in each side chain B. Some smaller fragments were detected by ESI-MS (see 2a–c, 3a, 3b, 4a–c in Table V), but they are most likely generated from the side chain B oligosaccharides when the RG-IIs are treated with warm 0.1 M TFA. B, The glycosyl residues that correspond to $R_1$ and $R_2$ in side chain B from RG-II synthesized by lycophytes, pteridophytes, and angiosperms. The number of *O*-acetyl (*O*-Ac) groups is also shown. The numbers used to designate each oligosaccharide fragment (1, 5, 6, and 7) correspond to those shown in Table V.

dimer are diminished by replacing L-fucosyl residues with L-galactosyl residues (O'Neill et al., 2001). Monomeric RG-II that lacks the L-Gal*p*-(1→2)-β-D-Glc*p*A-(1→) portion of side chain A has also been reported to form the dimer less readily than its wild-type counterpart (Iwai et al., 2002). Thus, the structural integrity of some but not all parts of the RG-II molecule is essential for formation of normal borate cross-links.

### Involvement of RG-II and Boron in the Growth of Pteridophytes, Lycophytes, and Bryophytes

Numerous studies have shown that seed plants require boron for their growth (Loomis and Durst, 1992; Brown et al., 2002) and that the boron requirement of angiosperms is correlated with the amount of pectin and RG-II in the cell wall (Hu et al., 1996; Matoh et al., 1996). In contrast, the boron requirements of lycophytes, pteridophytes, and bryophytes are poorly documented (Bowen and Gauch, 1965; Hoffman, 1966).

The sporophyte is the dominant stage in the life cycle of lycophytes, pteridophytes, and angiopserms. The walls isolated from the aerial portions (stems and leaves) of these plants contain comparable amounts of RG-II (2–36 mg $g^{-1}$ dry weight cell wall), and their RG-IIs have similar glycosyl residue and glycosyl-linkage compositions (Tables II–IV). Similar amounts of RG-II are present in the walls of *C. falcatum* gametophytes (10 mg RG-II $g^{-1}$ dry weight; Fig. 2A, profile 5) and *C. falcatum* sporophyte fronds (13 mg RG-II $g^{-1}$ dry weight; Fig. 2A, profile 6), and a RG-II with similar glycosyl residue composition is synthesized by *C. richardii* gametophytes (Table III). Pteridophytes and lycophytes may require boron for normal growth irrespective of the stage of their life cycle. In contrast, bryophyte gametophytes, which dominate the life cycle of these plants, are likely to require little, if any, boron to maintain their wall structure or for their growth because their walls contain much less RG-II than tracheophyte walls. The possibility cannot be discounted that the walls of bryophyte sporophytes contain quantitatively more RG-II than their gametophyte counterparts and that the two generations of the bryophyte life cycle differ in their boron requirements. However, there are no reports describing the boron requirements of sporophyte tissue or the glycosyl residue compositions of sporophyte walls. The lack of data on the composition of these walls is due in large part to the small size of the sporopohyte itself and the difficulties of obtaining sufficient tissue for cell wall analyses. Our ability to characterize such walls will be increased when monoclonal antibodies that recognize structurally well-defined portions of the RG-II molecule become available. A knowledge of the boron requirements of bryophyte gametophytes and sporophytes and the factors that resulted in increased RG-II synthesis in tracheophytes are likely to provide a greater understanding of the role of boron in vascular land plants.

### RG-II, Boron, and the Evolution of Land Plants

The first plants that colonized the land approximately 480 million years ago are believed to be related to extant bryophytes (Kenrick and Crane, 1997; Qiu and Palmer, 1999). These early land plants subsequently gave rise to the tracheophytes that now dominate the terrestrial environment. Many of the morphological and biochemical changes that allowed plants to adapt to life on land have been documented (Graham, 1993; Niklas, 1997). However, our understanding of the evolutionary origins of cell walls and the changes in wall structure that occurred during the evolution of land plants is limited by the lack of information on wall composition and architecture in non-flowering plants (Ligrone et al., 2002; Popper and Fry, 2003).

It is quite remarkable that the primary structure of RG-II has remained essentially unchanged in tracheophytes. The occurrence of RG-II in the walls of the Lycophytes (Lycopodiales and Sellaginales) and pteridophytes (Filicopsida, Equisetopsida, and Psilopsida) suggest that this polysaccharide must have originated in a common ancestor of all of these plants. It is unlikely that RG-II, which contains 12 different glycoses linked together by more than 20 different glycosidic linkages, would have evolved independently in each of these plant groups. Our data suggest that RG-II is also present, albeit it in small amounts, in bryophyte walls. These amounts are so small that borate cross-linking of RG-II is unlikely to have a structural role in bryophyte walls, although the possibility cannot be discounted that in these plants RG-II is required for wall structure in only a limited number of specialized cell types.

