

Borate-Rhamnogalacturonan II Bonding Reinforced by Ca^{2+} Retains Pectic Polysaccharides in Higher-Plant Cell Walls¹

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The extent of *in vitro* formation of the borate-dimeric-rhamnogalacturonan II (RG-II) complex was stimulated by Ca^{2+} . The complex formed in the presence of Ca^{2+} was more stable than that without Ca^{2+} . A naturally occurring boron (B)-RG-II complex isolated from radish (*Raphanus sativus* L. cv Aokubi-daikon) root contained equimolar amounts of Ca^{2+} and B. Removal of the Ca^{2+} by *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid induced cleavage of the complex into monomeric RG-II. These data suggest that Ca^{2+} is a normal component of the B-RG-II complex. Washing the crude cell walls of radish roots with a 1.5% (w/v) sodium dodecyl sulfate solution, pH 6.5, released 98% of the tissue Ca^{2+} but only 13% of the B and 22% of the pectic polysaccharides. The remaining Ca^{2+} was associated with RG-II. Extraction of the sodium dodecyl sulfate-washed cell walls with 50 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, pH 6.5, removed the remaining Ca^{2+} , 78% of B, and 49% of pectic polysaccharides. These results suggest that not only Ca^{2+} but also borate and Ca^{2+} cross-linking in the RG-II region retain so-called chelator-soluble pectic polysaccharides in cell walls.

Boron (B) is an essential element for higher plant growth, although its primary function is not known (Loomis and Durst, 1992). Determining the sites of B in cells is required to identify its function. In cultured tobacco cells more than 80% of cellular B is in the cell wall (Matoh et al., 1993), whereas the membrane fraction (Kobayashi et al., 1997) and protoplasts (Matoh et al., 1992) do not contain a significant amount of B. In radish (*Raphanus sativus* L. cv Aokubi-daikon) root cell walls, B cross-links two RG-II regions of pectic polysaccharides through a borate-diol ester (Kobayashi et al., 1995, 1996). The association of B with RG-II has been confirmed in sugar beet (Ishii and Matsunaga, 1996), bamboo (Kaneko et al., 1997), sycamore and pea (O'Neill et al., 1996), and red wine (Pellerin et al., 1996). In cultured tobacco cells the B associated with RG-II accounts for about 80% of the cell wall B (Kobayashi et al., 1997) and RG-II may be the exclusive carrier of B in higher plant cell walls (Matoh et al., 1996). Germanic acid, which partly substitutes for B in the growth of the B-deprived plants (Skok, 1957), also cross-links two RG-II chains (Kobayashi et al., 1997). These results suggest that the

physiological role of B is to cross-link cell wall pectic polysaccharides in the RG-II region and thereby form a pectic network.

It is believed that in the cell wall pectic polysaccharides are cross-linked with Ca^{2+} , which binds to carboxyl groups of the polygalacturonic acid regions (Jarvis, 1984). Thus, the ability of B and Ca^{2+} to cross-link cell wall pectic polysaccharides needs to be evaluated. In this report we describe the B-RG-II complex of radish root and the role of B-RG-II and Ca^{2+} in the formation of a pectic network.

MATERIALS AND METHODS

Preparation and Analyses of the Radish B-RG-II Complex

Radish (*Raphanus sativus* L. cv Aokubi-daikon) B-RG-II was prepared as described previously (Kobayashi et al., 1996). B-RG-II (3 mg) was dissolved in 0.3 mL of 10 mM Tris-HCl, pH 8.0, and passed through a column (1 × 1 cm) containing Chelex 100 resin (Bio-Rad) equilibrated with the same buffer. The effluent was then fractionated on a PD-10 size-exclusion column (Pharmacia) equilibrated with the same buffer. The high- M_r fraction was collected and its mineral composition determined.

Reconstitution of the B-RG-II Complex

Monomeric RG-II was prepared from the radish B-RG-II complex as described previously (Kobayashi et al., 1997). One-millimolar monomeric RG-II (assuming the M_r of the monomeric RG-II as 5000) and 10 mM boric acid were incubated in 50 mM sodium acetate buffer (pH 4.0) with or without 10 mM divalent cation, and an aliquot was subjected to size-exclusion HPLC (YMC-pack Diol 120, 8 × 300 mm, YMC, Karasuma-Oike, Kyoto, Japan) equipped with a refractive index detector (Kobayashi et al., 1997).

