Immunocytochemical analysis of subcellular localization of rhamnogalacturonan II, a pectic polysaccharide in plants

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Contents

Abbreviations

B	boron
B-RG-II	boron-rhamnogalacturonan II complex
BSA	bovine serum albumin
CCH	*Concholepas concholepas* hemocyanin
DAPI	4’,6-diamidino-2-phenylindole
DHA	3-deoxy-D-*lyxo*-2-heptulosaric acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	enzyme-linked immunosorbent assay
Fab	antigen binding fragment
GalA	α-D-galacturonic acid
GLU	glutaraldehyde
HG	homogalacturonan
IgG	immunoglobulin G
KDO	3-deoxy-D-*manno*-2-octulosonic acid
KLH	keyhole limpet hemocyanin
mBSA	methylated-bovine serum albumin
MES	2-(*N*-morpholino)ethanesulfonic acid
OVA	ovalbumin
PBS	phosphate-buffered saline
PBST	PBS containing 0.05% Tween 20
PGA	polygalacturonic acid
PLL	poly-L-lysine
RG-I	rhamnogalacturonan I
RG-II	rhamnogalacturonan II
TBS	tris-buffered saline
TEM	transmission electron microscope
Tobacco BY-2	*Nicotiana tobacum* L. cv. Bright Yellow 2
Tris	tris (hydroxymethyl) aminomethane
TVN	tubulo-vesicular membrane network

Introduction

Rhamnogalacturonan II (RG-II) is a pectic polysaccharide fragment that is released from plant cell walls through the action of *endo*-polygalacturonase (O'Neill et al., 2004). Although RG-II is a relatively small fragment with a size of approximately 5,000 Da, it is structurally very complex, consisting of a (1,4)-linked α-D-oligogalacturonic acid backbone with at least four side chains containing multiple rare sugars including aceric acid (3-*C*-carboxy-5-deoxy-L-xylose) (Spellman et al., 1983), D-apiose (Darvill et al., 1978), 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA) (Stevenson et al., 1988), 3-deoxy-D-*manno*-2-octulosonic acid (KDO) (York et al., 1985), 2-*O*-methyl-L-fucose (Darvill et al., 1978), or 2-*O*-methyl-D-xylose (Darvill et al., 1978). RG-II was first isolated from suspension-cultured cells of sycamore (*Acer pseudoplatanus*) (Darvill et al., 1978), and following the report RG-II has been found in all the plant species examined so far (Doco et al., 1993, 1997; Ishii and Matsunaga, 1996; Kaneko et al., 1997; Kobayashi et al., 1996, 1997; Matoh et al., 1996; Patrice et al., 1996; Spellman et al., 1983; Thomas et al., 1987, 1989; York et al., 1985). In spite of the complexity mentioned above, the overall structure of RG-II is highly conserved among species, except for the small species variation in the non-reducing end of side chain B (O'Neill et al., 2004), and some heterogeneity in side chain A within one species (Pabst et al., 2013). The ubiquitous occurrence and strong conservation of RG-II may suggest the importance of RG-II in plant cells (Pérez et al., 2003).

Kobayashi et al. (1996) found that RG-II enzymatically solubilized from the cell wall is a dimer that is covalently cross-linked by a borate diester bond (B-RG-II). In the cell wall, RG-II occurs as a substructure of macromolecular pectin and is connected with other substructures including homogalacturonan (HG) and rhamnogalacturonan I (RG-I) (O'Neill et al., 2004), indicating that RG-II serves as the site for cross-linking in pectin *in muro*. The network of pectin thus formed is considered to play multiple roles required for normal plant growth and development (Dumont et al., 2016). Pectins are retained in the cell walls unless the B-RG-II linkage is broken (Kobayashi et al., 1999). Insufficient cross-linking of RG-II with borate diester results in structurally abnormal and mechanically weak cell walls (Ishii et al. 2001; Matoh et al., 2000; Ryden et al., 2003). When RG-II cannot be cross-linked due to the lack of B in the external medium, immediate responses including extracellular Ca^{2+} influx, overproduction of reactive oxygen species, stress gene expression, or cell death are induced within 1 h (Koshiba et al., 2010; Oiwa et al., 2013). All these findings demonstrate the critical importance of pectin cross-linking at the RG-II domains. Thus, it is necessary to elucidate where RG-II is located within the cell walls of individual cells and tissues, and whether or not there is a change in its distribution during growth or in response to environmental stimuli. Immunocytochemical analysis using a RG-II-specific antibody is particularly suitable for the purpose.

So far, two antibodies were generated against RG-II, a monovalent antibody fragment (Fab) which was selected from a recombinant phage display library (Williams et al., 1996), termed CCRC-R1. In immunocytochemistry using this Fab, an alkaline treatment was necessary to detect signals, suggesting that the epitope recognized by the Fab is a structure masked by the esterification of carboxyl groups on uronic acid residues. A rabbit polyclonal anti-RG-II antibody was raised in our laboratory (Matoh et al., 1998), and gave signals in the cell walls without alkaline pretreatment. The polyclonal antibody has been used in many studies (Baluška

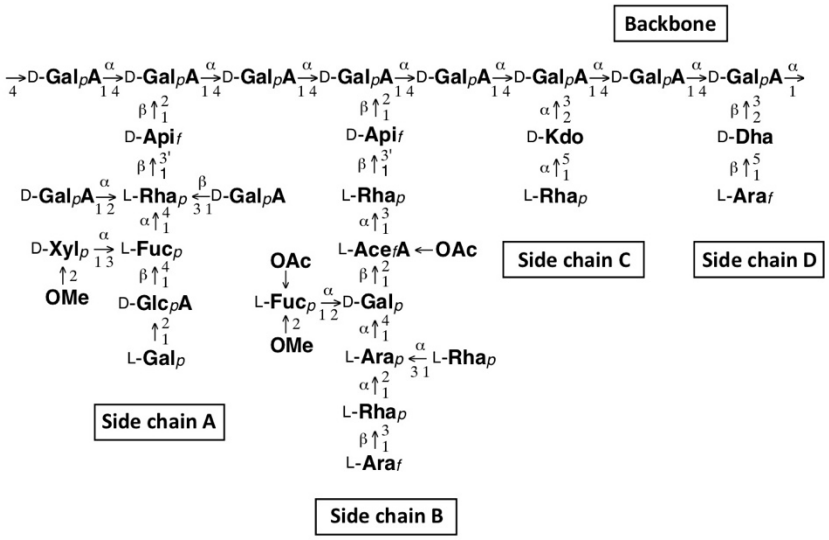

Figure Primary structure of rhamnogalacturonan II in cell walls
AceA, aceric acid; Api, apiose; Ara, arabinose; DHA, 3-deoxy-D-*lyxo*-hept-2-ulosaric acid; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; GlcA, glucuronic acid; KDO, 3-deoxy-D-*manno*-oct-2-ulosonic acid; OAc, *O*-acetyl group; OMe, *O*-methyl group, Rha, rhamnose; Xyl, xylose. The length of the backbone and the particular residue to which each side chain is attached have not been determined.

