The Pore Size of Non-Graminaceous Plant Cell Walls Is Rapidly Decreased by Borate Ester Cross-Linking of the Pectic Polysaccharide Rhamnogalacturonan II[1]

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The walls of suspension-cultured *Chenopodium album* L. cells grown continually for more than 1 year on B-deficient medium contained monomeric rhamnogalacturonan II (mRG-II) but not the borate ester cross-linked RG II dimer (dRG-II-B). The walls of these cells had an increased size limit for dextran permeation, which is a measure of wall pore size. Adding boric acid to growing B-deficient cells resulted in B binding to the wall, the formation of dRG-II-B from mRG-II, and a reduction in wall pore size within 10 min. The wall pore size of denatured B-grown cells was increased by treatment at pH ≤ 2.0 or by treatment with Ca^{2+}-chelating agents. The acid-mediated increase in wall pore size was prevented by boric acid alone at pH 2.0 and by boric acid together with Ca^{2+}, but not by Na^+ or Mg^{2+} ions at pH 1.5. The Ca^{2+}-chelator-mediated increase in pore size was partially reduced by boric acid. Our results suggest that B-mediated cross-linking of RG-II in the walls of living plant cells generates a pectin network with a decreased size exclusion limit for polymers. The formation, stability, and possible functions of a borate ester cross-linked pectic network in the primary walls of nongraminaceous plant cells are discussed.

The primary wall surrounding growing plant cells is a dynamic structure that determines cell shape and ultimately plant morphology (Carpita and Gibeaut, 1993; McCann and Roberts, 1994; Cosgrove, 1997). The primary wall of dicots and nongraminaceous monocots is believed to consist of a rigid, rod-like cellulose/xyloglucan load-bearing network that is embedded in and interacts with a compression-resistant pectin network (Carpita and Gibeaut, 1993). The possibility that covalent cross-links exist between wall polysaccharides is the subject of considerable debate (Carpita and Gibeaut, 1993; Needs et al., 1998), although compelling evidence for such cross-links has until recently been lacking.

Rhamnogalacturonan II (RG-II) is a complex pectic polysaccharide whose structure is conserved in the primary walls of all higher plants (O'Neill et al., 1990). RG-II is present in the wall predominantly as a dimer (dRG-II-B) that is cross-linked by a 1:2 borate:diol ester (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996; Kaneko et al., 1997). A single borate ester is believed to cross-link two of the four apiosyl residues present in dRG-II-B (Ishii and Matsunaga, 1996; O'Neill et al., 1996; Pellerin et al., 1996). These studies have led to the suggestion that a physiologically important role of B is to covalently cross-link wall pectins (O'Neill et al., 1996; Brown and Hu, 1997; Kobayashi et al., 1997; Matoh, 1997), since the B requirement and wall pectin content are correlated in many plants (Hu and Brown, 1994; Hu et al., 1996; Matoh et al., 1996). B deficiency results in walls with altered properties (Hirsch and Torrey, 1980; Hu and Brown, 1994; Findeklee and Goldbach, 1996; Dell and Huang, 1997; Matoh, 1997). Thus, the structural organization of a B-cross-linked pectin network may be a factor that determines the physical and biochemical properties of the wall (O'Neill et al., 1996; Brown and Hu, 1997).

Suspension-cultured *Chenopodium album* cells grow and divide rapidly in the absence of B (<0.1 μM), although their wall pore size is greater than that of cells grown at normal B levels (Fleischer et al., 1998). The wall pore size and cell diameter of B-deficient cells increase further during the transition to the stationary phase and, unless B is added, the cells die due to rupture of the weakened wall (Fleischer et al., 1998). In contrast, the wall pore size of *C. album* cells grown in the presence of B (100 μM) decreases at the beginning of the stationary phase and the cell diameter does not increase during the stationary phase. Moreover, the stationary phase cells remain viable for at least 3 weeks (Fleischer et al., 1998). Such results have led to the suggestion that the B-dependent decrease in wall pore size is correlated with the mechanical strength of the wall (Fleischer et al., 1998).

A cross-linked network of pectic polysaccharides determines the wall pore size of dicot cells (Read and Bacic, 1996), since the size of the molecules that can diffuse through the wall is increased by enzymatic and chemical fragmentation of wall-bound pectin (Baron-Epel et al., 1988; Ehwald et al., 1992). The cell wall cutoff size for dextran molecules is irreversibly increased by treating dehydrated walls with acetone-HCl (Koppitz et al., 1994). Such conditions are believed to alter the matrix structure of the wall by causing pectin and hemicellulose to condense onto cellulose microfibrils, and by the chemical cross-

linking of wall-bound pectin. These cross-links may prevent the normal re-swelling of wall pectins in aqueous media and thereby increase pore size (Koppitz et al., 1994). Nevertheless, there is little information on the in vivo mechanisms that affect the pectin structure controlling the permeability of cell walls to macromolecules.

We report the kinetics of B binding to the walls of growing B-deficient *C. album* cells and explore the effect of this binding on the formation of dRG-II-B and on wall pore size. We show that changes in the pore size of denatured walls are correlated with conditions (pH, concentration of boric acid, and divalent cation activity) that alter the ratio of dRG-II-B and monomeric RG-II (mRG-II).

MATERIALS AND METHODS

Chenopodium album L. cells (strain C.9.1. described by Knösche and Günther [1988]) were grown on Murashige and Skoog medium (Murashige and Skoog, 1962) containing KH_2PO_4 (0.4 g L^{-1}) and Suc (40 g L^{-1}). Cells were maintained at a high specific mean growth rate by subculturing every 2 d into fresh medium (1 volume of cells into 2.5 volumes of medium) containing boric acid (100 μM, control cells) or no added boric acid (B-deficient cells). The high-frequency subcultivation has been maintained continuously for more than 1 year (>220 subcultivations). Cultures (150 mL) were grown at 27°C in 500-mL flasks under dim light on a rotary shaker at 200 rpm (Fleischer et al., 1998). B-deficient cells were grown in quartz vessels and the B content of the B-deficient medium was shown to be <0.1 μM (Fleischer et al., 1998).