Sequential Extraction from Radish Root Cell Walls

Radish roots purchased at a local market were homogenized in ice-cold water with a kitchen blender and the homogenate was filtered under suction. The filtered cake was washed three times by suspension in 20 volumes (v/w) of ice-cold water and then used as a crude cell wall preparation. A 5-g (wet weight) aliquot of the crude cell

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Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; RG-II, rhamnogalacturonan II.

wall preparation was put into a column (3.2 cm diameter) and washed with 750 mL of a 1.5% (w/v) SDS solution buffered with 10 mM Mes-NaOH, pH 6.5, and then with 200 mL of water at a flow rate of 4 mL min⁻¹. The SDS-washed cell wall was treated for 18 h at 25°C with 100 mL of 50 mM CDTA, pH 6.5. The residue was further extracted for 18 h at 4°C and then for 2 h at 25°C with 2 mM CDTA dissolved in 50 mM Na₂CO₃. Aliquots were taken at each step, washed with distilled water, and freeze-dried before the uronic acids were determined. The SDS-washed cell wall was digested with Driselase (Kyowa Hakko, Tokyo) and the digest was chromatographed on a 1- × 20-cm column of DEAE-Sephacel (Cl⁻ form, Pharmacia) as described by Kobayashi et al. (1997).

Assay Methods

B was quantified by the chromotropic acid method (Matoh et al., 1997). Ca²⁺ was quantified by atomic absorption spectroscopy (AA-640, Shimadzu, Kyoto, Japan). Sr, Br, and Pb were quantified by atomic absorption spectroscopy (AA-660, Shimadzu) using a graphite furnace atomizer (GFA-4A, Shimadzu). Pectic polysaccharides in cell walls or extracted residues were determined as GalUA equivalents according to the method of Ahmed and Labavitch (1977). Wet samples were lyophilized before dissolution in H₂SO₄. The total sugar content was assayed by the phenol-sulfuric acid method (Dubois et al., 1956), using Glc as a standard. 2-Keto-3-deoxysugars were determined by the modified thiobarbituric acid method (York et al., 1985).

RESULTS

O'Neill et al. (1996) reported that B-RG-II isolated from the walls of sycamore cells and pea stems contains divalent metal cations, such as Mg²⁺, Ca²⁺, Sr²⁺, Ba²⁺, and Pb²⁺. These cations and Zn²⁺ are also present in B-RG-II isolated from beet and bamboo (Matsunaga et al., 1997). B-RG-II isolated from red wine also contains Ca²⁺ (Pellerin et al., 1996). The radish B-RG-II complex contains Ca²⁺, Sr²⁺, Ba²⁺, and Pb²⁺ at molar ratios to B of 1.2, 0.1, 0.01, and 0.04, respectively (Table I). The occurrence of Ca²⁺ at an equimolar concentration to B was also confirmed in the

Table I. Mineral contents of the radish B-RG-II complex

| Element | Treatment | |
|------------------|-------------------------------------|-------------------|
| | Native ^a | CDTA ^b |
| | <i>mol mol⁻¹ complex</i> | |
| B | 1.8 (1.0) ^c | 0.78 |
| Ca ²⁺ | 2.2 (1.2) | 0.05 |
| Sr ²⁺ | 0.20 (0.1) | - ^d |
| Pb ²⁺ | 0.06 (0.04) | - |
| Ba ²⁺ | 0.03 (0.01) | - |

^a Native radish B-RG-II complex was treated with the Chelex resin before mineral analyses. ^b Radish B-RG-II complex was incubated with 50 mM CDTA in sodium acetate (pH 6.5) for 3 h and then treated with the Chelex resin before analyses. ^c Values in parentheses designate mole ratios to B. ^d -, Not determined.

