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Immunocytochemistry of Rhamnogalacturonan II in Cell Walls of Higher Plants

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A polyclonal antibody against a borate-RG-II complex is raised in rabbits. The antibody recognized RG-II exclusively in cell wall polysaccharides. Immunocytochemical studies demonstrated that the epitope is ubiquitous in cell walls of all the cells in radish and rice roots, cultured tobacco cells, red clover root nodules, and lily growing pollen tubes. The label was denser in proximal to plasma membrane, and not detected in middle lamella, suggesting that borate may cross-link newly secreted pectic polysaccharides at the membrane-cell wall interface.

Key words: Antibody — Borate-rhamnogalacturonan II complex — Cell wall — *Oryza* — Pollen tube — *Rhaphanus*.

Although boron (B) is an essential element for higher plants (Warington 1923), its primary function is not known yet (Loomis and Durst 1992). We have proposed that determination of the B sites in a cell is a prerequisite if we are to identify the functions of B. In accordance with this proposal, our laboratory isolated a particular B-polysaccharide complex from radish root cell walls (Matoh et al. 1993) and revealed subsequently that the sugar component of the complex was rhamnogalacturonan II (RG-II) (Kobayashi et al. 1995) and that the complex was comprised of two identical chains of monomeric RG-II with two mol of borate (Kobayashi et al. 1996). Rhamnogalacturonan II was first isolated from the cell wall of cultured sycamore cells as a fragment of pectic polysaccharide which is resistant to endopolygalacturonase hydrolysis (Darvill et al. 1978). Our results suggest that pectic polysaccharide chains are covalently cross-linked together at the RG-II region through borate-diester bonding. Recently, Ishii and Matsunaga (1996), O'Neill et al. (1996), Pellerin et al. (1996) and Kaneko et al. (1997) confirmed our finding in sugar beet pulps, sycamore and pea cell walls, red wine, and bamboo shoots, respectively. The B-RG-II complex occurs in at least 24 species of mono- and dicotyledonous higher plants, and in these tissues contents of B and RG-II are closely related suggesting that RG-II exclusively provides the binding site for B in higher plant cell walls (Matoh et al. 1996). In order to identify the function of B in cell walls, information on the localization of the complex is useful. Therefore, an antibody toward the B-RG-II complex was raised and immunocytochemical analysis was performed.

Materials and Methods

Plant material—Seeds of radish (*Rhaphanus sativus* L. cv. Aokubidaikon, obtained from Takii Seed Co., Kyoto, 600-91 Japan) and rice (*Oryza sativa* L. cv. IR36) were sown on a sheet of gauze which covered plastic beads (diameter 2 mm) in a plastic tray (15 × 25 cm, 3 cm depth). Tap water was supplied so as to moisten the gauze. The tray was kept in dark at 25°C for three days, and then transferred to a growth chamber (25°C, 12 h light, light intensity 350 μE m^{-2} s^{-1}). Cultured tobacco BY-2 cells (*Nicotiana tabacum* L. cv. BY-2) were subcultured in 75 ml of a modified Linsmaier and Skoog medium with 3% sucrose and 0.2 mg $liter^{-1}$ 2,4-dichlorophenoxyacetic acid, pH 5.8, in a 300-ml conical flask maintained in the dark at 26°C shaken at 130 rpm (Nagata et al. 1981). Root nodules were taken from red clover (*Trifolium pratense* L.), which were raised in the university field of Kyoto University. Lily (*Lilium longiflorum* cv. Yamayuri) flowers were purchased at a local market. Opened anthers were collected every morning and the pollen grains were washed with petroleum-ether and air-dried. A batch of 350 mg of the grains was scattered in 100 ml of the culture medium of Dickinson (1968) except that B was supplied as boric acid at 100 mg $liter^{-1}$. After 12 h, germinating pollen grains were collected on a nylon mesh (60 μm).

Preparation of antibodies—Antigen, the B-RG-II complex, was purified from radish root cell walls following the method described previously (Kobayashi et al. 1996). In addition to the reported procedure, rechromatography with DEAE-Sepharose and Superdex 75 was supplemented to obtain a purer preparation. Antibody was raised in rabbits following the method of Moore et al. (1986). Briefly, 0.5 mg of the B-RG-II complex and 0.5 mg of methylated BSA (Sigma) were mixed and emulsified in 1 ml of the Freund's adjuvant and injected to a rabbit. The same dose in the incomplete Freund's adjuvant was injected 4 weeks later. After 8 weeks, the titer was determined by a blot assay described below. The antibody was partially purified by precipitation with ammonium sulfate and Protein-A (Pharmacia) column chromatography. The antisera was kept in 10 mM Na phosphate buffer (pH 7.2) containing 0.8% (w/v) NaCl (henceforth referred to as PBS) with 0.1% (w/v) NaN_3.

Antibody against methylated BSA was removed by incubating the sera with an equal volume of methylated BSA (0.5 mg ml^{-1}) for 4 h and centrifuged (5,000 × *g*, 15 min). When necessary, antibody toward the B-RG-II complex was removed by

Abbreviations: RG-II, rhamnogalacturonan II; vol, volume.

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incubating the sera with an equal volume of the B-RG-II complex (1 mg ml^{-1}) overnight.