Determination of the Pore Size of *C. album* Cell Walls

Cells were denatured by sequential treatment with aqueous 80% (v/v) ethanol containing 1% (v/v) acetic acid and aqueous 80% (v/v) ethanol, and then suspended in 96% (v/v) ethanol and stored at 6°C. The cells were rehydrated and the excess water removed by gentle suction. The cells were then incubated for 30 min in a polydisperse dextran solution and the size dependence of dextran partitioning was determined by size-exclusion chromatography (SEC) as previously described (Woehlecke and Ehwald, 1995; Titel et al., 1997; Fleischer et al., 1998).

Determination of the B Content of *C. album* Cells

Suspension-cultured cells (approximately 1 g fresh weight) were filtered using a polypropylene filter (35-μm pore size) contained in a polypropylene column. The cells were washed with "B-free" deionized water (5 × 3 mL) and frozen. The frozen-thawed cells were washed with B-free water (5 × 3 mL) and, together with the polypropylene filter, were transferred to polypropylene centrifuge tubes (50 mL). B was extracted by treating the cells for 24 h with a two-phase-system consisting of 1 N sulfuric acid (5 mL) and chloroform:hexanediol (9:1, v/v, 5 mL). The B content of the organic phase was determined colorimetrically using curcumin (Mair and Day, 1972). A portion of the chloroform-hexanediol phase (1.5 mL) was added to the

curcumin reagent (0.05%, w/v, 3 mL) in a polypropylene tube and kept for 20 min at room temperature. Sulfuric acid (0.9 mL, 36 N) was then added and the mixture kept for 15 min at room temperature. B-free water (35 mL) and chloroform (4 mL) were added, and the aqueous and organic phases separated by centrifugation. The A_{600} and A_{542} of the organic phase were then determined after suitable dilution with chloroform. The B concentration (below 20 μM) was proportional to A_{542} to A_{600}. The method has a detection limit of 0.03 μM B.

Solubilization of RG-II from the Alcohol-Insoluble Residue of B-Deficient, [10]B-Treated, and [11]B-Grown *C. album* Cells

C. album cells were grown continuously in B-deficient or B-containing (100 μM boric acid) medium. A portion of the B-deficient cells were grown for 10 min in the presence of [10]B-boric acid (100 μM). The cells were filtered and washed sequentially with aqueous 80% and 96% (v/v) ethanol. Cell suspensions in aqueous 80% (v/v) ethanol were homogenized for 5 min using a polytron blender (Kinematica, Luzern, Switzerland). The suspensions were filtered through nylon mesh and the alcohol-insoluble residue (AIR) washed with chloroform:methanol (1:1, v/v) and acetone and then vacuum dried at 30°C. AIR (1 g) was suspended in 100 mM potassium phosphate, pH 6.8 (100 mL), containing 0.02% (w/v) sodium azide, and treated for 24 h at room temperature with α-amylase (10 mg of *Bacillus subtilis* Type IIA, Sigma, St. Louis).

The suspensions were filtered through nylon mesh and the residues washed with water. The insoluble residues and phosphate-buffer-soluble fractions were separately dialyzed using 3.5-kD cutoff tubing against deionized water and freeze-dried. Approximately 10% of the weight of the AIR was solubilized by the phosphate buffer/α-amylase treatment. The insoluble wall residues were then treated for 4 h at 4°C with 0.1 N NaOH to hydrolyze the methyl and acetyl esters. The suspensions were adjusted to pH 5.0 with glacial acetic acid and then treated for 16 h at room temperature with a mixture containing homogeneous preparations of *endo*-polygalacturonase I (2 units, 1 unit releases 1 μmol of reducing sugar min^{-1} from a 1% [w/v] solution of polygalacturonic acid at pH 5.0 and 25°C), *endo*-polygalacturonase II (4 units), and *exo*-polygalacturonase (1 unit) from *Aspergillus niger*. The suspensions were filtered through nylon mesh and the residues washed with water. The insoluble residues and *exo*-polygalacturonase-soluble fractions were separately dialyzed (3.5-kD cutoff tubing) against deionized water and freeze-dried. The polygalacturonase treatment solubilized approximately 6% of the weight of the AIR.

Polygalacturonase Treatment of the Phosphate Buffer/α-Amylase-Soluble Material from *C. album* AIR

Portions (15 mg) of the phosphate-buffer-/α-amylase-soluble materials in 50 mM sodium acetate, pH 5.0 (2 mL), containing 0.02% (w/v) sodium azide were treated for 24 h at room temperature with homogeneous preparations of

endo-polygalacturonases I (1 unit) and II (1 unit) and *exo*-polygalacturonase (1 unit) from *A. niger*. The solutions were dialyzed (1-kD cutoff) and freeze-dried. In a second experiment the phosphate-buffer-soluble materials (20 mg) were treated for 4 h at 4°C with 0.1 N NaOH. The solutions were adjusted to pH 5.0 with glacial acetic acid, and the resulting precipitate removed by centrifugation. The solutions were then treated for 24 h at room temperature with a mixture of *endo*-polygalacturonases I and II and *exo*-polygalacturonase (1 unit each), dialyzed (1-kD cutoff), and freeze-dried.

Determination of the Effect of pH, EDTA, CDTA, and Boric Acid on the Formation of mRG-II from dRG-II-B

Dimeric RG-II was isolated from red wine as previously described (Pellerin et al., 1996). The effect of boric acid on the generation of mRG-II from dRG-II-B at pH 2.0 was determined by treating solutions of dRG-II-B (500 μg) in 100 mM KCl/HCl, pH 2.0 (200 μL), containing no boric acid, 1 mM boric acid, or 10 mM boric acid for 24 h at room temperature. In a second series of experiments, solutions of dRG-II-B (500 μg) in 100 mM KCl/HCl, pH 2.0 (200 μL), containing boric acid (1 or 10 mM) and either $Pb(NO_3)_2$ or $CaCl_2$ (0.5 mM) were kept for 24 h at room temperature. The amounts of mRG-II and dRG-II-B present were determined by SEC.

Solutions of dRG-II-B (500 μg) in 50 mM EDTA, pH 5.5 (200 μL), or 25 mM CDTA, pH 6.5 (200 μL), containing no boric acid, 1 mM boric acid, or 10 mM boric acid were kept for 24 h at room temperature. The amounts of mRG-II and dRG-II-B present were determined by SEC.