et al., 2002; Bárány et al., 2010; Dhonukshe et al., 2006; Dumont et al., 2014; Goldbach et al., 2002; Iwai et al., 2006; Kasprowicz et al., 2009; Shi et al., 2017). The immunocytochemical studies using this polyclonal antibody indicated a ubiquitous distribution of RG-II in primary cell walls (Matoh et al., 1998). It also demonstrated the denser localization of RG-II near the plasma membrane than in the other layers. These findings demonstrate the usefulness of immunocytochemical technique to reveal the localization of RG-II. Thus, in this study I investigated the subcellular localization of RG-II immunocytochemically.

Upon investigating the distribution of RG-II within the cells, I focused on cytokinesis. Cytokinesis in terrestrial plants is associated with cell plate formation.

The process involves the formation at the cell equator of a tubulo-vesicular membrane network (TVN). TVN evolves into a tubular network and a planar fenestrated sheet, which extends at its periphery until it fuses to the parent cell wall (Fabien et al., 2014). New primary cell walls are then completed on this structure, via deposition of polysaccharides including cellulose. The cell plate is, therefore, considered to be the precursor of the cell wall. Then it would be interesting to know how the cell plate is similar to the primary cell walls, for example in terms of the polysaccharide composition. Northcote et al. (1989) used the antibodies to locate the arabinogalactans, xylan, callose, L-arabinofuranose in dividing and differentiating cells of bean (*Phaseolus vulgaris L.*) root, bean callus tissue and cells of *Zinnia elegans L*, and the results showed that arabinogalactans and callose were present in the cell plate, while xylan was not present in the cell plate. Fabien et al. (2014) showed that cellulose, and three distinct

cellulose synthases have already accumulated in the cell plate at the TVN stage. Moore et al. (1988) showed xyloglucan was present in the Golgi stacks and cell plate in root tip cells of red clover (*Trifolium pratense L.*). Rodriguez-Gfilvez et al. (1995) showed that RG-I was present on the cell plate using antibody CCRC-M2, suggesting pectin might be present on the cell plate. However, there is almost no information about whether RG-II, the domain critical for pectin cross-linking, is present in the cell plate. Hence, the aim of this study was to investigate whether RG-II is present in the forming cell plates, as a necessary step toward elucidating whether or not the pectin in the cell plate exists as a network cross-linked with B.

In this study, I first established the B-RG-II immobilization methods for the RG-II antibodies evaluation (Chapter 1). Then I used the rabbit polyclonal anti-RG-II antibody to analyze the subcellular localization of RG-II in the cultured tobacco cells which were undergoing cell division, and found RG-II was present on the forming cell plates from the early stage of their assembly (Chapter 2). Considering the advantages of monoclonal antibodies over the polyclonal antibodies, a novel rabbit monoclonal antibody against RG-II was generated, and it was used to localize RG-II in the Arabidopsis root tips (Chapter 3). RG-II signals were found in developing cell plates and cell walls. These results together demonstrate that RG-II is present in forming cell plates, suggesting the importance of B-RG-II as the cross-linker of pectin in cell wall assembly.

Chapter 1

Establishment of the methods for quantitative evaluation of the performance of anti-rhamnogalacturonan II antibodies

Introduction

In this study, I used a rabbit polyclonal anti-RG-II antibody produced previously in our laboratory, but the specificity of the antibody had not been characterized in detail. In a following study, production and application of a new rabbit monoclonal anti-RG-II antibody was described, which also required quantitative assessment of the titer and specificity of the antibody. Such characterizations of antibodies are usually conducted using enzyme-linked immunosorbent assay (ELISA). However, unlike protein antigens, RG-II as a small and hydrophilic polysaccharide fragment does not bind efficiently to polystyrene surfaces via hydrophobic interaction. The character makes it difficult to hold RG-II on microtiter plates as the immobilized antigen in ELISA, thus quantitative assays of antibodies against RG-II had been difficult. Therefore, in this chapter, I tried to develop methods to immobilize RG-II onto solid surfaces via conjugation with proteins or using a plate with chemically modified surfaces.

Materials and methods

1.2.1 Preparation of B-RG-II

B-RG-II was isolated from radish root cell walls as described previously (Kobayashi et al., 1996). Briefly, radish root cell walls were treated with Pectinase-SS (0.1% w/v; Kyowa Chemical Products, Osaka, Japan) in 400 mL of 20 mM sodium acetate, pH 4.0 for 48 h at 25ºC on a rotary shaker (130 rpm). The suspension was centrifuged at 10,000×*g* for 15 min. The supernatant was adjusted to pH 8.0 with 2 M Tris and applied to a DEAE-Sepharose column (1.6×30 cm, Cl- form, GE Healthcare, Piscataway, NJ, USA) equilibrated with 20 mM Tris-HCl, pH 8.0. The column was eluted with a 1-liter linear gradient of 0 to 0.5 M NaCl in the column buffer, and the fractions containing 2-keto-3-deoxysugars were pooled and dialyzed against distilled water. The sample was further purified by a re-chromatography on the same DEAE-Sepharose column. Fractions containing 2-keto-3-deoxysugars were pooled, dialyzed against water and lyophilized, then subjected to gel-filtration using a Superdex 75 column (1.6×60 cm, GE Healthcare), equilibrated with 20 mM Tris-HCl, pH 8.0 containing 0.1 M NaCl. Fractions containing 2-keto-3-deoxysugars were pooled, dialyzed against water and lyophilized.