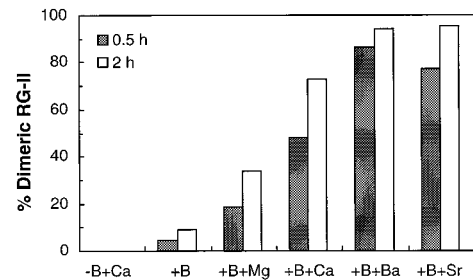


Figure 1. Effect of divalent cations on the rate of reconstitution of the B-RG-II. Monomeric RG-II (1 mM) was incubated in 50 mM sodium acetate buffer (pH 4.0) with 10 mM H₃BO₃ and 10 mM metal cations. The treatment is designated on the abscissa. The metal cations were added as chloride salts. At 0.5 and 2 h, an aliquot (10 μL) was subjected to size-exclusion HPLC with a refractive index detector. The figures on the ordinate indicate the mole percentage of RG-II converted into the dimeric form.

B-RG-II complex isolated from celery, cultured tobacco cells, and cabbage (data not presented).

The radish B-RG-II complex was reconstituted *in vitro* by mixing B and monomeric RG-II at pH 4.0; however, the extent of dimerization was low (Fig. 1; Kobayashi et al., 1997). Following the report by O'Neill et al. (1996), we confirm that Sr²⁺, Ba²⁺, and Pb²⁺ promote B-RG-II complex formation *in vitro* and that Ca²⁺ is also effective (Fig. 1). The complex reconstituted in the presence of Ca²⁺ contained 1.1 to 1.3 mol of Ca²⁺ per mol of B (data not presented). Divalent cations alone did not bring about dimer formation (Fig. 1), as O'Neill et al. (1996) demonstrated. The B-RG-II complex generated in the absence of Ca²⁺ gradually decomposed into a monomeric one when the RG-II concentration in the reconstitution solution was reduced by dilution and free B and free Ca²⁺ were removed by ion-exchange resins (Fig. 2). However, the B-RG-II complex reconstituted in the presence of Ca²⁺ was stable under the same conditions.

Removal of the Ca²⁺ from the native B-RG-II with CDTA induced release of B (Table I) and hence decay of the complex. The spontaneous decomposition of B-RG-II generated without Ca²⁺ (Fig. 2) suggests that removing Ca²⁺ from the dimer renders the dimer unstable and allows it to decompose spontaneously. These results are compatible with the notion that Ca²⁺ is a native constituent of the B-RG-II complex.

To establish the significance of Ca²⁺ in the B-RG-II formation, the distribution of cell wall Ca²⁺ was examined by sequential extraction of Ca²⁺ from radish root cell walls. When freshly prepared crude cell walls were treated with NaCl solutions of various concentrations to solubilize pectic polysaccharides and Ca²⁺, reproducible results were not obtained. The use of organic solvents such as ethanol, methanol, or a chloroform-methanol mixture was avoided because dehydration may alter the solubility of the polysaccharides. Therefore, a 1.5% (w/v) SDS solution, adjusted to pH 6.5 with 10 mM Mes-NaOH buffer, was used throughout this study to reduce autolytic activities. Pectic polysaccharides were released from crude cell walls more slowly than was Ca²⁺ (data not presented), perhaps be-

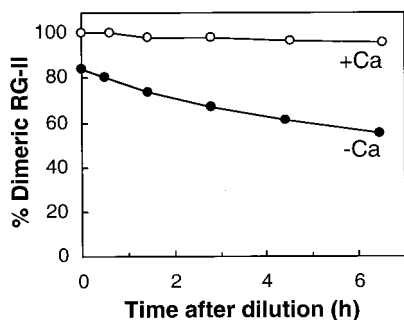


Figure 2. Effect of Ca^{2+} on the stability of the reconstituted B-RG-II complex. Boric acid (50 mM) and monomeric RG-II (5 mM) were incubated in 50 mM sodium acetate buffer (pH 4.0) with (○) or without (●) 50 mM CaCl_2 . After 20 h, the reaction mixture was diluted 10 times with the acetate buffer and free boric acid and Ca^{2+} were removed with Amberlite IRA-743 resin (SO_4^{2-} form) and Dowex 50W-X8 resin (Na^+ form), respectively. At intervals an aliquot (10 μL) was subjected to size-exclusion HPLC with a refractive index detector. The figures on the vertical axis indicate the mole percentage of RG-II converted into the dimeric form.

cause of a steric factor, as pointed out by Jarvis (1982). Therefore, SDS washing was performed on column-packed crude cell walls.