Dot blotting—A titer of the antibody was determined using a dot blot assay. Nylon membrane (Amersham Hybond N^+) was set in a blotting apparatus (San Platec, Osaka, 530 Japan) and 50 μl of samples in 10 mM Tris-HCl buffer (pH 8.0) was applied to each well. After blotting, non-specific protein binding was blocked by soaking the membrane in Block Ace (Dai Nihon Seiyaku, Osaka, 565 Japan) for 30 min. The membrane was incubated for 1 h in the sera diluted appropriately in a mixture of 9 vol of 10 mM Na phosphate buffer (pH 6.0) containing 0.01% (v/v) Tween 20 and 1 vol of Block Ace. The membrane was washed by soaking in a medium consisted of 9 vol of PBS containing 0.1% Tween 20 and 1 vol of Block Ace, three changes each 5 min. Antibody binding was detected by goat anti-rabbit IgG sera conjugated with horseradish peroxidase (Biomakor, 2,000-fold diluted similarly as for the rabbit antisera described above) and the diaminobenzidine assay (Liners et al. 1989).

Specificity of the antibody—Monomeric RG-II was prepared by incubating the B-RG-II complex (1 mg) in 0.1 M HCl (5 ml) for 10 min, then neutralized with NaOH. The mixture was passed thorough a prepacked Sephadex G-25 column (PD-10, Pharmacia) equilibrated with water and polysaccharides recovered in the void volume was lyophilized.

Radish root cell walls (1 g) were hydrolyzed with 0.1% (w/v) Pectinase SS (Kyowa Chemical Products Co. Ltd.) in 100 ml of 20 mM Na acetate buffer (pH 4.0) for 48 h, and the pH of the digest was adjusted to 8.0 with 2 M Tris. Insoluble material was removed by centrifugation (10,000 × *g*, 20 min) and the supernatant was applied to a column of DEAE-Sepharose (1.6 × 36 cm, Cl^- from). The column had been equilibrated with 20 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient of NaCl (0 to 0.5 M) made in the column buffer (2 liter) at a flow rate of 1.0 ml min^{-1}. An aliquot of 20 ml of the eluate was collected per tube, and the contents of sugars and 2-keto-3-deoxysugars were determined. The sugar peaks were assayed for their activity toward the antisera using the dot blot assay. The positive fractions (about 200 ml) were combined and conc. HCl was supplemented to achieve 0.1 M HCl at a final concentration to hydrolyze the B-dimeric RG-II complex. After 15 min at a room temperature, the solution was neutralized with NaOH, and then dialyzed against water overnight. The monomeric RG-II produced was chromatographed again on the same column under the same condition.

Pectic polysaccharides were extracted from radish root cell walls following the method of Jarvis (1982). A batch of 2 g of the cell walls was incubated in 150 ml of Na acetate buffer containing 50 mM cyclohexanediamine tetraaceteic acid (CDTA) (pH 6.5) for 18 h. The mixture was centrifuged (16,000 × *g* for 20 min) and the supernatant was designated as CDTA-pectin. The precipitate was further extracted with 2 mM CDTA in 50 mM Na_2CO_3 for another 20 h at 4°C, and the supernatant (16,000 × *g*, 20 min) was obtained (alkaline CDTA-pectin). The CDTA- and alkaline CDTA-pectin were precipitated by adding 4 vol of ethanol, and collected by centrifugation (10,000 × *g*, 5 min) and dissolved in 20 mM Na phosphate buffer (pH 7.0) supplemented with 100 mM NaCl. The suspension was dialyzed against 100 mM NaCl overnight then against water two nights, and a portion of the dialysate was applied to a column of Superdex 75 (1.6 × 70 cm) equilibrated with 0.3 M Na acetate buffer (pH 5.2) to remove CDTA completely (Mort et al. 1991). Pectic polysaccharides at a concentration of 1 mg ml^{-1} were deesterified by incubating in 10 mM NaOH for 3 h at 4°C, and then dialyzed extensively against water.

Oligomers of galacuturonic acid were prepared by hydrolysis of commercially available citrus pectin through endopolygalacturonase (Megazyme, Australia) and purified with column chromatography on Bio-gel P2 (1.6 × 63 cm).

Analyses—Boron was determined using chromotropic acid as described previously (Matoh et al. 1997). Total sugars and uronic acids were quantitated by the phenol-sulfuric acid method (Dubois et al. 1956) with glucose as the standard and the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) with galacturonic acid as the standard, respectively. 2-Keto-3-deoxysugars were quantitated by the method of York et al. (1985) with 3-deoxy-D-manno-2-octulosonic acid as the standard. Glycosyl-residue composition was analyzed as described previously (Kobayashi et al. 1996).

Microscopy—The radish and rice seedlings were gently picked up from the gauze and the root was cut into small pieces. Roots of red clover were taken up from soil and the root nodules were washed extensively with distilled water. Cultured tobacco cells were collected on a filter paper under suction. Root segments, nodules and cultured cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 100 mM Na phosphate buffer (pH 7.4) overnight at 4°C, then washed with the buffer (four times, 15 min each). Germinating pollen tubes were fixed similarly except that the fixative was made in the germinating medium. The tissues were passed through an ethanol series and embedded in LR White resin (London Resin Co. Ltd., London) and polymerized in gelatin capsules overnight at 60°C.

In some experiments, tissues were fixed in 3% glutaraldehyde in 100 mM Na phosphate buffer (pH 7.4) overnight at 4°C, then washed with the phosphate buffer (four times, 15 min each). The tissues were postfixed in 1% OsO_4 in the phosphate buffer for 2 h on ice, washed four times with the buffer, and then processed as described above.