1.2.2 Immobilization of B-RG-II as covalent conjugate with poly-L-lysine

Cyanuric chloride-mediated conjugation of B-RG-II and poly-L-lysine (PLL; Sigma-Aldrich, St Louis, MO, USA) was conducted as described for the conjugation of bacterial polysaccharide and PLL (Gray 1979). Briefly, B-RG-II was dissolved in autoclaved distilled

water at 1 mg/mL. A 40-μL aliquot of the B-RG-II solution was mixed with 200 μL of 0.01 M NaOH containing 0.001% (w/v) phenolphthalein. After mixing by vortex for 10 s, the pink solution was transferred to a tube containing 0.2 mg cyanuric chloride and mixed by vortex for 10 s while monitoring the solution until it turned colorless. The mixture was then added to 40 μL of 1 mg/mL PLL, mixed by vortex for 10 s, and incubated at 4°C for 2 h. The reaction was diluted by mixing with 0.72 mL of 50 mM sodium phosphate buffer (pH 7.0). Wells of Maxisorp plate (Nunc, Roskilde, Denmark) were added with 100 μL of this solution and incubated at 4ºC overnight.

1.2.3 Immobilization of B-RG-II as covalent conjugate with hemocyanin

B-RG-II and *Concholepas concholepas* hemocyanin (CCH; Thermo Scientific) or keyhole limpet hemocyanin (KLH) were conjugated via carbodiimide coupling. Conjugation of B-RG-II with CCH was carried out following the manufacturer's instruction. Briefly, a 3-mg aliquot of B-RG-II was dissolved in 675 μL conjugation buffer (0.1 M 2-[*N*-morpholino]ethanesulfonic acid [MES] pH 4.7 containing 0.9 M NaCl and 0.02% sodium azide) and mixed with 300 μL of 10 mg/mL CCH solution. The mixture was added with 75 μL of freshly prepared 10 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Dojin Laboratories, Kumamoto) in distilled water, and incubated for 2 h at room temperature. At this point a blue-white precipitate was formed, which probably contained the B-RG-II-CCH conjugate. Since the supernatant might also contain the conjugate, the reaction was centrifuged at 12,000×*g* for 5 min and the supernatant (1 mL) was fractionated with PD-10 desalting column (GE Healthcare) equilibrated with 80 mM sodium phosphate buffer (pH 7.2) containing 0.9 M NaCl and 0.1 M sorbitol. The high molecular weight fraction was recovered in 2 mL and then mixed again with the blue-white precipitate. As these treatments were not expected to degrade any linkage in B-RG-II, all the B-RG-II used for the conjugation reaction should be recovered in the final suspension. Total B-RG-II concentration of the suspension is therefore expected to be 1.5 mg/mL. Conjugation with KLH was conducted in the same manner except that 0.1 M MES (pH 4.5) was used for conjugation. The suspension containing B-RG-II-CCH or B-RG-II-KLH conjugate was diluted with 50 mM sodium phosphate buffer (pH 7.0) to 100 μg/mL (B-RG-II-CCH) or 50 μg/mL (B-RG-II-KLH). Wells of Maxisorp plate were added with 100 μL of the solution and incubated at 4ºC overnight.

1.2.4 Immobilization of B-RG-II via direct cross-linking to surface-modified microtiter plate

A 50-μL aliquot of B-RG-II solution was added to the wells of an amino-group modified microtiter plate (NH Covalink; Nunc). The wells were subsequently added with 50 μL of 0.5 mg/mL EDC. The microtiter plate was incubated at room temperature for 1.5 h.

1.2.5 ELISA

Wells coated with B-RG-II were washed 3 times with 250 μL of phosphate buffered saline (PBS; 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl). The wells were blocked with 0.5% (w/v) ovalbumin (OVA, Wako, Osaka, Japan) in PBS containing 0.05% (w/v) Tween 20 (PBST) at 4ºC for 1 h. After washed 3 times with PBST, wells were added with

100 µL of PBST containing rabbit polyclonal anti-RG-II antibody and 0.1% (w/v) OVA and incubated for 1 h at room temperature with gentle shaking. The antibody was produced in our laboratory, and its characters are described in Chapter 2. The antibody was used at 0.5 µg/mL unless otherwise stated. The wells were washed for 3 times with PBST and then added with peroxidase-conjugated goat anti-rabbit secondary antibody (Nacalai Tesque, Kyoto, Japan) diluted 5,000-fold with PBST containing 0.1% (w/v) OVA. After incubated for 1 h at room temperature, the wells were washed for 3 times with PBST, and then the signals were detected using an ELISA POD substrate TMB kit (Nacalai Tesque).

1.2.6 Dot-blot analysis

B-RG-II was dissolved in 20 mM Tris-HCl (pH 8.0) at appropriate concentrations, and 1-µL aliquots were spotted onto the membrane (Hybond-N+, GE Healthcare). The air-dried membrane was blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan) for 30 min. The blot was incubated with 1 µg/mL rabbit polyclonal anti-RG-II antibody in binding buffer (a 9:1 [v/v] mixture of 10 mM phosphate buffer, pH 7.4, containing 0.05% Tween 20 and Block Ace solution) for 1 h at room temperature. The blot was washed three times with washing buffer (a 9:1 [v/v] mixture of 10 mM phosphate buffer, pH 7.4, containing 0.1% Tween 20 and Block Ace solution) for 5 min each time.

The blot was then incubated with peroxidase-conjugated anti-rabbit secondary antibody (Nacalai Tesque, Kyoto, Japan), diluted 20,000-fold in binding buffer for 1 h at room temperature. After washing the blot 3 times for 5 min each, the signal was detected using a chemiluminescence substrate kit (Chemi-Lumi One Super, Nacalai Tesque).

Results

1.3.1 Immobilization of B-RG-II on positively charged nylon membrane

Since B-RG-II is charged negatively, it may be associated ionically with positively charged solid surfaces. Thus, I first evaluated the efficiency of B-RG-II immobilization on positively charged nylon membrane, which had been used in characterization of B-RG-II antibody in our laboratory (Matoh et al., 2000). Aliquots of serially diluted B-RG-II solution was spotted onto positively charged nylon membrane, and the retention of B-RG-II was examined by rabbit polyclonal anti-RG-II antibody, which was previously produced in our laboratory and is described in the following chapter. Signals increasing with the amount of B-RG-II were obtained (Fig. 1.1), suggesting that B-RG-II was immobilized on the nylon membrane successfully. However, the retention was rather labile, as the signal was lost when sodium chloride at 150 mM was included in the solutions for antibody binding or washing during the detection procedure. Therefore, it was necessary to use 20 mM Tris-HCl buffer (pH 8.0) in these steps, instead of using Tris-buffered saline or PBS that are used in standard immunological techniques. In addition, the signals from dot-blotting was not very suitable for quantitative analysis. These points warranted the necessity of establishing the method to immobilize B-RG-II stably on microtiter plate surfaces.