Jarvis (1982) demonstrated that during preparation of cress, cucumber, and celery cell walls with 0.5% SDS, negligible amounts of pectin are lost. A similar result was obtained for radish roots. The SDS washing of the homogenized root tissue removed 98% of Ca^{2+} from the tissues (Table II), probably because of an ion-exchange action of Na^+ , since the SDS solution contains approximately 50 mM Na^+ . Judging from the Ca^{2+} concentrations in the crude cell walls and in the fresh material, and the yield of crude cell walls from fresh material, we surmised that at least 70% of Ca^{2+} of intact radish tissues is localized in the cell walls. A molar ratio of Ca^{2+} to B of the SDS-washed cell walls was less than 1.5, which was in the same range as that of the purified B-RG-II complex (Table I). Concomitantly, the SDS treatment removed 17% of B and 22% of pectic polysaccharides (Table II). Extraction of the SDS-washed cell walls with CDTA removed all of the remaining Ca^{2+} and 78% of B, as well as 49% of pectic polysaccharides (Table II). After the CDTA treatment, the walls retained a portion of 29% of pectic polysaccharides, and one-half of

the remaining pectic polysaccharides was released with the alkaline-CDTA treatment. SDS-, CDTA-, and alkaline-CDTA solubilized pectic polysaccharides all contain the RG-II region, because 2-keto-3-deoxysugars, which are the diagnostic monosaccharides for RG-II (York et al., 1985), also decreased concomitantly (Table II). RG-II may occur mainly in the CDTA fraction, judging from the contents of 2-keto-3-deoxysugars in the three fractions. Occurrence of the RG-II region in both CDTA- and alkaline-CDTA-solubilized pectic polysaccharides is also demonstrated using an antibody toward RG-II (Matoh et al., 1998).

The SDS-washed cell walls were digested with Driselase and the digest was fractionated on DEAE-Sepharose. As presented in Figure 3, Ca^{2+} comigrated with B and 2-keto-3-deoxysugars. Ca^{2+} in the RG-II peak accounted for 80% of the Ca^{2+} present in the SDS-washed cell walls. These results suggest that almost all of the Ca^{2+} retained in the SDS-washed cell walls occurs in the RG-II region of the pectic polysaccharides. We detected another peak in the chromatogram for 2-keto-3-deoxysugars around fraction no. 27, just before the peak for the B- Ca^{2+} -RG-II complex. The eluting position coincided with that for the monomeric RG-II. The CDTA treatment eliminated not only Ca^{2+} but also B from the purified RG-II complex (Table I), which may have resulted in decomposition of the complex into monomeric RG-II. Driselase digestion of the CDTA extract of the SDS-washed cell walls yielded only monomeric RG-II, which was free of B (Fig. 4). This is compatible with the result that CDTA released B from cell walls (Table II). The pectic polysaccharides that are not released with SDS but are released with CDTA are probably retained in cell walls through Ca^{2+} -B cross-linking at the RG-II regions.

DISCUSSION

In a previous report (Kobayashi et al., 1997), we suggested that in vitro formation of B-RG-II from B and monomeric RG-II may not reflect the mode of the B-RG-II bonding in vivo, and we pointed out that another factor(s) may be involved. We now suggest that Ca^{2+} is a constituent of the naturally occurring B-RG-II complex and promotes the rate of dimer formation in vitro. O'Neill et al. (1996) reported that low concentrations (0.5 mM) of Ca^{2+} did not stimulate dimer formation effectively, that Mg^{2+}

Table II. Sequential extraction of Ca^{2+} , B, and pectic polysaccharides from radish root cell walls

| Sample | Ca^{2+} | B | Pectic Polysaccharides ^a | 2-Keto-3-Deoxysugars ^b |
|---|-----------------------|------------|-------------------------------------|-----------------------------------|
| Crude cell walls | 100 (13) ^c | 100 (0.25) | 100 (25) | 100 (1.0) |
| SDS-treated cell walls | 2 | 83 | 78 ± 0.4 | 90 ± 2.8 |
| SDS- and CDTA-treated cell walls | 0 | 5 | 29 ± 2.1 | 34 ± 0.7 |
| SDS-, CDTA-, and alkaline-CDTA-treated cell walls | — ^d | — | 14 ± 0.5 | 29 ± 2.8 |

^a Determined by the *m*-hydroxydiphenyl method using GalUA as a standard. ^b Determined by modified thiobarbituric acid method using 3-deoxy-D-*manno*-2-octulosonic acid as a standard. ^c Values in the parentheses are $\mu\text{mol Ca}^{2+}$, $\mu\text{mol B}$, $\mu\text{mol 2-keto-3-deoxysugars}$, and mg uronic acid g^{-1} crude cell walls. ^d —, Not determined.