Analytical Methods

SEC was performed with a Superdex-75 HR10/30 column (Pharmacia, Uppsala) eluted at 0.6 mL min^{-1} with 50 mM ammonium formate, pH 5.0 (O'Neill et al., 1996). SEC in combination with inductively coupled-plasma mass spectrometry (SEC-ICP-MS) was performed with a Superdex-75 HR10/30 column connected to the ICP source of a mass spectrometer (Plasma-Quad ICP, VG Elemental, Franklin, MA). The column was eluted at 0.6 mL min^{-1} with 10 mM ammonium formate, pH 5.0. The mass spectrometer was operated according to the manufacturer's instructions to selectively detect ^{10}B and ^{11}B. The B content of the AIR was determined according to the manufacturer's instructions using an ICP atomic absorption spectrometer (Thermo Jarrell-Ash, Franklin, MA). The ^{10}B to ^{11}B ratio of the AIR was determined by ICP-MS.

The neutral and acidic glycosyl residue compositions of the AIR and the neutral glycosyl residue compositions of RG-II were determined by gas-liquid chromatography-MS analysis of the trimethylsilyl methyl glycoside derivatives and alditol acetate derivatives, respectively (York et al., 1985). The amounts of uronic acid solubilized by treating

denatured cells at pH 2.0 and with EDTA were determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973).

RESULTS

B Binding to the Cell Wall Is Correlated with a Rapid Decrease in Wall Pore Size

The term "wall pore size" as used in this paper is derived from the size-exclusion function of ethanol-denatured cells. It is quantitatively expressed as a mean size limit (MSL) in nanometers. The MSL is determined chromatographically as the Stokes' radius of that fraction of a polydisperse dextran solution that reaches 50% of the equilibrium concentration in the lumen of denatured cells within 30 min (see Fig. 1). The MSL of different batches of continuously growing *C. album* cells showed slight variability (5.1–6.2 nm for B-deficient cells and 3.3–3.7 nm for B-grown [100 μM] cells). This most likely results from oscillations in the physiological state of the semi-continuous cultures. However, duplicate MSL values obtained for cells harvested at the same time and treated identically differed by no more than 0.1 nm (Fleischer et al., 1998).

The MSL of the walls of growing B-deficient cells was rapidly reduced after the addition of 100 μM boric acid, and by 60 min a value of approximately 3.9 nm was obtained (Fig. 1). In a second experiment the dependence of the decrease in MSL on boric acid concentration was determined. The decrease in MSL occurred more slowly in the presence of 10 μM boric acid, although after 1 h the reduction in pore size was comparable to that obtained with 100
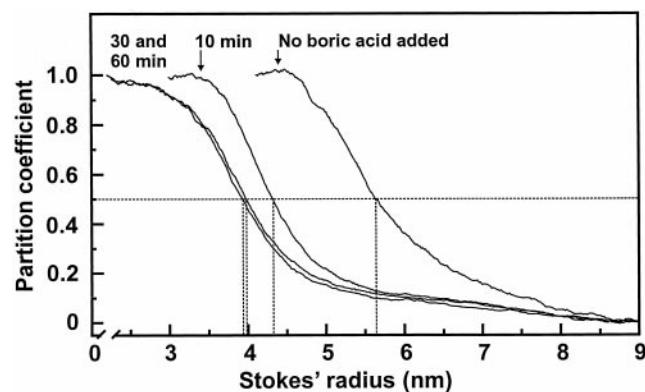

Figure 1. The B-dependent decrease in the MSL of growing B-deficient *C. album* cells. Boric acid (100 μM) was added to a growing suspension of B-deficient *C. album* cells 2 d after their subcultivation. The cells were collected at the times shown and then denatured with ethanol. The denatured and rehydrated cells (from 1 g fresh weight) were equilibrated for 30 min with a polydisperse dextran probing solution (1 mL). The molecular size distribution of the dextrans was modified by their partial diffusion into the cell lumina. The modified dextran solution was fractionated by SEC and the eluate monitored with a polarimetric detector. The dextran partition curves were generated by computer analysis of the size-exclusion chromatograms (Dautzenberg et al., 1999). The curves show the dependence of the dextran partition coefficient on Stokes' radius of the dextran. The Stokes' radius obtained from each curve at a partition coefficient of 0.5 is designated as the MSL of the walls.
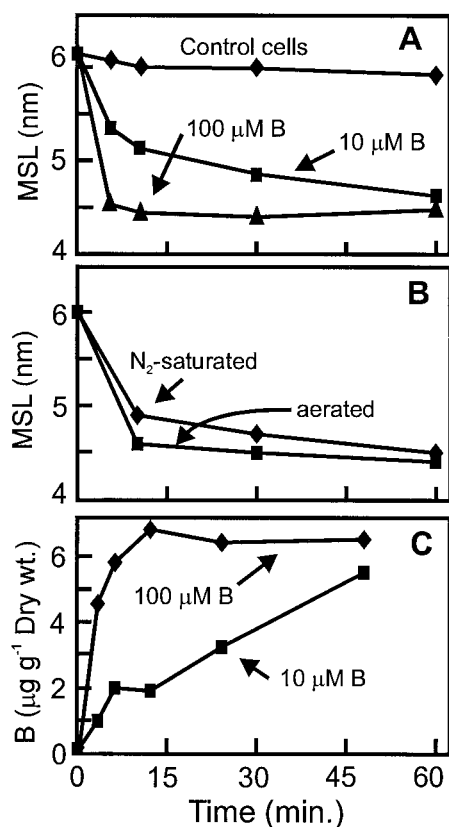
Figure 2. The decrease in wall pore size of growing B-deficient *C. album* cells and the binding of B to B-deficient cells after the addition of boric acid. A, Cells were grown continuously in the absence of added B. Boric acid (10 [■] or 100 μM [▲]) was added at time zero. No boric acid was added to control cells (♦). At the indicated times the cells were collected by centrifugation and denatured by treatment with ethanol. The MSL of the walls was determined by permeation of polydisperse dextrans into the lumen of the denatured cells. B, Cells were grown continuously in the absence of added B. One portion of the cells was saturated for 30 min with N_2 gas (♦) and a second portion saturated with air (■). Boric acid (100 μM) was added and at the specified times the MSL of the walls was determined. C, Cells were grown continuously in the absence of added B. Boric acid (10 [■] and 100 μM [♦]) was added at time zero and at the indicated times, the cells were collected by centrifugation and washed with water to remove the soluble B. The wall-bound B was released by treatment with 1 N sulfuric acid and chloroform-hexanediol and then quantified colorimetrically.