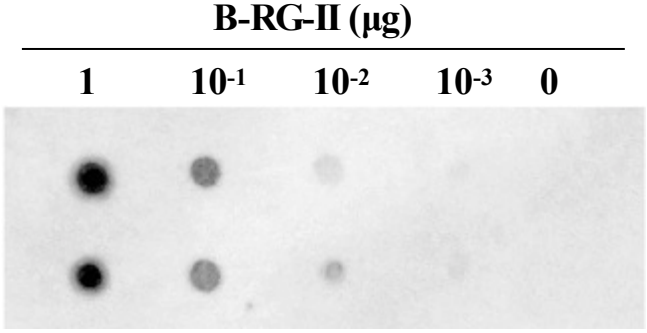

Figure 1.1 Immobilization of B-RG-II on positively charged nylon membrane. The membrane was spotted in duplicate with 1 μL of 20 mM Tris-HCl (pH 8.0) containing various amount of B-RG-II, and then subjected to chemiluminescent immunodetection with the rabbit polyclonal anti-RG-II antibody.

1.3.2 Immobilization of B-RG-II on microtiter plate as a conjugate with poly-L-lysine

In standard ELISA assays with protein antigen, the proteins are immobilized on the surfaces of polystyrene microtiter plates through a hydrophobic interaction between proteins and plastics. Thus, here I tried to conjugate B-RG-II with poly-L-lysine (PLL), so that the polysaccharide could be anchored on microtiter plate surfaces via an interaction of the protein moiety with polystyrene. Since B-RG-II and PLL are charged negatively and positively, respectively, they may associate through ionic interaction. However, it was unknown if the ionically associated B-RG-II-PLL conjugate, if any, could be stable enough to be used in downstream applications. Therefore, a covalently-linked B-RG-II-PLL conjugate was prepared, then compared its retention on microtiter plate surfaces with that of the complex without covalent cross-linking. The retention of B-RG-II on the surface was assessed immunologically as the amount of rabbit polyclonal anti-RG-II antibody bound to the wells. The wells after coating were applied with the antibody, washed and subjected to the detection using peroxidase-linked secondary antibody and color reaction. In the wells applied with the B-RG-II-PLL covalent conjugate, signals increasing with the amount of B-RG-II-PLL conjugate were observed (Fig. 1.2), indicating that B-RG-II was immobilized successfully on microtiter plate surfaces. On the other hand, wells applied with the mixture of B-RG-II and PLL without cross-linking, gave only very weak signals (Fig. 1.2). The result suggests that the complex formed through ionic interaction, if any, is not stable enough, and the covalent cross-linking between B-RG-II and the protein is necessary to conduct downstream experiments.

Next, I tried to apply this method of B-RG-II immobilization for the titration of antibody. As shown in Fig. 1.3, a change in signal consistent with the serial dilution of antibody was obtained. Essentially no signal was obtained from the wells coated with PLL alone, confirming that the signal from the wells coated with B-RG-II-PLL conjugate was due to B-RG-II immobilized. Consistent with the result in Fig. 1.2, signals from the wells coated with B-RG-II-PLL mixture without cross-linking were much lower than that from the wells coated with B-RG-II-PLL conjugate (Fig. 1.3).

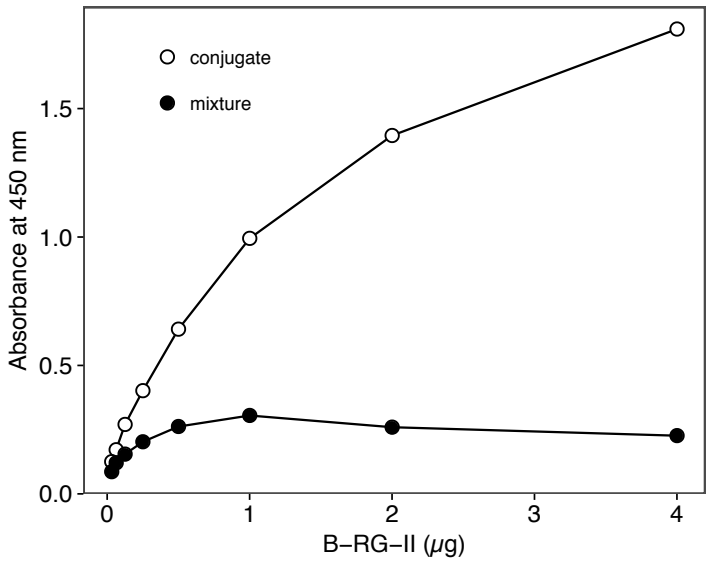

Figure 1.2 Immobilization of B-RG-II on microtiter plate as B-RG-II-PLL conjugate. Microtiter plate wells were coated with either covalently cross-linked B-RG-II-PLL conjugate (open circles) or the mixture of B-RG-II and PLL without covalent cross-linking (closed circles). After washing with buffer, B-RG-II present in the wells were quantified immunologically; the wells were added with 100 µL of 0.5 µg/mL rabbit polylclonal antibody, then the amount of antibody bound to the wells was quantified using a peroxidase-conjugated secondary antibody. Each point represents the average of duplicate.