For light microscopy, one μm thick sections were cut with a microtome (ULTRACUT, Reichert-Jung, Vienna, Austria) and mounted on glass slides. The section was held on the glass slide by brief girdling from the back side with flame of a cigarette lighter. Following procedure was carried out at room temperature keeping the sections on glass slide in an air-tight small container having a moistened filter paper. A droplet of PBS containing 0.1% (w/v) gelatin and 0.5% (w/v) BSA (henceforth referred to PBSG) was placed on glass slide and left 1 h for blocking non-specific binding. If necessary, a droplet of 10 mM NaOH was put onto the section, kept for 15 min and washed out with water, before the blocking. Then, a droplet containing antisera diluted with PBSG was placed on the section and left overnight at 4°C. The antisera was washed out by flushing with PBSG, and then the section was labelled with goat anti-rabbit IgG conjugated to 15 nm colloidal gold (Amersham), diluted 25-fold with PBSG, for 30 min. For post-fixation, a droplet of 2% glutaraldehyde in PBS was placed on the section. The signal was enhanced with silver precipitation (IntenSE M kit, Amersham) following the manufacture's instruction.

Ultrathin sections of 60–90 nm thick were prepared using the ultramicrotome and the sections were put on a nickel grid coated with Bioden Meshcement (Ohken-Shoji, Tokyo, 104 Japan) at a final concentration of 0.5% in toluene. The section on the grid was hydrated by floating on water for 10 min, and then on-grid alkaline treatment was carried out with 10 mM NaOH for 15 min. After rinsing the grid in distilled water, the grid was then floated on a droplet of PBSG for 30 min. The section was labelled with the antisera diluted appropriately with PBSG overnight at 4°C, followed by treatment with the gold-conjugated secondary antibody, diluted 25-fold with PBSG, for 30 min. The section was post-fixed for 10 min with 2% glutaraldehyde in PBS, then with

2% uranyl acetate at 45°C for 30 min and lead citrate at room temperature for 3 min. Preimmune sera was used for a control. A light microscope, Olympus BH-2, and an electron microscope, JEM-2000EX (JEOL), were used throughout this study.

Results and Discussion

Fig. 1 shows dot blot analyses of diluted antisera with the B-dimeric RG-II complex and the monomeric RG-II. The sera recognized both B-dimeric RG-II complex and monomeric RG-II, suggesting that the antisera recognizes the sugar component of the complex. The signal was enhanced according to the increase in the antigen concentration. Antisera previously immunoprecipitated with the B-RG-II complex did not react with the antigen. The antisera also recognized the purified B-RG-II complex from cabbage leaves and cultured tobacco BY-2 cells (data not presented).

Fig. 2A shows a result of DEAE-Sepharose column chromatography for the hydrolysate of radish root cell walls with a pectinase enzyme. Fractions indicated by the arrows were all negative toward the antisera, except for the B-RG-II complex which eluted around the fraction No. 70. The positive fractions, No. 66 to 75, were combined and the B-RG-II complex was hydrolyzed to remove B, and the resulted monomeric RG-II was applied to the same column again (Fig. 2B). The antibody reacted only with the fractions around No. 60, where the monomeric RG-II eluted as judged by the occurrence of 2-keto-3-deoxysugars. Fractions around No. 70 contained polysaccharides, however, these were negative to the antibody. Thus, the epitope of the antibody was on the RG-II fragment, not on the fragments co-migrated with the B-RG-II complex.

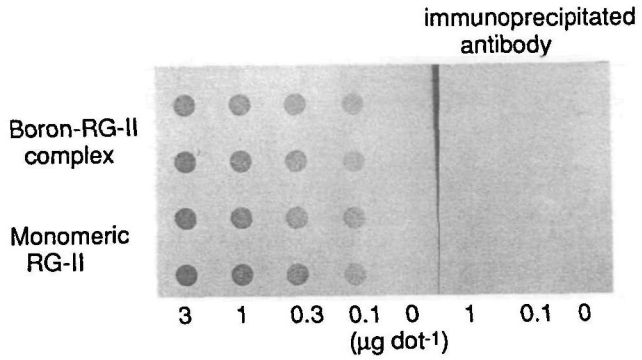

Fig. 1 Immunoblot assay toward the borate-dimeric RG-II complex (top two raw, duplicate) and the monomeric RG-II (bottom two row, duplicate). Each dot contained polysaccharide at an amount determined gravimetrically specified in the bottom row. The right three dots of each row received antisera previously immunoprecipitated with the antigen.