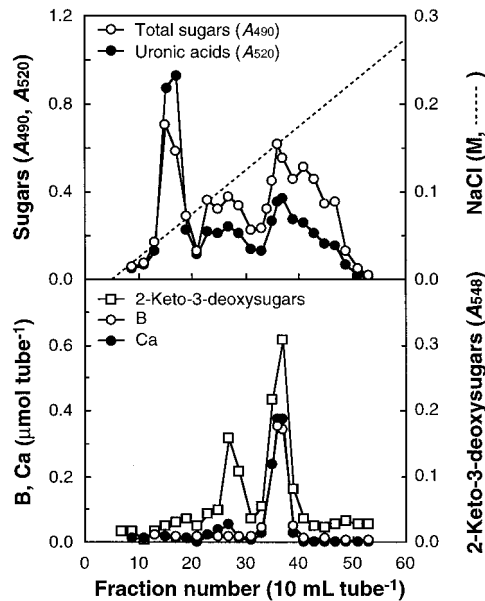


Figure 3. Chromatogram of a Driselase digest of the SDS-washed cell walls on DEAE-Sepharose. About 10 g of the SDS-washed cell walls was hydrolyzed with Driselase and the digest was chromatographed on a DEAE-Sepharose column (1.6 × 17 cm), that had been equilibrated with 20 mM Tris-HCl (pH 8.0). The pectic fragments were eluted with a 1-L gradient of 0 to 0.5 M NaCl in 20 mM Tris-HCl (pH 8.0).

and Cu²⁺ were inhibitory, and that monovalent cations had no discernible effect. We have shown, however, that higher concentrations (10 mM) of Ca²⁺ do stimulate dimer formation and that Mg²⁺ and Cu²⁺ are also effective, although the effects were small. We observed that high concentrations (about 100 mM) of monovalent cations such as Na⁺, K⁺, and NH₄⁺ also promoted, to a certain extent, reconstitution under our conditions (data not shown). The apparent discrepancies regarding the effects of Ca²⁺ and other cations may be due to different experimental conditions. In our experimental conditions, monomeric RG-II and boric acid were mixed at a final concentration of 1 and 10 mM, respectively, and divalent cations were added at 10 mM. These concentrations are higher than those used by O'Neill et al. (1996) (0.5 mM monomeric RG-II, 1.2 mM boric acid, and 0.5 mM divalent cation). Thus, the reactivity and effectiveness of coexisting cations may vary significantly according to the concentrations used.

The effect of monovalent cations on reconstitution may be due to the masking of the negative charge owing to GalUA residues of the RG-II backbone (see discussion in Kobayashi et al. [1997]). Although some part of the effect of divalent metal ions may be due to the cationic nature of the elements, the effects of divalent cations such as Sr²⁺, Ba²⁺, Pb²⁺, and Ca²⁺ are superior to the monovalent ones. The ratio of the dimer and the monomer is under equilibrium in the reconstituting solution; therefore, the divalent metal cations may stabilize the formed dimeric B-RG-II complex and hence prevent the breakdown of the dimer. O'Neill et al. (1996) suggested that divalent metal cations might form metal coordination complexes with B-RG-II, because the

cations were not removed by treatment with Chelex resin and they were not bound to monomeric RG-II. Smaller cations such as Ca²⁺ may not interact strongly enough with the ligands on the two chains to stabilize the complex. However, higher concentrations of the smaller cations, such as Ca²⁺, may compensate for their lower affinity. The Chelex and the Dowex 50 resins do not remove the Ca²⁺ in the naturally occurring and reconstituted B-RG-II complex. Therefore, the Ca²⁺ may not only bind to the carboxyl groups ionically but may also form coordinate bonds, as has been suggested for Sr²⁺. Van Duin et al. (1987) reported that Ca²⁺ forms coordination complexes with borate diesters of polyhydroxy carboxylates. In their model the Ca²⁺ is thought to interact directly with the borate anion. However, O'Neill et al. (1996) suggested that the metal cations in B-RG-II do not interact with the borate anion, because the cations do not affect the 11B-NMR spectrum of the complex. Some carboxyl or hydroxyl groups along the RG-II sugar moiety may provide sites that are favorable to coordinate the cations.