μM boric acid (Fig. 2A). The B-dependent decrease in wall MSL does not require cellular respiration, since the decrease was similar in aerated cells and in cells saturated with N_2 gas (Fig. 2B).

The addition of boric acid to growing B-deficient *C. album* cell cultures resulted in the rapid binding of B (Fig. 2C). The B present in the water-washed cells was covalently linked in the wall, since it was only solubilized by treatment with acid; low pH converts dRG-II-B to mRG-II and boric acid (Kobayashi et al., 1996; O'Neill et al., 1996). Binding of B occurred more rapidly with 100 μM boric acid than with 10 μM boric acid, but after 50 min the amount of bound B was similar (Fig. 2C). The kinetics of the increase

in wall-bound B (Fig. 2C) and the B-dependent decrease in wall MSL (Fig. 2A) were similar.

The Addition of Boric Acid to B-Deficient Cells Results in the Rapid Formation of the Borate Ester Cross-Linked RG-II Dimer

We have shown that the addition of boric acid (100 μM) to growing B-deficient *C. album* cells results in B binding to the walls and a decrease in the pore size of the wall within 10 min. We now show that in living cells the formation of dRG-II-B from mRG-II also occurs within this time period, and that the stoichiometry of wall-bound B and dRG-II-B are comparable. We also provide evidence that the conversion of half of the mRG-II to dRG-II-B is sufficient to decrease the wall MSL of B-deficient cells to nearly normal values.

The AIR prepared from B-deficient, [10]B-treated, and [11]B-grown cells, containing <0.1, 1.0, and 1.6 μM g^{-1} B, respectively, had similar glycosyl residue compositions and contained similar amounts of RG-II (Table I). [10]B accounts for approximately 80% of the B found in the [10]B-treated cells. Somewhat unexpectedly, approximately 95% of the B was solubilized by treating the AIR with phosphate buffer/α-amylase. This fraction was shown by glycosyl-residue composition analysis to contain the glycosyl residues (2-*O*-Me xylosyl, 2-*O*-Me fucosyl, apiosyl, and aceryl acid) that are diagnostic for RG-II (O'Neill et al., 1990). At least 90% of

Table I. *Glycosyl residue composition and RG-II and B contents of the AIR isolated from C. album cells grown in the presence or absence of boric acid*

The values given are for those glycosyl residue that accounted for >1 mol % of the AIR. The glycosyl residues that are diagnostic of RG-II (2-*O*-Me Fuc, 2-*O*-Me Xyl, and apiose) each accounted for <0.5 mol % of the AIR.

Glycosyl Residue	B Normal[a]	B-Deficient[b]	[10]B-Treated[c]
	mol %[b]		
Rha	9	8	9
Fuc	2	2	2
Ara	18	17	19
Xyl	7	11	11
Man	1	1	1
Gal	13	11	10
Glc	27	30	30
GalA	23	19	19
	μM g^{-1} dry wt AIR		
mRG-II[d]	0.6	4.3	2.3
dRG-II-B[d]	1.8	0	1.0
B[e]	1.6	<0.1	1.0

[a] B, AIR from cells grown in the presence of [11]B boric acid (100 μM). [b] AIR from cells grown in the absence of added boric acid. [c] AIR from B-deficient cells grown for 10 min in the presence of [10]B boric acid (100 μM). [d] The RG-II content was estimated from the 2-*O*-Me Fuc and 2-*O*-Me Xyl contents of the AIR. The relative proportions of dRG-II-B and mRG-II were determined by SEC of the phosphate-buffer-soluble extracts of the AIR (see Fig. 3), and these values were used to estimate the mRG-II and dRG-II-B contents using molecular masses of 5 and 10 kD, respectively (O'Neill et al., 1996). [e] The B content of the AIR was determined by ICP-AES.
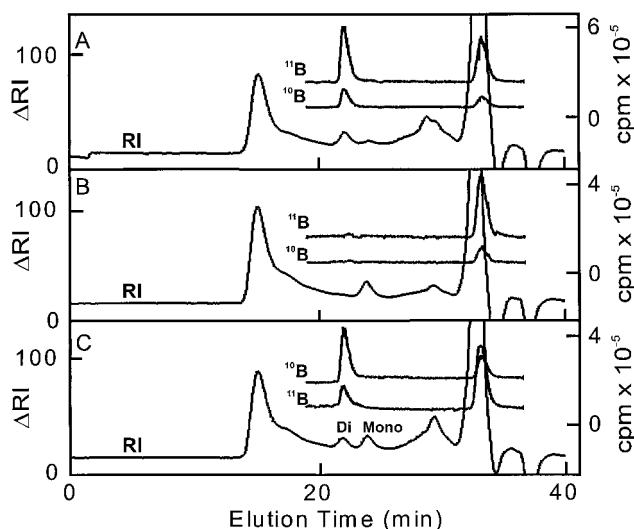
Figure 3. SEC with refractive index (RI) detection and SEC with ICP-MS detection of the saponified and polygalacturonase-treated phosphate buffer-soluble extracts of the AIR from *C. album* cells grown in the presence or absence of boric acid. A, SEC-RI profile of the extract from cells grown in the presence of boric acid (100 μM). B, SEC-RI profile of the extract from cells grown in the absence of boric acid. C, SEC-RI profile of the extract from growing B-deficient cells that had been treated for 10 min with ^{10}B boric acid (100 μM). The RG-II dimer (dRG-II-B) and monomer (mRG-II) eluted at 21.9 and 23.8 min, respectively. The insets in A through C show the ^{11}B and ^{10}B profiles obtained by SEC-ICP-MS analysis of the extracts. The ICP-MS was operated in the selected ion mode to detect only the ^{11}B and ^{10}B isotopes. The peak at approximately 36 min corresponds to boric acid that originated from a contaminant in the eluant used for chromatography. The Superdex-75 column was calibrated with red wine dRG-II-B (approximately 9.4 kD) and red wine mRG-II (approximately 4.7 kD), which have retention times of 22.8 and 24.7 min, respectively. Dextrans of 40 and 25 kD eluted at 17 and 19.6 min, respectively. The V_i of the column using Glc was 35 min.

the RG-II was solubilized by phosphate buffer treatment irrespective of whether the cells were grown in the presence or absence of boric acid.