The CDTA-pectin reacted weakly (Fig. 3a), while alkaline CDTA-pectin reacted strongly toward the antisera (Fig. 3c). The CDTA- and alkaline CDTA-pectin contained 2-keto-3-deoxysugars at 0.36 and 0.48% (w/w), respectively. Therefore, both pectic polysaccharides may contain the RG-II region (Stevenson et al. 1988) at nearly the same concentration, even though the latter signal was stronger (Fig. 3a versus c). The signal toward the CDTA-pectin was intensified when the pectin was deesterified with alkaline (Fig. 3b). Therefore, the weak reaction of the antibody toward the CDTA-pectin may be due to the methylesterifi-

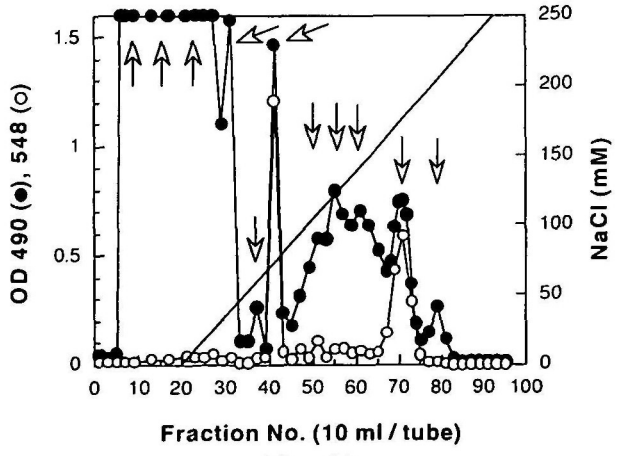

Fig. 2A

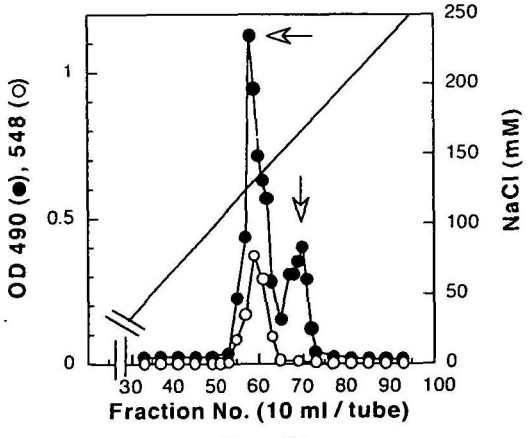

Fig. 2B

Fig. 2 Chromatogram for the hydrolysate of radish root cell walls with pectinase enzymes on a DEAE-Sepharose column. A batch of 1 g of radish root cell walls was hydrolyzed with 0.1% (w/v) Pectinase SS for 48 h, and the digest was applied to a DEAE-Sepharose column (1.6×36 cm) and the polysaccharide fragments were eluted with a linear gradient of NaCl (0–0.5 M) (A). Fractions containing B-dimeric RG-II (No. 66 to 75) were combined and hydrolyzed with 0.1 M HCl. The resulting monomeric RG-II was subjected to chromatography under the same condition for the cell wall hydrolysate (B). Fractions indicated by the arrows were assayed for their immunoreactivity toward the antisera for RG-II. Total sugars were determined with the phenol-sulfuric acid method at OD 490 nm, and 2-keto-3-deoxysugars with the method of York et al. (1985) at OD 548 nm.

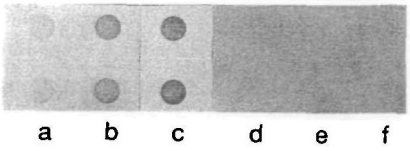

Fig. 3 Immunoblot assay toward pectic polysaccharides extracted from radish root cell walls. Results were presented in duplicate. Pectic polysaccharides were extracted with a sodium acetate buffer (pH 6.5) containing 50 mM CDTA (CDTA-pectin) (a). Following the CDTA extraction, cell walls were further extracted with 2 mM CDTA in 50 mM Na_2CO_3 for another 20 h at 4°C (alkaline CDTA-pectin) (c). The CDTA-pectin was treated with 10 mM NaOH for 3 h at 4°C (b). The alkaline-treated CDTA-pectin, and the alkaline CDTA-pectin were reacted with antisera which had been immunoprecipitated with purified B-RG-II complex (d and e, respectively). Dots (f) received no antigen.

cation of the uronic residues of the RG-II region. Inclusion of Na_2CO_3 in the extraction medium stimulated deesterification, accordingly, the signal might have been intensified. A similar result that alkaline treatment of pectic polysaccharides enhances reactivity toward the antibody was noticed by Moore et al. (1986) and Williams et al. (1996). Even though the signals were enhanced with alkaline treatment, the signal disappeared when the antisera had been immunoprecipitated with the B-RG-II complex (Fig. 3d, e), indicating that the antisera only recognizes the RG-II region and that other epitope than RG-II is not present in both pectic fractions.

When pectic polysaccharides were extracted first with CDTA, then hydrolyzed with pectinase enzymes, all the RG-II was recovered in the monomeric form. This is because of the removal of not only Ca ions but also B by the CDTA treatment (Kobayashi et al. results under submitted). This monomeric RG-II was not recognized by the antibody. Component sugar analyses of the RG-II prepared from cell walls and that from CDTA-extracted pectin revealed a significant and reproducible difference in the sugar composition in the uronic residues (Table 1). The main chain of the RG-II backbone is comprised of galacturonic residues (Whitcombe et al. 1995). Therefore, the difference in the number of the galacturonic residues along the main chain may be the determinant for the epitope activity. However, the antibody did not react with mono-, di-, and trimers of galacturonic acid. Therefore, the antibody may recognize the stereometric difference around the terminal galacturonic residue on the RG-II main chain. Performance of pectinase enzymes may be influenced by a steric factor of the substrate pectic polysaccharides mediated by Ca ions and B, which cross-link pectic polysaccharide chains.

In conclusion, these results demonstrate that the antibody recognizes RG-II exclusively. Furthermore, a detectable amount of the monomeric RG-II does not occur in the cell-wall hydrolysate (Fig. 2A). Together with our previous results that almost all the cell-wall RG-II occurs as a B-RG-II complex (Matoh et al. 1996), the location of the RG-II epitope may indicate the localization of B in the form of B-dimeric RG-II complex in cell walls.