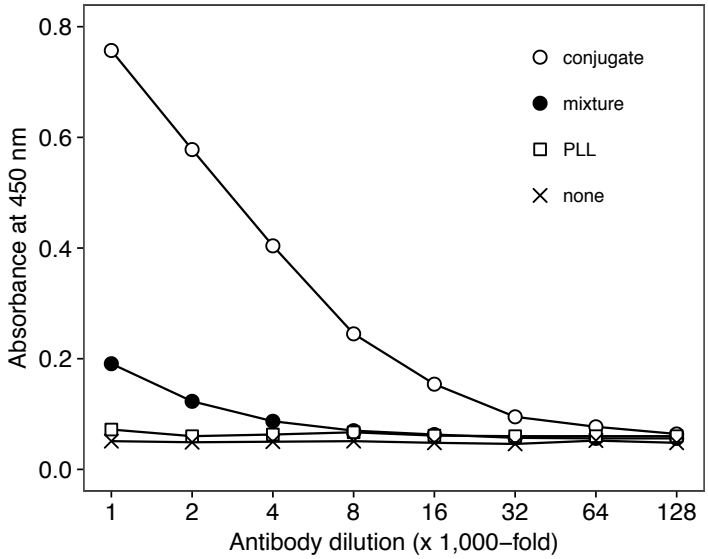

Figure 1.3 Titration of rabbit polyclonal anti-RG-II antibody using microtiter plates coated with B-RG-II-PLL conjugate. B-RG-II was immobilized on microtiter plate as either the covalently cross-linked B-RG-II-PLL conjugate (open circles) or the mixture of B-RG-II and PLL without covalent cross-linking (closed circles). The amount of B-RG-II-PLL used for coating was 4 µg per well as total B-RG-II (including both PLL-bound and unbound form). Wells coated with PLL alone (4 µg/well, open squares) or no coating (crosses) were also included in the analysis. The wells were applied with two-fold dilution series of the rabbit polyclonal anti-RG-II antibody, prepared from the stock solution of 2 mg/mL. The antibody bound to the wells were quantified using peroxidase-mediated color reaction. Each point represents the average of triplicates.

1.3.3 Immobilization of B-RG-II on microtiter plate as a conjugate with CCH or KLH

The results of B-RG-II-PLL immobilization demonstrated that conjugation with protein was useful to immobilize B-RG-II onto microtiter plate surfaces. Hence, I also tried conjugating B-RG-II to CCH or KLH, which are the proteins frequently used as the carriers in preparation of antibodies against small molecules. A carbodiimde coupling was employed to cross-link B-RG-II to the proteins. Microtiter plate wells were coated with the conjugate, then used to titrate the rabbit polyclonal anti-RG-II antibody. In the wells coated with B-RG-II-CCH conjugate, signals decreasing with the dilution of antibody were obtained (Fig. 1.4), whereas essentially no signal was generated in the wells coated with CCH alone (Fig. 1.4). The result indicates that the signals were due to B-RG-II moiety. Signals from the wells added with B-RG-II alone were faint (Fig. 1.4), indicating that B-RG-II itself could not be hold efficiently on the microtiter plate surface. These results together demonstrate that B-RG-II could be immobilized on the microtiter plate through the conjugation with CCH. A similar result was obtained using KLH as the conjugation partner (Fig. 1.5).

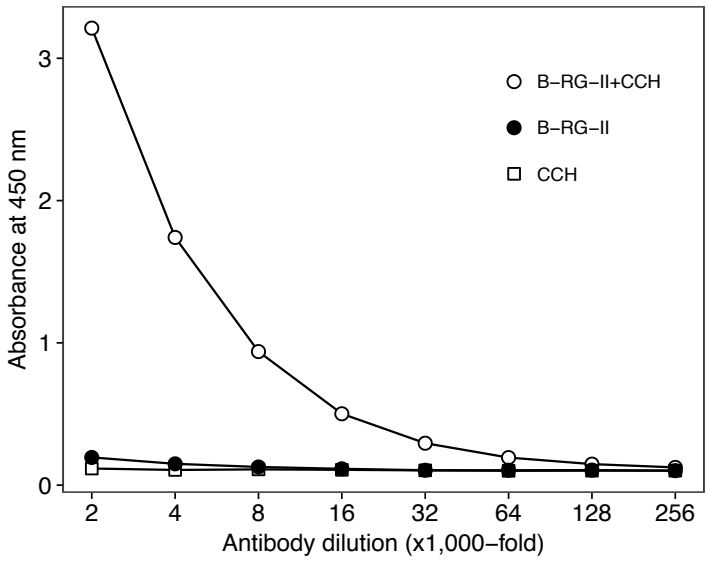

Figure 1.4 Titration of rabbit polyclonal anti-RG-II antibody using microtiter plates coated with B-RG-II-CCH conjugate. B-RG-II was immobilized on microtiter plate as the B-RG-II-CCH conjugate prepared via a carbodiimide coupling (open circles). The amount of B-RG-II-CCH used for coating was 10 μg per well (as the B-RG-II subjected to the conjugation reaction). Wells applied with B-RG-II alone (20 μg/well, closed circles) or CCH alone (10 μg/well, open squares) were also included in the analysis. The wells were applied with two-fold dilution series of the rabbit polyclonal anti-RG-II antibody, prepared from the stock solution of 2 mg/mL. The antibody bound to the wells were quantified using peroxidase-mediated color reaction.

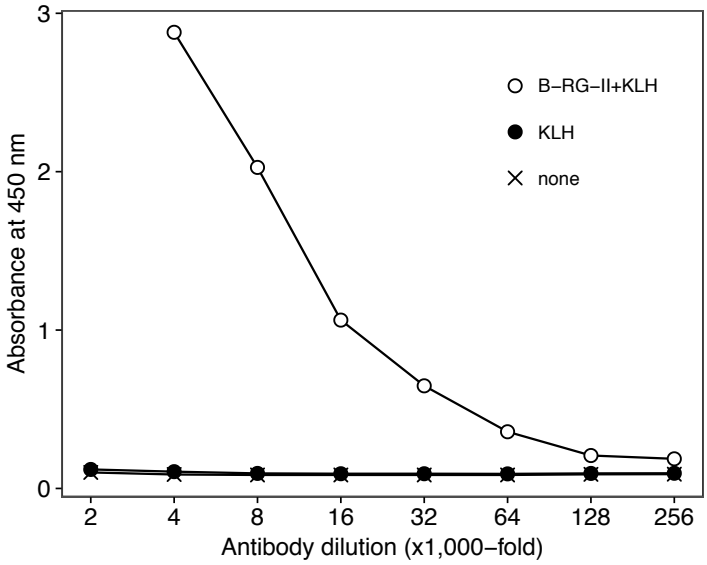

Figure 1.5 Titration of rabbit polyclonal anti-RG-II antibody using microtiter plates coated with B-RG-II-KLH conjugate. B-RG-II was immobilized on microtiter plate as the B-RG-II-KLH conjugate prepared via a carbodiimide coupling (open circles). The amount of B-RG-II-KLH used for coating was 5 μg per well (as the B-RG-II subjected to the conjugation reaction). Wells applied with KLH alone (10 μg/well, closed circles) or no coating (crosses) were also included in the analysis. The wells were applied with two-fold dilution series of the rabbit polyclonal anti-RG-II antibody, prepared from the stock solution of 2 mg/mL. The antibody bound to the wells were quantified using peroxidase-mediated color reaction.