Tracheophytes are believed to have evolved approximately 400 million years ago during the Silurian period (Kenrick and Crane, 1997). The selection pressures that have maintained the structure of RG-II in diverse taxonomic groups remain to be determined. However, it is known that a seemingly small change in the structure of RG-II can dramatically reduce its ability to form a borate cross-linked dimer and that these structurally changes adversely affect plant growth and development (O'Neill et al., 2001; Iwai et al., 2002).

A major innovation in the evolution of tracheophytes from their bryophyte-like ancestors was the ability to form supporting and conducting tissues that contain cells with lignified walls (Niklas, 1997). The metabolic pathways to lignin are believed to have arisen from pre-existing primary metabolic pathways in charophytes and bryophytes (Kenrick, 2000). Reduced lignification and abnormal development of xylem and phloem cells are often associated

with boron deficiency in angiosperms, although the role of borate in these processes is not understood (Dell and Huang, 1997). Lewis (1980) proposed that boron has a primary role in lignin biosynthesis and xylem differentiation and thus was required for tracheophyte evolution. In contrast, Lovatt (1985) suggested that the evolution of xylem allowed borate to be transported to and accumulate in the growing regions of stems and leaves. The accumulation of borate in apical meristems may then have led to the evolutionary acquisition of an essential role for boron. In seed plants, one essential role of borate is to covalently cross-link primary cell wall pectin (Fleischer et al., 1999; Ishii et al., 2001; O'Neill et al., 2001). We have shown that bryophyte gametophyte walls contain much less borate cross-linked RG-II than tracheophyte walls, which suggests that the amount of RG-II synthesized by plants increased dramatically during the evolution of vascular plants from their bryophyte-like ancestors. Thus, the acquisition of a boron-dependent growth habit may be correlated with the ability of vascular plants to maintain upright growth and to form lignified secondary walls.

Specialized water-conducting cells (hydroids) are formed by some bryophytes but the hydroid walls are not lignified (Ligrone et al., 2002). The RG-II content of bryophyte walls is not correlated with the formation of conducting tissues because comparable amounts of RG-II are present in the walls of Polytrichales species (*Pogonatum japonicum*, 0.13 mg RG-II $g^{-1}$ cell wall; and *Polytrichum juniperinum*, 0.08 mg RG-II $g^{-1}$ cell wall) that form hydroids and bryophytes that do not form these cellular structures. The results of immunocytochemical studies suggest that the hydroid walls of some but not all mosses contain partially methyl-esterified homogalacturonan (Ligrone et al., 2002). However, it is not known whether these walls contain RG-II. The peristome teeth of moss sporophytes and the elaters of liverwort sporophytes also have specialized thickened walls (Bell and Coombe, 1965), but the glycosyl residue compositions of these walls have not been determined. Characterization of such walls is challenging because of the difficulty of obtaining tissue in amounts sufficient for chemical analyses and because of the lack of monoclonal antibodies that specifically recognize RG-II.

The results of preliminary experiments suggest that no borate cross-linked RG-II is present in the Driselase-soluble material from *Chara* sp. cell walls. Thus, a essential role for boron in wall structure may have appeared during the period that plants adapted to life on the land and before the evolution of lignin and vascular tissues. Interestingly, *Chara* sp. walls have been reported to contain no xyloglucans, which has led to the suggestion that wall structure changed dramatically during the period that plants colonized and adapted to life on land (Popper and Fry, 2003).

In conclusion, we have shown that borate cross-linked RG-II is present, albeit in different amounts, in the primary walls of all major groups of land plants. Our study also demonstrates that the primary structure of RG-II is conserved in tracheophytes, which adds support to the hypothesis that the structure and organization of cell wall pectic polysaccharides are important for normal plant growth and development. Additional insight into the evolutionary origin and function of RG-II in plants will be accessible when the genes responsible for the biosynthesis of angiosperm RG-II are identified, and the complete genomic sequences of *Physcomitrella* and *Chara* become available. A comprehensive description of the changes in wall composition and organization that have occurred during the evolution of land plants will require extensive knowledge of the structures of all of the polymers that are present in the walls of charophytes and in the walls of different bryophyte and tracheophyte groups.