No discernible amounts of RG-II were solubilized by polygalacturonase treatment of the phosphate-buffer-treated residue or by polygalacturonase treatment of this residue after saponification with 0.1 N NaOH, although some rhamnogalacturonan I (RG-I) and oligogalacturonides were solubilized (data not shown). The results of preliminary studies suggest that a mixture of partially methyl-esterified oligogalacturonides that are terminated at their non-reducing end with a Δ-4,5-unsaturated uronic acid residue are also solubilized by phosphate buffer irrespective of the presence of α-amylase (M.A. O'Neill, A. Fleischer, and R. Ehwald, unpublished results). Such fragments may be generated by pectin/pectate lyase or by non-enzymatic β-elimination of methyl-esterified pectin.

The RG-II solubilized by phosphate buffer/α-amylase treatment eluted in the void volume of the Superdex-75 SEC column and thus had an apparent molecular mass of >25 kD (data not shown). However, peaks corresponding to dRG-II-B (approximately 10 kD) and mRG-II (approxi-

mately 5 kD) were detected after the phosphate-buffer-soluble materials had been saponified and then treated with a mixture of *endo*- and *exo*-polygalacturonases (see Fig. 3). Glycosyl residue composition analysis confirmed that each peak contained RG-II (Table II).

Both dRG-II-B and mRG-II were present in the walls of *C. album* cells grown continuously in the presence of ^{11}B boric acid (Fig. 3A; Table II). The dimer accounts for >85% of the mass of RG-II and accounts for all of the B present in the wall (Table I). The walls of *C. album* cells grown in the absence of boric acid contained mRG-II but no dRG-II-B (Fig. 3B; Tables I and II). The soluble mRG-II was converted to dRG-II-B (Fig. 4, A and B) by treatment with boric acid and lead nitrate (O'Neill et al., 1996), showing that all of the mRG-II synthesized by B-deficient cells was capable of forming a dimer. Both dRG-II-B and mRG-II were present in the walls of B-deficient *C. album* cells treated for 10 min with ^{10}B boric acid (Fig. 3C; Table II). The peak corresponding to the soluble dimer was converted to mRG-II by treatment for 30 min with 0.1 M HCl (Fig. 4, C and D), thereby providing additional evidence that the dimer had been formed. The walls of the ^{10}B-treated cells contained B, dRG-II-B, and mRG-II in molar ratios of 1.0:1.0:2.3 (Table I), showing that approximately 50% of the mRG-II had been converted to dRG-II-B. Nevertheless, the walls of the ^{10}B-treated cells had a MSL (approximately 4.4 nm) only slightly greater than that of of cells grown continuously in the presence of B (3.3–3.7 nm).

Table II. *Glycosyl residue compositions of the mRG-II and dRG-B-II isolated from C. album cells grown in the presence or absence of boric acid*

RG-II was isolated by SEC from the phosphate buffer-soluble extracts of the AIR of *C. album* cells grown in the presence or absence of boric acid (100 μM). The neutral glycosyl residue compositions were determined by gas-liquid chromatography analysis of the alditol acetates.

Glycosyl Residue	B Normal[a]		B-Deficient[b]	^{10}B-Treated[c]	
	dRG-II-B[d]	mRG-II[d]	mRG-II[d]	dRG-II-B[d]	mRG-II[d]
	mol %				
2-MeFuc	8	7	7	8	9
Rha	15	14	13	13	11
Fuc	4	3	2	4	3
2-MeXyl	8	7	8	9	8
Ara	24	26	29	24	30
Api	9	8	11	9	8
AceA	7	5	6	6	6
Gal	25	30	24	27	25
^{11}B/^{10}B[e]	3.8	–[f]	–[f]	0.6	–[f]

[a] Cells grown continuously in the presence of ^{11}B boric acid (100 μM). [b] Cells grown continuously in the absence of added boric acid. [c] B-deficient cells that were grown for 10 min in the presence of ^{10}B boric acid (100 μM). [d] mRG-II and dRG-II-B were isolated from the phosphate-buffer-soluble fractions by SEC (see Fig. 3). [e] The relative abundance of ^{11}B and ^{10}B in dRG-II-B were determined by SEC-ICP-MS analysis of the phosphate-buffer-soluble fractions. [f] mRG-II contains no detectable amounts of B.
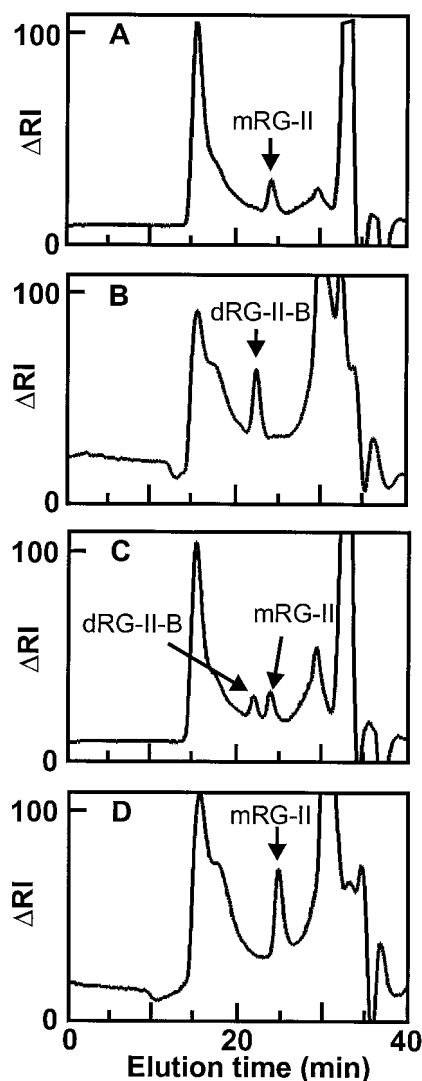
pH and Divalent Cations Modulate the B-Dependent Increase in Cell Wall Pore Size of Denatured *C. album* Cells and the Inter-Conversion of Soluble dRG-II-B and mRG-II

We have shown that the B-dependent decrease in the wall MSL of growing B-deficient *C. album* cells (Figs. 1 and 2) is correlated with the formation of dRG-II-B (Fig. 3C). We now provide evidence that the MSL of denatured walls is increased by conditions that convert dRG-II-B to mRG-II.