Fig. 4A to E show localization of the RG-II epitope under a light microscope in seedling root tips (0–5 mm) of radish (A, B) and rice (C), cultured tobacco cells (D), and root nodules of red clover (E). The antibody bound to cell walls of all cells in all tissues. The RG-II epitope detected

Table 1 Glycosyl composition of RG-II

Residue	RG-II (cell wall)	RG-II (CDTA-pectin)
	(mol %)	
Rhamnose	9.0	10.3
Fucose	2.7	2.8
2-*O*-Methylfucose	3.6	3.6
Arabinose	9.6	9.8
Xylose	—	0.9
2-*O*-Methylxylose	3.5	3.8
Apiose	13.2	12.2
Galactose	6.9	6.9
Mannose	0.2	0.2
Aceric acid	1.5	1.6
2-Keto-3-deoxysugar	6.0	7.0
Uronic acids	43.8	40.9

Cell-wall RG-II (the left column) was prepared by hydrolysis of radish root cell walls with Pectinase SS, and CDTA-pectin RG-II (the right column) by hydrolysis with Pectinase SS of pectin which had been extracted with CDTA from the cell walls. Determination was made for three independent samples and the representative result is presented here.

was based on the immunoreaction. When preimmune sera was used instead, cell walls, i.e. the shape of cells, were not visible at all in light microscopy (data not presented) and no label was found in electron microscopy (Fig. 5D).

In mature cells of the cortex tissues of radish root tips, immunogold particles labeled relatively stronger the inner

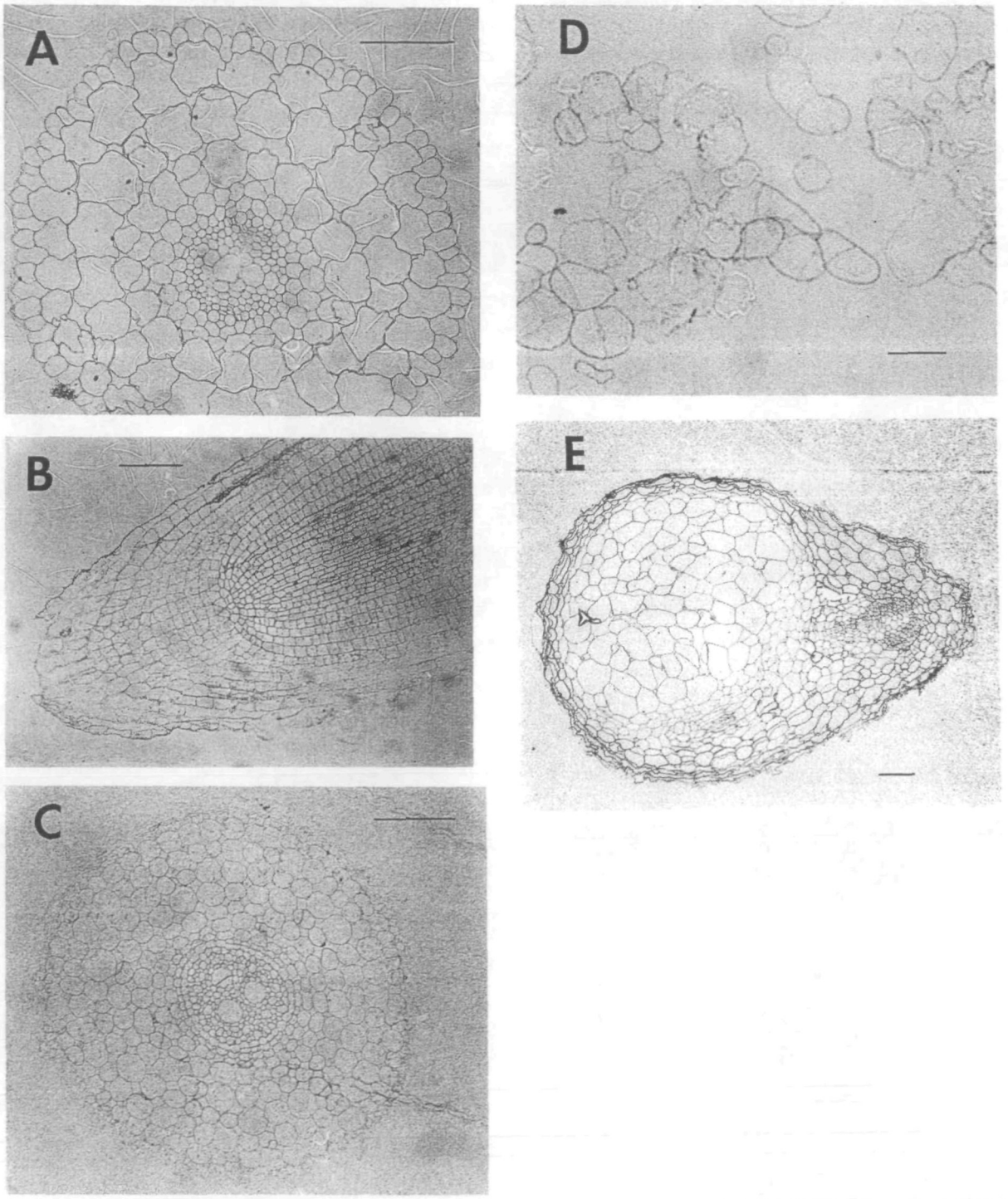

Fig. 4 Immunogold labeling with the RG-II antisera of transverse section of radish root tip (A) and longitudinal section (B), and transverse section of rice root tip (C), and cultured tobacco cells (D), and root nodules of red clover (E). The gold particles were visualized by enhancement with silver. Measurement bars are 20 μm.