1.3.4 Immobilization of B-RG-II to amino-derivatized microtiter plate surfaces

Results of trial using CCH or KLH as the conjugation partners indicate that carbodiimide coupling could be used to covalently cross-link B-RG-II to other molecules. Thus, I also examined the carbodiimide coupling of B-RG-II directly to the wells of a microtiter plate with amino group-modified surfaces. B-RG-II were added to the wells of amino-modified plate together with carbodiimide (EDC), then incubated at room temperature. Detection with the anti-RG-II antibody gave signals dependent on the dose of B-RG-II added to the wells (Fig. 1.6). Wells added with EDC alone did not produce any signal (data not shown). When B-RG-II was applied to the wells without EDC, signals were much lower than those from the wells added with B-RG-II and EDC (Fig. 1.6). The result suggests that at least a part of B-RG-II added to the wells was covalently cross-linked with the amino groups on the plate, and the linkage could retain B-RG-II efficiently.

To examine the usability of B-RG-II immobilization on amino plate, titration of the antibody was carried out. As shown in Fig. 1.7, signals consistent with the concentration of the antibody was observed, whereas no signal was observed with preimmune serum. The result demonstrates that this method of immobilization is useful for the characterization of antibodies against B-RG-II.

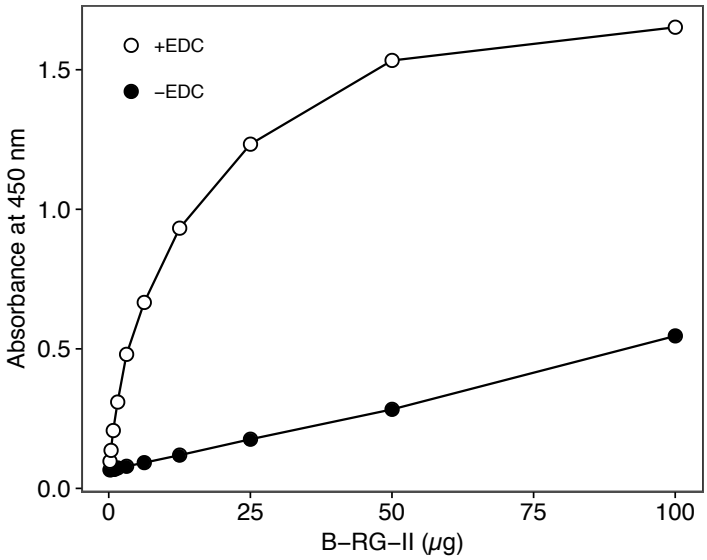

Figure 1.6 Immobilization of B-RG-II onto amino plate via carbodiimide coupling. Aliquots of 50 µl B-RG-II solution was added to the wells with (open circles) or without (closed circles) EDC. After washing, the wells were added with 100 µL of 0.5 µg/mL rabbit polyclonal anti-RG-II antibody, then the amount of antibody bound to the wells was quantified using a peroxidase-conjugated secondary antibody. Each point represents the average of the signals from four (+EDC) or two (–EDC) wells.

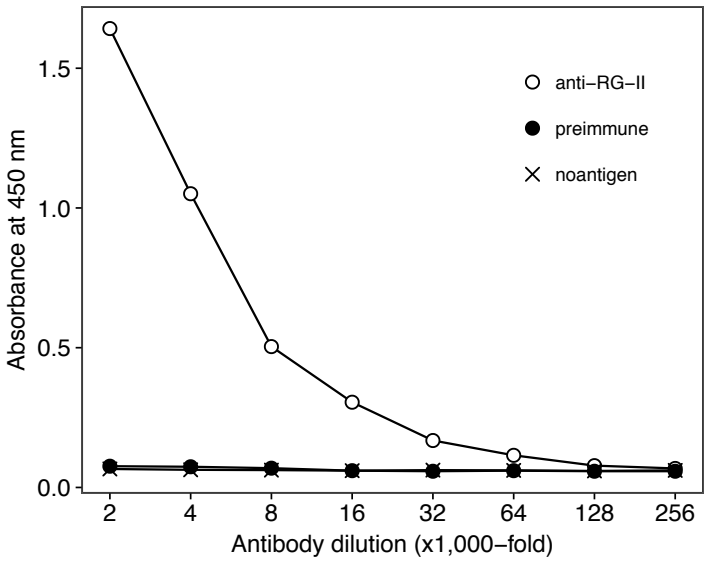

Figure 1.7 Titration of rabbit polyclonal anti-RG-II antibody using amino group-modified microtiter plates. B-RG-II was cross-linked to the amino groups on the microplate surface via a carbodiimide coupling. The amount of B-RG-II used for coating was 3 µg per well. The wells were applied with two-fold dilution series of the rabbit polyclonal anti-RG-II antibody (open circles) or preimmune serum (closed circles), prepared from the stock solutions of 2 mg/mL. The antibody bound to the wells were quantified using peroxidase-mediated color reaction. Wells without B-RG-II coating were also included in the analysis as negative control (crosses).

Discussion

Establishing antibodies against RG-II had been difficult for several reasons. Firstly, as a relatively small, hydrophilic polysaccharide fragment, RG-II does not efficiently elicit an immune response, thus raising the antibody against RG-II is more difficult when compared with protein antigens. In addition, unlike protein antigens, RG-II adsorbs poorly to plastic surfaces via hydrophobic interaction. The property makes the use of ELISA for the characterization of anti-RG-II antibody difficult, since the immobilization of the antigen on polystyrene microtiter plate is necessary in the method. The problem in immunogenicity has been solved to some extent by immunizing animals with a conjugate of RG-II and methylated bovine serum albumin (mBSA), and a rabbit polyclonal anti-RG-II antibody was raised indeed (Matoh et al., 1998). On the other hand, the lack of convenient method for quantitative characterization of anti-RG-II antibody remained unsolved. To get reliable insights from immunocytochemical analysis, thorough characterization of the antibody is critically important. Thus, here I tried to establish the method to immobilize B-RG-II on the polystyrene microtiter plate surface, so that a quantitative characterization of anti-RG-II antibodies became possible. Immobilization of B-RG-II-protein conjugates through plate-protein hydrophobic interaction, and direct coupling of B-RG-II onto an amino group-modified plate surface were examined, and both principles were found to be effective.