## MATERIALS AND METHODS

### Plant Material

The plants used in this study (see Table I) were obtained from the greenhouses of the National Science Museum (Tsukuba, Japan) or the University of Georgia or were collected from fields in Japan and China. Tissue from the actively growing aerial portions of each plant were harvested, rinsed with deionized water, and stored at −80°C. Except where stated otherwise, tissue was obtained from actively growing aerial portions (stem and leaf) of the gametophyte generation of the bryophytes and the sporophyte generation of lycophytes and pteridophytes. *Ceratopteris richardii* ("C-Fern") spores, *Huperzia lucidula*, and *Lycopodium tristachyum* plants were obtained from the Carolina Biological Supply Company. *C. richardii* spores were germinated in liquid "C-Fern" medium (Carolina Biological Supply Company), and the gametophytes were grown for 12 d at 28°C under constant light. *Cyrtomium falcatum* gametophytes were grown on agar containing 0.1% (w/v) Hyponex.

### Preparation of Cell Wall Material

Plant tissue was suspended in aqueous 80% (v/v) ethanol and homogenized using a Polytron blender. The suspensions were centrifuged, and the insoluble residues were then washed with aqueous 80% (v/v) ethanol, aqueous 95% (v/v) ethanol, 99.5% (v/v) ethanol, chloroform:methanol (1:1, v/v), and acetone. The alcohol-insoluble residues were air dried and used as the source of cell walls.

### Driselase Treatment of Cell Walls

Cell walls (5–10 mg) were suspended in 15 mM ammonium acetate (1 mL), pH 5.0, containing Driselase (10 $\mu$L of a 500 mg $mL^{-1}$ solution in 75 mM ammonium acetate, pH 5.0; Kyowa Hakko Kogyo Co., Tokyo). The mixture was shaken for 20 h at 30°C and then centrifuged. The soluble fraction was filtered through a 0.45-$\mu$m membrane and then analyzed by SEC/ICP-MS (Matsunaga et al., 1997).

### Isolation of RG-II from the Cell Walls of Pteridophytes and Lycophytes

Cell walls (1–5 g) isolated from *Ceratopteris thalictroides* and *Salvinia molesta*, were treated for 4 h at 4°C with 0.1 M NaOH to saponify the methyl and acetyl esters. The suspensions were adjusted to pH 5.0 with glacial acetic acid and then treated for 16 h at 30°C with EPG from *Aspergillus niger* (2.5 units; Megazyme, Wicklow, Ireland; 1 unit releases 1 $\mu$mol of reducing

sugar $min^{-1}$ from a 1% (w/v) solution of poly-GalUA at pH 5.0 and 25°C). The suspensions were centrifuged, and the insoluble residues were washed with water. The EPG-soluble fractions were dialyzed (1 kD $M_r$ cut-off tubing) against deionized water and freeze dried.

The EPG-soluble fractions from *C. thalictroides* and *S. molesta* was treated with α-amylase (*Bacillus subtilis*, Sigma-Aldrich, St. Louis) to remove starch. RG-II-enriched material was then isolated by SEC on a Sephadex G-75 column (2.5 × 90 cm; Sigma-Aldrich), followed by anion exchange chromatography on a DEAE-Sepharose column (1.6 × 20 cm; Amersham Biosciences, Uppsala) using a 50 mM to 1 M ammonium formate, pH 5.3, gradient and then SEC on a Superdex 75 prep grade column (1.6 × 38 cm) eluted with 50 mM ammonium formate, pH 5.3, at 0.6 mL $min^{-1}$. The column eluants were assayed colorimetrically for uronic acid (Blumenkrantz and Asbo-Hansen, 1973) and for their boron content by ICP-MS as described (Matsunaga et al., 1997). The uronic acid and boron-containing fractions were combined, dialyzed (1 kD $M_r$ cut-off tubing) against deionized water, and freeze dried.

In a separate series of experiments, cell walls (1–5 g) from the aerial portions of *L. tristachyum*, *Selaginella kraussiana*, *Platycerium bifurcatum*, *Equisetum hyemale*, *Psilotum nudum*, and Arabidopsis rosette leaves were suspended in 50 mM $KPO_4$, pH 6.8, and treated for 24 h at 23°C with α-amylase (10 mg; from *B. subtilis*, Sigma-Aldrich). The suspensions were filtered through nylon mesh. Suspensions of the residues in 50 mM NaOAc, pH 5, were treated for 24 h at 23°C with *A. niger* EPG (10 units; 1 unit releases 1 μmol reducing sugar $min^{-1}$ from a 1% [w/v] solution of poly-GalUA at pH 5.0 and 25°C) and *A. niger* pectin methyl esterase (15 units; 1 unit releases 1 μmol methanol $min^{-1}$ from a 1% [w/v] solution of 90% methyl esterified pectin at pH 5.0 and 25°C). The suspensions were filtered, and the solutions were dialyzed and freeze dried. The EPG-soluble materials were fractionated by SEC using a Superdex 75 HR10/30 column eluted with 50 mM ammonium formate, pH 5. The column was calibrated using a mixture of dimeric and monomeric RG-II isolated from red wine (O'Neill et al., 1996).