half of the cell walls of radish (Fig. 5A). When cell wall was thin in immature cells close to the cambium tissues, the gold particle was found very close to the plasma membrane (Fig. 5B). In cultured tobacco cells (Fig. 7), the label was denser in proximal to plasma membrane. As B does not occur in the cytoplasm at a significant amount (Matoh 1997), these results suggest that the B-RG-II complex is formed immediately after secretion of RG-II into cell walls. Williams et al. (1996) also demonstrated that the RG-II epitope is located close to the plasma membrane. In mature cells of the roots of *A. thaliana*, the RG-I antibody labeled the quarter of the cell wall closest to the plasma membrane (Freshour et al. 1996). Knox et al. (1990) noted that their monoclonal antibody which recognizes the deesterified

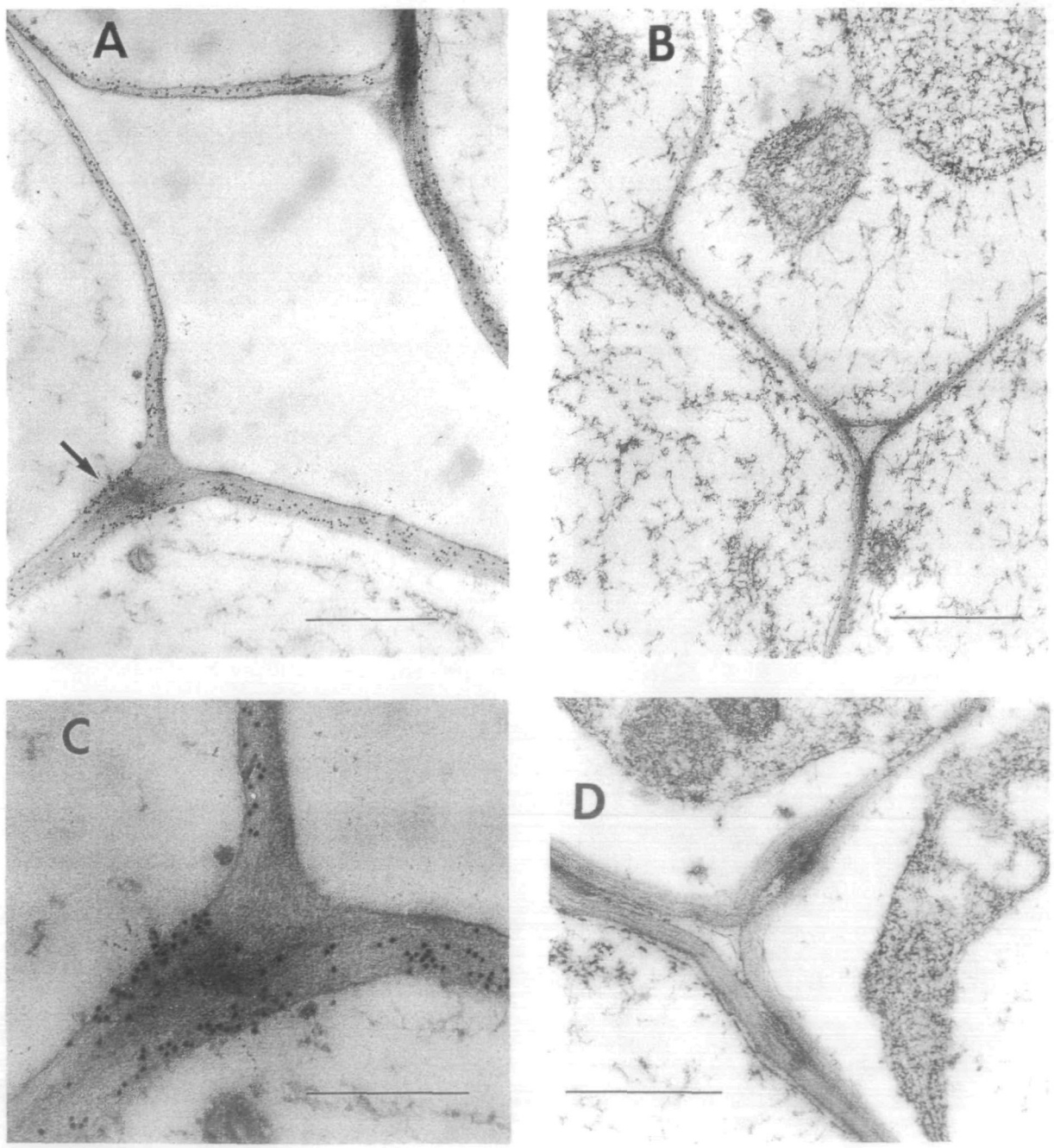

Fig. 5 Immunogold labeling with the RG-II antisera of radish root tips. A, Mature cells in the cortex tissues. B, Immature cells close to cambium cells in the stele tissues. C, A part designated by the arrow in A is enlarged. D, Preimmune sera was used. Measurement bars in A, B and D are 1 μm, and that in C is 0.5 μm.