C. album cells grown continuously in the presence of boric acid (100 μM) were denatured with cold aqueous 80% (v/v) ethanol. These cells were then treated with buffers between pH 1.0 and 7.0 in the presence or absence of boric acid, and the MSL of the walls was determined. Only treatments at <pH 2.5 increased the MSL of the wall (Table III). The MSL of walls treated at pH 1.0 was above the limit of the assay irrespective of the presence of boric acid.

The role of divalent cations in mediating changes in the wall pore size was investigated by treating denatured cells with 50 mM EDTA, pH 5.5, in the presence or absence of boric acid (1 and 10 mM). EDTA treatment alone resulted in an increase in wall MSL over a 20-h period (Table IV). The addition of boric acid (1 and 10 mM) reduced but did not completely prevent the increase in MSL.

We have provided evidence that low pH and EDTA treatment result in an increase in the MSL of denatured walls, and that boric acid partially prevents this increase. However, such results do not demonstrate unequivocally that the low-pH- and EDTA-dependent increase in MSL result from the conversion of dRG-II-B to mRG-II. Thus, dRG-II-B isolated from red wine was treated at pH 2.0 and with Ca^{2+} chelators, and the amount of mRG-II that formed was then determined. The dimer was almost completely converted to mRG-II at pH 2.0, although in the

Figure 4. Chemical interconversion of mRG-II and dRG-II-B from B-deficient and [10]B-treated *C. album* cells. A, SEC-RI profile of the saponified and polygalacturonase-treated phosphate-buffer-soluble material from the AIR of *C. album* cells grown in the absence of added boric acid. B, SEC-RI profile of the extract shown in A after treatment for 24 h at pH 3.5 with boric acid (1 mM) and lead nitrate (1 mM). C, SEC-RI profile of the saponified and polygalacturonase-treated phosphate-buffer-soluble extract from the AIR of B-deficient *C. album* cells grown for 10 min in the presence of [10]B boric acid (100 μM). D, SEC-RI profile of the extract shown in C after treatment for 30 min at pH 1.0.

To establish that adding [10]B boric acid to B-deficient cells results in the formation of dRG-II-[10]B, the phosphate buffer/α-amylase-soluble/polygalacturonase-treated materials were analyzed by SEC in combination with ICP-MS (see Fig. 3). The dRG-II-B of cells treated with [10]B boric acid was enriched with [10]B (see Fig. 3C; Table II), whereas the dRG-II-B from cells grown in the presence of [11]B boric acid contained B in the expected natural abundance ratio (see Fig. 3A; Table II). No [10]B or [11]B was detected in the region corresponding to RG-II from *C. album* cells grown in the absence of added boric acid (see Fig. 3B; Table II).

Table III. *The effect of pH in the presence or absence of boric acid on the wall pore size of C. album cells*

C. album cells were grown in the presence of boric acid (100 μM) and then denatured with ethanol. The denatured cells (4 g) were treated for the specified times in 100 mM HCl/KCl or Na phosphate (20 mL) of different pH and in the presence or absence of boric acid. The cells were then transferred to 20 mM Na phosphate, pH 6.5, and the MSL of their walls determined.

Time/Treatment	MSL				
	pH 7.0	pH 2.5	pH 2.0	pH 1.5	pH 1.0
			nm		
1 h					
No boric acid	3.5[a]	3.5	3.7	3.9	>7.0[b]
1 mM Boric acid	3.5	3.5	3.5	3.5	>7.0
2.5 h					
No boric acid	ND[c]	ND	3.7	5.0	ND
1 mM Boric acid	ND	ND	3.7	3.8	ND
24 h					
No boric acid	3.5	3.5	4.5	>7.0	>7.0
	ND	ND	5.0	>7.0	ND
1 mM Boric acid	3.5	3.5	3.5	4.1	>7.0
	ND	ND	3.8	5.9	ND

[a] MSL of walls at time zero.　[b] MSL beyond the limit of the assay.　[c] ND, Not determined.

Table IV. *The effect of EDTA in the presence or absence of boric acid on the wall pore size of C. album cells*

C. album cells were grown in the presence of boric acid (100 μM) and then extracted with ethanol. The denatured cells (5 g) were rehydrated in water and then incubated for the indicated times in 20 mM Na phosphate, pH 5.5. (45 mL), containing 50 mM EDTA in the presence or absence of boric acid. The cells were then transferred to 20 mM Na phosphate, pH 6.5, and the MSL of their walls determined.

Time/Treatment	MSL
	nm
2.5 h	
pH 5.5	3.6
EDTA	4.3
EDTA + 1 mM B	3.9
EDTA + 10 mM B	3.8
20 h	
pH 5.5	3.8
	3.9
EDTA	6.4
	7.1
EDTA + 1 mM B	5.4
	4.7
EDTA + 10 mM B	4.2
	ND[a]

[a] ND, Not determined.

presence of boric acid alone or in combination with Pb^{2+}, the amount of mRG-II that formed was somewhat reduced (Table V). Boric acid also reduced the amount of mRG-II formed by treating wine dRG-II-B in vitro with EDTA and CDTA (Table V). Thus, the pore size of the wall and the conversion of soluble dRG-II-B to mRG-II are both affected, albeit to a different extent, by low pH and divalent cation chelators. These results, together with the stabilizing effect of boric acid on wall pore size, provide evidence that hydrolysis of the borate ester in dRG-II-B is involved in both the acid-and chelator-mediated MSL increase.

At least 90% of the wall-bound B was solubilized by treating the denatured cells for 18 h at pH 2.0 and by treatment with EDTA at pH 5.5. These treatments also solubilized uronic acid-containing material. Treating the cells at pH 5.5 solubilized some uronic acid-containing polysaccharides (3.0 mg uronic acid g^{-1} dry weight of cells, approximately 2% of total uronic acid). The amount of pectic material solubilized was increased by treatment at pH 2.0 (13.5 mg uronic acid g^{-1} dry weight of cells) and by treatment with EDTA (8.1 mg uronic acid g^{-1} dry weight of cells). Boric acid (10 mM) reduced the amount of pectin solubilized at pH 2.0 more than 2-fold (5.9 mg uronic acid g^{-1} dry weight of cells), and also reduced the amount of pectin solubilized (3.1 mg uronic acid g^{-1} dry weight of cells) by 50 mM EDTA, pH 5.5. Boric acid also reduced the amount of pectin solubilized by treating mechanically ruptured cells at low pH and with EDTA (data not shown).