## Generation of Side Chain B by Selective Acid Hydrolysis of the Apiosyl Residues of RG-II

*C. thalictroides* RG-II (1 mg) was treated for 16 h at 40°C with 0.1 M TFA (Spellman et al., 1983). The hydrolysate was separated by SEC on a Superdex 75 prep grade column. Side chain B eluted as a broad peak (approximately 88 min). This material was collected and then chromatographed on a Superdex Peptide HR 10/30 column to give two fractions (B-1, 22.4 min; and B-2, 27.5 min). Fractions B-1 and B-2 were analyzed by ESI-MS in the negative ion mode, and their glycosyl residue compositions were determined.

The EPG-soluble RG-IIs from *L. tristachyum*, *S. kraussiana*, *P. bifurcatum*, *E. hyemale*, *P. nudum*, and Arabidopsis and RG-II from red wine RG-II (5–10 mg) were treated for 16 h at 40°C with 0.1 M TFA (Spellman et al., 1983). The hydrolysates were separated by SEC on a Superdex 75 HR10/30 column (O'Neill et al., 1996). Side chain B eluted between 26 and 32 min and was collected manually. This material was analyzed by ESI-MS and ESI/MS-MS in the positive ion mode, and the glycosyl residue and glycosyl-linkage compositions were determined.

## Analytical Methods

SEC/ICP-MS was performed with a PU-1580i HPLC (Jasco, Tokyo) interfaced to a SII SPQ9000 ICP mass spectrometer (Seiko Instruments Inc., Chiba, Japan) and a YMC-Pack Diol-120 column (8 × 300 mm; YMC Co., Kyoto) eluted at 1.0 $mL^{-1}$ with 0.2 M ammonium formate, pH 6.5 (Matsunaga et al., 1997). A column (7.5 × 50 mm) containing Amberlite IRA743 was placed between the pump and sample injector to remove borate from the eluant. The SEC/ICP-MS was calibrated using known amounts of boric acid. The total boron content of the Driselase digests (20 μL) was determined by flow injection ICP-MS (Matsunaga et al., 1997). ESI-MS analysis was performed with a mass spectrometer (LCQ DUO, Thermo Quest Ltd., Tokyo) operated in the negative-ion mode and with a Q-TOF-2 (Micromass, Waters, Milford, MA) operated in the positive ion mode. Positive ion ESI/MS-MS was performed with argon as the collision gas. $^{11}$B-NMR spectra were recorded on a spectrometer (JNM-A500, JEOL, Akishima, Japan) operated at 160.3 MHz.

Uronic acid was determined colorimetrically using the *m*-hydroxy biphenyl method (Blumenkrantz and Asbo-Hansen, 1973). The neutral and acidic glycosyl residue compositions of the cell walls and RG-II were determined by GC and GC/EI-MS analysis of the alditol acetate and trimethylsilyl methyl glycoside derivatives, respectively (York et al., 1985).

The absolute configurations of the galactosyl residues in side chain A were determined by GC/EI-MS analysis of the trimethyl silyl (+)-sec-butyl glycosides (Gerwig et al., 1979) using D- and L-Gal as standards. Material containing side chain A (approximately 1 mg) was treated for 1.5 h at 120°C with 2 M TFA (1 mL). The solution was then concentrated to dryness under a flow of nitrogen gas. A solution of the residue in 10 mM ammonium formate (1 mL), pH 7, was loaded onto a Q-Sepharose column (1 × 2 cm), and the neutral monosaccharides were eluted with 10 mM ammonium formate (4 mL), pH 7. The neutral fraction was repeatedly freeze dried to remove the ammonium formate. The residue was then treated for 16 h at 80°C with (+)-sec butanol (Sigma-Aldrich) containing 1M HCl. The mixture was concentrated to dryness, and the residue was treated for 20 min at 80°C with 100 μL of Tri-Sil (Sigma-Aldrich). The trimethyl silyl (+)-sec-butyl glycosides were extracted into hexane and analyzed by GC/EI-MS using a 30 M DB-1 column.

3-*O*-Methyl rhamnose was generated by partial *O*-methylation of L-rhamnose. Glycosyl linkage compositions were determined by GC/EI-MS analysis of the methylated alditol acetate derivatives generated from per-*O*-methylated RG-II by a modified Hakomori procedure (Ishii et al., 1999) and from per-*O*-dueteriomethylated side chain B oligosaccharides that were methylated with solid NaOH and dueteriomethyl iodide (Ciucanu and Kerek, 1984).

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