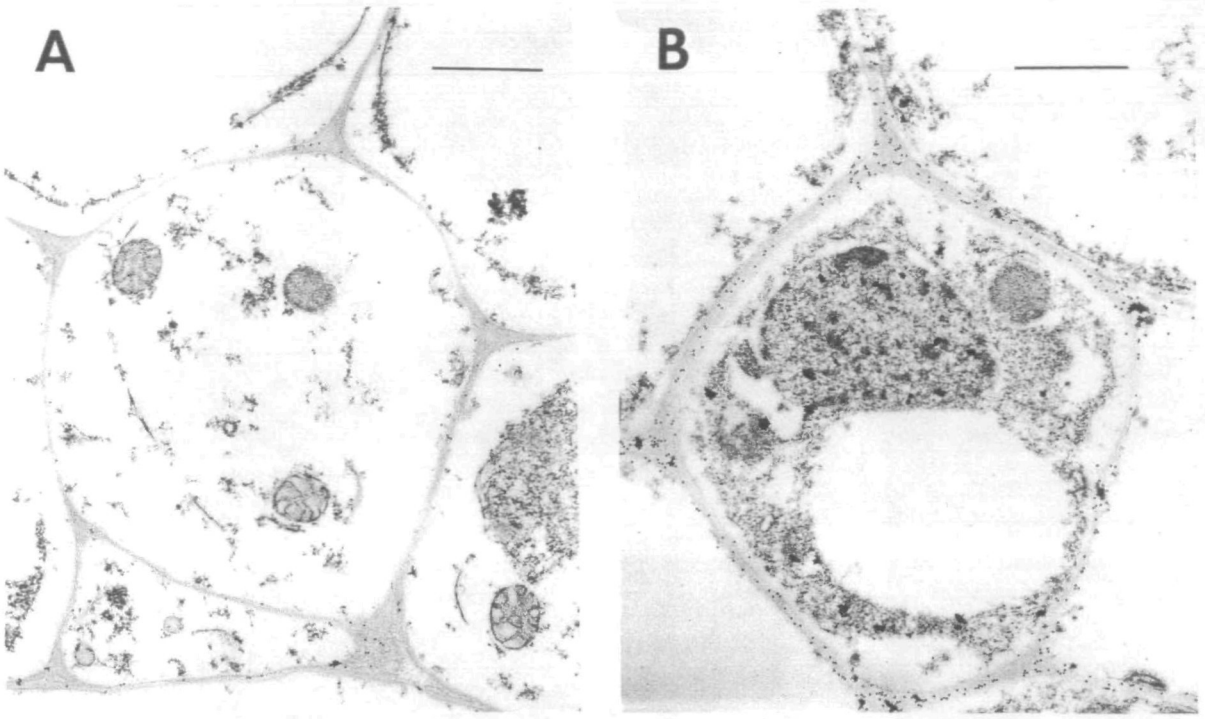

Fig. 6 Immunogold labeling with the RG-II antisera of rice root tips. The section without on-grid alkaline treatment (A) and with the treatment (B). Measurement bars are 1 μm.

epitope of pectin was located on the inner surface of the primary cell walls adjacent to the plasma membrane in the root apex of carrot. Such pectic polysaccharides are considered to be cross-linked with Ca ions through the carboxyl group of uronic residues. Similar distribution of RG-II, RG-I, and deesterified epitope of pectic polysaccharides in the primary cell walls suggests that cross-linking with Ca ions and B is important for the network formation of newly produced pectic polysaccharides. Boron has been claimed to work for the maintenance of membrane integrity (Loomis and Durst 1992). Localization of the RG-II epitope proximal to the plasma membrane suggests that the complex may influence the membrane activities.

The RG-II epitope was not detected in the middle lamella nor in the intercellular spaces (Fig. 5A, B, C), even though the epitope of RG-I and of deesterified pectin are localized in these regions (Moore et al. 1986, Moore and Staehelin 1988, Knox et al. 1990, Liners and Von Cutsem 1992). The absence of the RG-II epitope in the middle lamella and in the intercellular spaces suggests that pectic polysaccharides in the primary cell walls and those in the middle lamella may be different in their constituents, and that RG-II and B may not work to cement cells together.

In radish root segments, alkaline treatment did not enhance the labeling significantly (data not presented). However, on-grid alkaline treatment enhanced the labeling significantly in rice roots (Fig. 6A versus B). Cultured tobacco cells (Fig. 4D, 7A) and growing pollen tubes of lily (Fig. 7B) also needed alkaline treatment to obtain intensive labeling. As described above, the epitope for the antibody may be the uronic residues along the main chain of the RG-II, therefore, it is likely that deesterification of the uronic residues with the alkaline treatment enhances the reactivity of the antibody toward the epitope. Williams et al. (1996) also noticed that their monoclonal antibody recognizes the antigen RG-II of *A. thaliana* plants poorly without on-grid alkaline treatment. The extent of enhancement of the reactivity of the epitope to the antibody with on-grid alkaline treatment differed among plant species. This suggests that the degree of esterification of the carboxyl group of polygalacturonic acid residues around the RG-II region may differ from species to species in situ.

In the present study, the epitope was seldom found in cytoplasmic region. At present, we are not sure whether this is because of the absence of the epitope in cytoplasm or the poor fixation of the cytoplasmic constituents. Surveying the optimum condition for tissue fixation using OsO_4 is now under progress.