The EDTA- and low-pH-dependent increase in wall pore size is a relatively slow process (Tables III and IV). In contrast, treating cell walls with EDTA or low-pH buffers

displaced most of the Ca^{2+} ions from the walls within minutes (data not shown). Nevertheless, the presence of Ca^{2+} ions reduced the increase in MSL at low pH (Table VI). Ca^{2+} ions and B acted synergistically, because together they prevented an increase in the wall MSL even at pH 1.5 (Table VI). Mg^{2+} and Na^+ ions were considerably less effective than Ca^{2+} irrespective of the presence of boric acid (Table VI). These results suggest that the formation of borate ester cross-linked RG-II combined with Ca^{2+} cross-linking may regulate both wall pore size and the solubility of wall-bound pectin.

DISCUSSION

Conversion of mRG-II to dRG-II-B in Living B-Deficient Cells

There is increasing evidence that a primary function of B is as a structural component of the cell wall (Loomis and Durst, 1992; Brown and Hu, 1997; Dell and Huang, 1997; Matoh, 1997; Matoh and Kobayashi, 1998). The results of our study support this notion and are also consistent with a previous report (Goldbach and Amberger, 1986) that B-deficient cells and cells grown in the presence of B have walls with similar glycosyl residue compositions (see Table I). Nevertheless, our results do not preclude the possibility that other wall components, including glycoproteins, are affected by B deficiency (Bonilla et al., 1997).

We have shown that *C. album* cell walls contain similar amounts of RG-II irrespective of whether they are grown in the presence or absence of B, and that the relative proportions of dRG-II-B and mRG-II are correlated with the amount of water-insoluble wall B (Tables I and II; Fig. 3). Approximately half of the mRG-II in the walls of growing B-deficient cells was converted to dRG-II-B within 10 min of the addition of boric acid (Fig. 3, B and C). Long-term (approximately 1 year) B deficiency did not result in the

Table V. *The effect of pH, divalent cations, and divalent cation chelators in the presence or absence of boric acid on the amount of mRG-II formed from dRG-II-B*

Solutions (200 μL) containing dRG-II-B (500 μg) were treated for 24 h at the specified conditions and the amounts of mRG-II that had formed then determined by SEC.

Treatment	mRG Formed
	% of total RG-II
None	2
pH 2.0	100
pH 2.0 + 1 mM boric acid	95
pH 2.0 + 10 mM boric acid	68
pH 2.0 + 1 mM boric acid + 0.5 mM Pb^{2+}	42
pH 2.0 + 10 mM boric acid + 0.5 mM Pb^{2+}	11
pH 2.0 + 1 mM boric acid + 0.5 mM Ca^{2+}	95
pH 2.0 + 10 mM boric acid + 0.5 mM Ca^{2+}	62
50 mM EDTA pH 5.5	13
50 mM EDTA pH 5.5 + 1 mM boric acid	9
50 mM EDTA pH 5.5 + 10 mM boric acid	8
25 mM CDTA pH 5.5	33
25 mM CDTA pH 5.5 + 1 mM boric acid	28
25 mM CDTA pH 5.5 + 10 mM boric acid	18

Table VI. *The effect of pH and selected cations in the presence or absence of boric acid on the pore size of C. album cell walls*

C. album cells were grown in the presence of boric acid (100 μM) and then denatured with ethanol. The denatured cells (5 g) were rehydrated in water and then incubated in 100 mM KCl/HCl, pH 1.5 and 2.0 (20 mL), containing 50 mM CaCl$_2$, 50 mM MgCl$_2$, or 75 mM NaCl. At the indicated times the cells were transferred to 20 mM Na phosphate, pH 6.5, and the MSL of their walls determined.

Time/Treatment	MSL			
	Ca^{2+}	Mg^{2+}	Na$^+$	No Cation
	nm			
2.5 h				
No boric acid				
pH 2.0	3.6	3.6	3.8	3.7
pH 1.5	4.5	5.1	5.2	5.0
1 mM Boric acid				
pH 2.0	3.6	3.7	3.7	3.7
pH 1.5	3.7	3.8	3.9	3.8
24 h				
No boric acid				
pH 2.0	3.7	4.7	4.9	5.0
pH 1.5	>7.0[a]	>7.0	>7.0	>7.0
1 mM Boric acid				
pH 2.0	3.7	3.8	3.8	3.8
pH 1.5	3.9	5.4	5.6	5.7

[a] MSL beyond the limit of the assay.

synthesis of RG-II incapable of forming dimers, since the mRG-II solubilized from B-deficient cell walls was completely converted to dRG-II-B in vitro (see Fig. 4, A and B). We conclude that a primary effect of B deficiency in *C. album* cells is to prevent the formation of borate ester cross-linked RG-II in muro.

The Relationship between Cell Wall Pore Size and the Putative dRG-II-B Cross-Linked Pectic Network

Cell wall pore size, as defined by Carpita et al. (1979), is the Stoke's-radius of a neutral hydrocolloid that is sufficient to prevent its free permeation through the cell wall. Therefore, wall pore size in this context is the molecular size cutoff of the cell wall and is most likely determined by the density and structure of the matrix polysaccharides in the cell wall layer that controls permeation. The results of previous studies have shown that the pore size of pectin-rich dicot walls is increased when the pectin is partly depolymerized by endo-polygalacturonase treatment (Baron-Epel et al., 1988) or by β-elimination (Ehwald et al., 1991, 1992).

A range of values (1.6–4.6 nm) have been published for the cell wall cutoff size (Carpita et al., 1979; Woehlecke et al., 1995; Read and Bacic, 1996; Fleischer et al., 1998), and are in part dependent on the analytical method used. Nevertheless, using the same method, differences can be demonstrated in the wall pore size of different plant tissues and plant cells in different physiological states (Carpita et al., 1979; Carpita, 1982; Woehlecke and Ehwald 1995; Titel et al., 1997). The method used to determine wall pore size in this study has also been used to show that the walls of *C.*

album cells have a higher pore size in the growth phase than in the stationary phase (Titel et al., 1997), and that the wall pore size of growing B-deficient cells is higher than that of cells growing in the presence of 100 μM B (Fleischer et al., 1998).