The conditions that promote dimer formation in the cell wall are not known. However, we suggest that Ca²⁺ stabilizes the B-RG-II complex *in vivo*, based on the following considerations. The concentration of water-soluble Ca²⁺ in root tip cell walls is approximately 2.5 mM (Bjorkman and Cleland, 1991), whereas the content of Sr²⁺ in angiosperm is three orders lower than that of Ca²⁺ (Bowen, 1966), even though the effect of Ca²⁺ on the stabilization is not as high as Sr²⁺; Ca²⁺ is more abundant in soil than Sr²⁺; the nutritional requirement of Sr²⁺, Ba²⁺, and Pb²⁺ has not been reported unequivocally for higher plants (Bowen, 1966), except for the substitution effect of Sr²⁺ under Ca²⁺-deficient conditions (Da Silva, 1962); and, finally, Sr²⁺, Pb²⁺, and Ba²⁺ account for only 0.15 mol per mol of B in the native radish B-RG-II (Table I) and less than 0.21 mol per mol of B-RG-II (O'Neill et al., 1996), in contrast to the higher content of Ca²⁺ (1.2 mol Ca²⁺ to 1.0 mol B) in native B-RG-II.

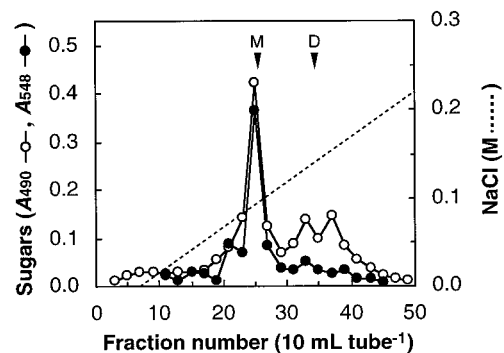


Figure 4. Chromatogram of a Driselase digest of the CDTA extract of the SDS-washed residues on DEAE-Sepharose. About 15 g of the SDS-washed cell walls was extracted with 100 mL of 50 mM CDTA, pH 6.5, at 25°C for 18 h. The extract was dialyzed against distilled water and subjected to hydrolysis with Driselase. The column (1.6 × 17 cm) was equilibrated with 20 mM Tris-HCl (pH 8.0) and eluted with a 1-L gradient of 0 to 0.5 M NaCl in 20 mM Tris-HCl (pH 8.0). The arrowheads indicate the elution position of the radish monomeric (M) and dimeric (D) RG-II on the same column.

In cell walls, pectic polysaccharides are believed to be cross-linked by Ca^{2+} bridges, phenolic coupling, galacturonate ester bonds (Fry, 1986), and borate-diester bonds (Kobayashi et al., 1996). Jarvis (1982) demonstrated that CDTA solubilizes Ca^{2+} -bound pectic polysaccharides. However, the data presented in this report suggest that the RG-II regions provide the sites for linkages through borate-diester and Ca^{2+} bonding and the linkages help to retain so-called chelator-soluble pectic polysaccharides in cell walls. During this series of experiments we noticed that SDS washing increased the packed volume of cell wall preparations, that the apparent volume of cell walls increased 2-fold after washing with SDS, and that the change was reversed by incubation of the swollen cell walls with 50 mM CaCl_2 (data not presented). This suggests that the SDS-extractable Ca^{2+} , which accounts for the 98% of the cell wall Ca^{2+} that is probably associated with the polygalacturonic acid regions, may control the properties of the pectic polysaccharides in cell walls.

The RG-II regions provide cross-linking sites for pectic polysaccharides through borate-diester bonding, and the residue responsible for the bonding is believed to be the apiosyl (O'Neill et al., 1996). Ca^{2+} also cross-links pectic chains through ionic and coordinate bonding in the polygalacturonic acid region. Although formation of the Ca^{2+} cross-linking is controlled by the esterification/de-esterification of the carboxyl groups on the uronic residues (Fry, 1986), the Ca^{2+} bridge may be more arbitrary than the B bridge; the latter may be more site specific. This may give a clue for elucidating the physiological significance of B as an essential element in plants.

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