It is well recognized that matrix polymers of the gramineous monocotyledonous plants are characterized by very low levels of pectic polysaccharides (McNeil et al. 1984, Jarvis et al. 1988). Boron is essential for these plants

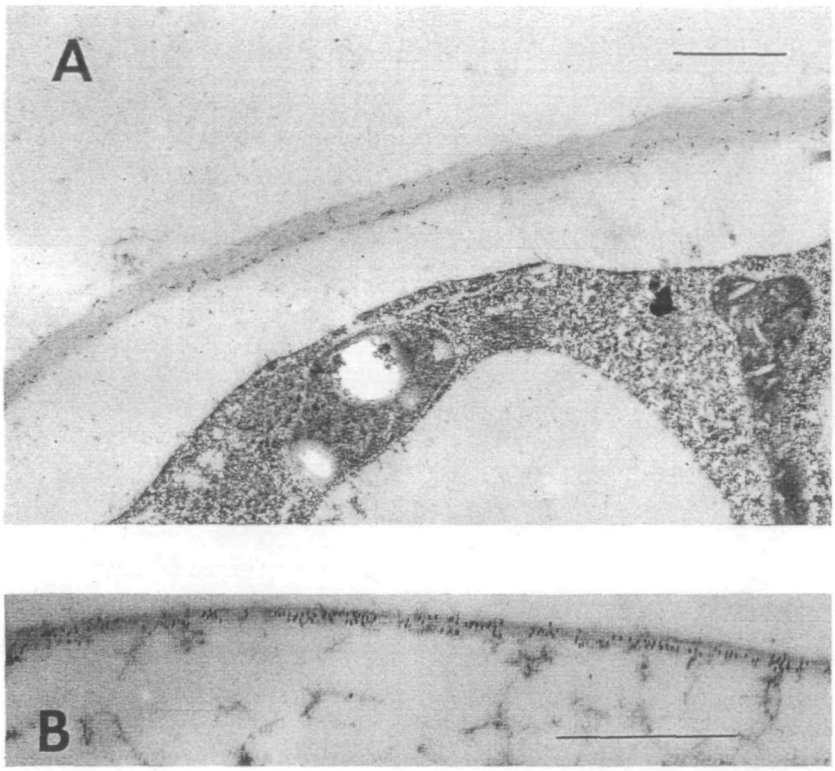

Fig. 7 Immunogold labeling with the RG-II antisera of the cell walls of cultured tobacco cells (A) and the cell walls of elongating pollen tubes of lily (B). Measurement bar is 1 μm.

as well as dicotyledonous plants, while the content is low (Mengel and Kirkby 1982). Results presented in figures 4C and 6 demonstrate the omnipresence of the epitope in the cell walls of rice root cells. In the root nodules of red clover plants, all the cell walls are labeled with the antibody, and the epidermal cell walls were labeled always more intensely compared to the cell walls of the inner ones (Fig. 4E). This may explain the significant requirement of B by nitrogen-fixing leguminous nodules (Bolaños et al. 1994). The most dramatic manifestation of B requirement is seen in pollen tube growth. The requirement of B for pollen tube elongation was first demonstrated by Schumacker (1933) and thereafter the requirement for pollens of a wide range of higher plants has been confirmed by several authors. As presented in Fig. 7B, the cell walls of elongating pollen tubes were densely labeled with the antibody. Omnipresence of the B-RG-II complex in cell walls may substantiate the essential requirement of B by higher plants.

Boron deficiency brings about structural abnormalities, especially in cell walls. Spurr (1957) demonstrated that B apparently affects the rate and process of carbohydrate condensation into wall material. In our research, slightly denser label (arrows in Fig. 5A, C) was found in the primary wall at cell corner regions, which reminds us that some of the collenchyma cells in B deficient celery plants fail to develop typical "corner" thickening (Spurr 1957). Hirsh and Torrey (1980) also pointed out that cell wall thickening was observed as early as 6 h after the removal of B. They confirmed that cell wall thickening with B deficiency was associated with increased vesicle formation at the cell wall-plasma membrane interface. They speculated that these vesicles are most similar to paramural bodies which are thought to be associated with cell wall synthesis. These vesicular aggregations were especially apparent at the corner edges of three-way junctions, where a slightly denser label was detected (arrows in Fig. 5A, C). Thus, involvement of B in the cell wall intactness is likely.

Skok (1958) discussed the possibility that if B is required for some other more basic physiological function than that of cell wall synthesis, it is reasonable to expect B requirement to be more widespread among living organisms. Loomis and Durst (1992) concluded that the rapid response of pollen tube elongation to withdrawal of B from the culture medium indicates that neither nucleic acid metabolism nor protein synthesis is involved in the function of B, rather, it is most likely that B's role is in the assembly of cell walls. Hu and Brown (1994) provided evidence that B is involved in the structural role in cell wall by binding to pectic polysaccharides. For the sake of maintaining the overall shape of a plant, B may participate in such controll-

ed and vectorial expansion by mediating the cross-linkage between matrix polysaccharides. Boric acid works to form a network of pectic polysaccharides in cell walls, however, direct evidence for the function of B is still lacking, since the significance of the integrity of cell walls to cell viability is little understood. Future studies will be focused on the determination of the role of cell walls on the cell activity analyzing the function of B in the assembly of cell walls.

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