To our knowledge, our data are the first to show that borate ester cross-linking of RG-II from pre-existing mRG-II results in a rapid decrease in the wall pore size of living cells. The addition of boric acid to B-deficient suspension-cultured carrot and *Larix decidua* cells also results in a reduction of wall pore size, and B prevents an increase in wall pore size of denatured parenchyma cells from a range of dicot species when these tissues are incubated at low pH or in neutral EDTA solution (A. Fleischer and R. Ehwald, unpublished results).

The results of this study, along with the ubiquitous occurrence and conserved structure of RG-II and the correlation between B requirement and wall pectin content (Hu et al., 1996; Matoh et al., 1996), provide strong evidence that dRG-II-B cross-links in plant cell walls are required for the formation and stabilization of a distinct macromolecular structure of the pectic network.

The Stability and Function of dRG-II-B in Muro

The borate ester cross-link of RG-II in the cell wall has a high stability, since it formed rapidly in living cells even in the presence of low concentrations (10 μM) of boric acid (see Fig. 2A). Moreover, the wall pore size of denatured cells did not increase even in the presence of the B-complexing solvent hexanediol-chloroform, unless the pH was significantly lower than the pK$_a$ of hexuronic acid (approximately 2.9).

The increase in wall pore size at pH 2.0 involves the hydrolysis of the borate ester and was prevented by the presence of boric acid. The EDTA-mediated increase in cell wall pore size may involve cleavage of the borate ester, since it was significantly diminished by 10 mM boric acid. However, low pH and EDTA treatment also solubilize a portion of the wall pectin and this may itself increase the wall MSL. Nevertheless, the ability of B to prevent or diminish the low-pH- and EDTA-dependent increase in MSL (Tables III and IV) and to reduce the amount of pectin solubilized by such treatments suggest that dRG-II-B cross-links in large part prevent or minimize changes in the structure and solubility of the pectic network that determines pore size. It has not been possible to solubilize wall-bound B without also solubilizing a portion of the wall pectin. Thus, additional studies are required to demonstrate unequivocally that cleavage of the borate ester cross-links alone is sufficient to increase the wall pore size of denatured cells.

The concentration of boric acid required to prevent the pH- and chelator-dependent decrease in wall pore size was 10-fold lower than that required to reduce mRG-II formation from soluble dRG-II-B (compare Tables III, IV, and V). Thus, the stability of the borate ester in denatured walls and in solubilized dRG-II-B differ. Furthermore, boric acid and Ca^{2+} together prevent the increase in wall pore size even at pH 1.5 (Table VI), but they are considerably less

effective than boric acid and Pb^{2+} at preventing the low-pH-dependent hydrolysis of soluble dRG-II-B (Table V). Such results confirm that Pb^{2+} is more effective than Ca^{2+} in promoting dimer formation from soluble mRG-II (O'Neill et al., 1996), and suggest that the role of divalent cations in regulating borate ester cross-linking of wall-bound and soluble mRG-II differ.

The results of preliminary experiments suggest that >95% of the wall-bound dimer is converted to the monomer by treatment with 50 mM CDTA, pH 6.5, whereas only 30% to 40% of the soluble dRG-II-B is converted to the monomer (M.A. O'Neill, A. Fleischer, and R. Ehwald, unpublished results). Such differences may be due to the covalent linkage of wall-bound mRG-II to homogalacturonan chains (O'Neill et al., 1990). Wall-bound mRG-II molecules inserted within homogalacturonan chains may be structurally constrained in a manner that favors borate ester formation, and the cross-link, once formed, may be stabilized by the interaction of Ca^{2+} ions with both dRG-II-B and homogalacturonan. Soluble mRG-II has a backbone composed of between eight and 15 1,4-linked α-D-galacturonosyl residues (Whitcombe et al., 1995; Pellerin et al., 1996). Consequently, soluble dimer formation and stability are in large part promoted by the interaction of divalent cations with RG-II (O'Neill et al., 1996).

The EDTA-induced increase in MSL was considerably slower than the removal of most wall-bound Ca^{2+} ions. Such a result suggests that most of the pectin-bound Ca^{2+} ions do not have a strong direct effect on wall pore size. Nevertheless, Ca^{2+} ions stabilize the borate ester cross-links in denatured walls even at low activity. Kobayashi et al. (1999) have provided evidence that Ca^{2+} chelators promoted the hydrolysis of isolated dRG-II-B, and that the release of the most tightly bound wall Ca^{2+} was correlated with the hydrolysis or solubilization of wall-bound dRG-II-B. Our data confirm the chelator-mediated hydrolysis of soluble dRG-II-B in vitro and wall-bound dRG-II-B in denatured walls. These results, along with the ability of divalent cations to promote soluble dRG-II-B formation in vitro (O'Neill et al., 1996; Matoh and Kobayashi, 1998), suggest that in muro Ca^{2+} has a role at specific, but as yet unidentified, sites close to the borate ester cross-link.

Dividing plant cells typically have a lower B requirement than cells in the differentiating or stationary phases (Torsell, 1956; Slack and Whittington, 1964; Birnbaum et al., 1974; Kouchi and Kumazawa, 1976; Behrendt and Zoglauer, 1996; Dell and Huang, 1997; Fleischer et al., 1998). Our data confirm that *C. album* cells divide and grow in the absence of added B (Fleischer et al., 1998) and show that their walls contain mRG-II but no discernible amounts of dRG-II-B. Thus, borate ester cross-linking of RG-II is unlikely to be required for cell division and growth in these cells. However, the B-deficient *C. album* cells, in contrast to B-grown cells, are unable to reduce their wall pore size and thereby prevent cell enlargement and wall rupture during their transition to the stationary phase (Fleischer et al., 1998). In *C. album* cells, the size-exclusion effects of the pectic network may become essential for cell stability only when growth has ceased. The inability to form a pectic network with small pore size may influence physiologically important processes, including the incorporation of polymers into the wall, the access of wall-modifying enzymes or proteins to their substrates, and the transport of polymers from the protoplast into the wall.

In summary, we have shown that the walls of growing B-deficient *C. album* cells contain mRG-II but no dRG-II-B. The rapid conversion of mRG-II to dRG-II-B following the addition of boric acid to living cells is accompanied by a rapid decrease in wall pore size. Our data provide additional support for the hypothesis that B is a structural component of the cell wall and suggest that B is required for the formation of a covalently cross-linked pectic network. This network determines wall pore size and may participate in regulating cell wall mechanics and extensibility.

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