Since the polyclonal anti-RG-II antibody used in this study was raised using a B-RG-II-mBSA conjugate as the antigen and hence contains a fraction recognizing the mBSA moiety, the conjugate for immobilization needs to be made with proteins other than mBSA. Thus, cyanuric chloride-mediated coupling with PLL, and carbodiimide coupling with KLH or CCH were examined and found to be effective as the immobilization method. In addition, direct coupling of B-RG-II to the amino group on a chemically modified microtiter plate also immobilized B-RG-II effectively.

It remains unknown how much percent of the B-RG-II applied to the wells were immobilized onto the plate surfaces. The information might be significant if the ELISA is used with the aim of B-RG-II quantification. Nonetheless, all the methods described here gave sufficient signals in ELISA, indicating that the immobilized amounts were enough to be detected with immunological methods. It also remains to be confirmed if the structure of B-RG-II is kept intact during the immobilization. Several glycosidic linkages and the borate diester in B-RG-II are acid-labile, whereas there has been no report on the effect of alkaline pH on B-RG-II structure. The conjugation with PLL involves a brief exposure of B-RG-II to alkaline pH, and the effects of the treatment on B-RG-II structural integrity remains elusive. On the other hand, the carbodiimide coupling used for the conjugation of B-RG-II with CCH, KLH, or the amino group-modified plate was carried out under pH 4.5 at room temperature. The condition is comparable to that used for B-RG-II isolation from cell wall (pH 4.0 at room temperature). Thus, it is unlikely to break any linkage in B-RG-II. Taken together, I concluded that the immobilization methods enabled a quantitative characterization of anti-RG-II antibodies in ELISA format. The methods were used in the following chapters to check the reactivity of rabbit serum against B-RG-II during the antibody production and the substrate specificities of the produced antibodies.

Chapter 2

Immunocytochemical analysis of subcellular localization of rhamnogalacturonan II in cultured tobacco cells

Introduction

Cell wall pectin plays multiple roles as a hydrogel in cell walls, and B-RG-II is one of the major cross-linkers of pectin to form the gel. The importance of the B-RG-II linkage has been demonstrated by multiple studies. However, still only limited information is available on the formation of B-RG-II, including where in the cells or when during the assembly of new cell wall B-RG-II structure is formed.

Cell plate is the structure formed during cytokinesis to separate the two daughter cells, and it is considered as a precursor of the primary cell wall. Previous studies have demonstrated that antibodies against HG and RG-I label the forming cell plate (Moore et al., 1988; Rodriguez-Gfilvez et al., 1995), suggesting that pectin is already present in this structure. However, there is almost no information about whether RG-II, the domain critical for pectin cross-linking, is present in the cell plate. It would be significant to know whether the pectin in the cell plate forms the same B-cross-linked network as that in primary cell walls. Thus, as the necessary step to figure out whether the pectin in the cell plate exists as a network cross-linked with B-RG-II, a polyclonal antibody against RG-II was raised in rabbit and used to investigate whether RG-II was present on the forming cell plates in cultured tobacco cells. The antibody used for the analysis was characterized using ELISA established in the previous chapter.

Materials and methods

2.2.1 Plant materials

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/L 2,4-dichlorophenoxyacetic acid, pH 5.8, in a 300-mL conical flask maintained in the dark at 25ºC shaken at 130 rpm (Nagata et al., 1981).

2.2.2 Preparation of antibody

B-RG-II was isolated from radish root cell walls as described in the previous chapter. A polyclonal antibody against B-RG-II was raised in rabbit by immunizing rabbits with a B-RG-II-mBSA conjugate and purified as described previously (Matoh et al., 1998). The purified antibody was dialyzed against water, lyophilized, and dissolved in PBS at 2 mg/mL as a stock solution.

2.2.3 Competitive ELISA

B-RG-II was immobilized on the amino plate as described in 1.2.4. Briefly, 50 μL B-RG-II (62.5 μg/mL) was added to each well, followed by 50 μL 0.5 mg/mL EDC. The plate was then incubated with gentle shaking at room temperature for 2 h. The wells were washed twice with 250 μL PBST and blocked with 0.5% (w/v) OVA in PBST at 4 ºC overnight.

Aliquots of 62.5-ng rabbit polyclonal anti-RG-II antibody were incubated in 250 μL PBST containing different concentrations of competitors for 2 h at room temperature. Then, 100 μL of each mixture was added in duplicate to the wells coated with B-RG-II and blocked, and then the plate was incubated for 1.5 h at room temperature with gentle shaking. The wells were washed three times with PBST before adding goat anti-rabbit immunoglobulin (IgG) antibody conjugated with peroxidase (Nacalai Tesque) diluted 5,000-fold with PBST. After a 1-h incubation at room temperature, the wells were washed three times with PBST, and then signals were detected using an ELISA POD substrate TMB kit (Nacalai Tesque).

Monomeric RG-II was prepared by incubating B-RG-II in 0.1 M HCl at a concentration of 1 mg/mL for 15 min at room temperature followed by neutralization with 1 M Tris. Rhamnogalacturonan I was prepared from radish cell wall as described previously (Darvill et al., 1978), except that a DEAE-Sepharose FF and a HiLoad Superdex 75 column (GE Healthcare) were used as the media for ion exchange and gel-permeation chromatography, respectively. Polygalacturonic acid (PGA) and esterified pectin, both were from citrus, was obtained from Nacalai Tesque and Sigma-Aldrich, respectively.

2.2.4 Immunofluorescence microscopy

To increase the number of cells undergoing cell division, 7-day-old cells were allowed to sediment. Then, approximately 15 mL sedimented cells was transferred to 75 mL medium and then cultured for 22-24 h. Progression of the cell cycle was estimated by staining the cells with 4′,6-diamidino-2-phenylindole (DAPI). Cells were fixed when a significant proportion of the cells was in anaphase or telophase (typically 23 h).

Cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde (GLU) in microtubule-stabilizing buffer (50 mM piperazine-*N,N'*-bis(ethanesulfonic acid), 5 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 5 mM $MgSO_4$, pH 7.0) at room temperature. After washing four times by suspending cells in the buffer for 15 min, the cells were dehydrated in a graded ethanol series (10 min each in 30, 50, 70, 80% ethanol, 10 min in 95% ethanol twice, and 10 min in 99.5% ethanol three times). Then, the cells were gradually infiltrated with 50% LR White resin (Electron Microscopy Sciences, Fort Washington, PA, USA) in ethanol at room temperature overnight, followed by 100% LR White resin for 12 h. After transferring the suspension to a gelatin capsule, the LR White resin was left to polymerize at 60ºC overnight.

An Ultracut E microtome (Reichert-Jung, Vienna, Austria) was used to cut 1-μm sections from the blocks, and then the sections were mounted on glass slides. For double labeling with rabbit polyclonal anti-RG-II antibody and the mouse monoclonal anti-(1→3)-β-glucan antibody (Biosupplies, Parkville, Australia), the sections were blocked with 3% (w/v) skim milk in tris-buffered saline (TBS; 20 mM Tris-HCl, 154 mM NaCl, pH 8.2) for 30 min at room temperature. The sections were incubated with the mixture of anti-RG-II (40 μg/mL) and anti-(1→3)-β-glucan (30 μg/mL) at 4ºC overnight. As the control, sections were incubated with 80 μg/mL IgG from an unimmunized rabbit (Sigma-Aldrich). The sections were washed three

times with TBS for 5 min before adding a mixture of 20 μg/mL goat anti-mouse IgG conjugated to Alexa Fluor 488 (Thermofisher Scientific, Waltham, MA, USA) and 20 μg/mL goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Thermofisher Scientific), followed by incubation for 2 h at room temperature. The slides were washed 6 times with TBS (5 min each) and once with deionized water (5 min). After drying, the sections were mounted using Pristine Mount (Falma, Tokyo, Japan), and observed under a fluorescence microscope (BX50 with BX-FLA fluorescent light attachment; Olympus, Tokyo, Japan) using U-MNIBA (Olympus) and TXRED-4040B (Semrock, Rochester, NY, USA) filter sets.

Double labeling with the anti-RG-II and mouse monoclonal anti-β-tubulin antibodies was carried out in the same way as that described above for the anti-RG-II and anti-(1→3)-β-glucan antibodies, except that the section was blocked with 3% (w/v) BSA, and the primary antibody mixture consisted of the rabbit anti-RG-II antibody (40 μg/mL) and mouse monoclonal anti-β-tubulin antibody (1.3 μg/mL, clone N357; Amersham, Little Chalfont, UK).

2.2.5 Immunoelectron microscopy

Cells were synchronized using aphidicolin and propyzamide (Nagata et al., 1992). To prepare cells, 10 mL of 7-day-old cell suspension was transferred to 75 mL new medium containing aphidicolin at a concentration of 3 μg/mL. After culturing for 24 h under standard conditions, the cells were collected on a 20-μm autoclaved nylon mesh, washed 5 times by suspending in 250 mL fresh medium and agitating for 15 min on a shaker, and then cultured in 75 mL medium. At 3 h after removing aphidicolin, the culture was fed with propyzamide at a concentration of 6 μM and then cultured for a further 6 h. The cells were then washed 3 times by suspending in 200 mL fresh medium and agitating on a shaker for 10 min.

At 2 h after the removal of propyzamide, cells were fixed with 2.5% GLU in 0.1 M phosphate buffer (pH 7.2) overnight at 4ºC. After washing 4 times by suspending in 0.1 M phosphate buffer (pH 7.2) for 15 min, cells were dehydrated and embedded in LR White resin as described above in 2.2.4 for immunofluorescence microscopy. Ultrathin sections were cut from the embedded blocks and mounted on nickel grids (Nisshin EM Co., Ltd. Tokyo, Japan; 100 mesh). The section was blocked by soaking in 50 μL blocking buffer containing 1% (v/v) normal goat serum (Dako, Glostrup, Denmark) and 0.1% (w/v) NaN_3 in TBS for 30 min at room temperature. The sections were soaked in 50 μL anti-RG-II antibody (1 μg/mL) in blocking buffer for 2 d at 4ºC. For the control, IgG from unimmunized rabbit (Sigma-Aldrich) was used at 2 μg/mL instead of the anti-RG-II antibody. Labeled sections were washed with blocking buffer 3 times (15 min each time). The sections were then incubated at 35ºC for 2 h with goat anti-rabbit secondary antibody conjugated to 15-nm gold particles (British Biocell International, Cardiff, UK) diluted 100-times in blocking buffer. The sections were washed with blocking buffer 6 times for 15 min each time, and then once with deionized water. Washed sections were stained with 2% uranyl acetate for 10 min and washed thoroughly with deionized water. A JEM-1400 transmission electron microscope (TEM; JEOL, Tokyo, Japan) operated at 100 kV was used for observations.

Results

2.3.1 Production of rabbit polyclonal antibody against B-RG-II

A polyclonal antibody was raised in rabbits against B-RG-II isolated from radish root cell walls. The purity of B-RG-II used for antibody immunization was confirmed by a size-exclusion chromatography (Fig. 2.1). The B-RG-II preparation gave a peak at 8.8 min (Fig. 2.1 Panel A). A tailing shoulder associated with the peak was due to small amount of monomeric RG-II produced during the storage. A large negative peak eluted after 11 min was due to water in the sample. No other peak was apparent between the void volume and the water peak. The hydrolyzed B-RG-II was shown in Fig. 2.1. Panel B, which showed a shift of main peak from B-RG-II to monomeric RG-II due to the reduction of molecular weight by half. The peak with a white arrowhead shows the salts generated by acid treatment and subsequent neutralization. No peak remained around 8.8 min, indicating that B-RG-II in panel A was not co-eluted with any other polysaccharide. Based on these results, I concluded that the B-RG-II preparation used for the immunization did not contain any other polysaccharide.

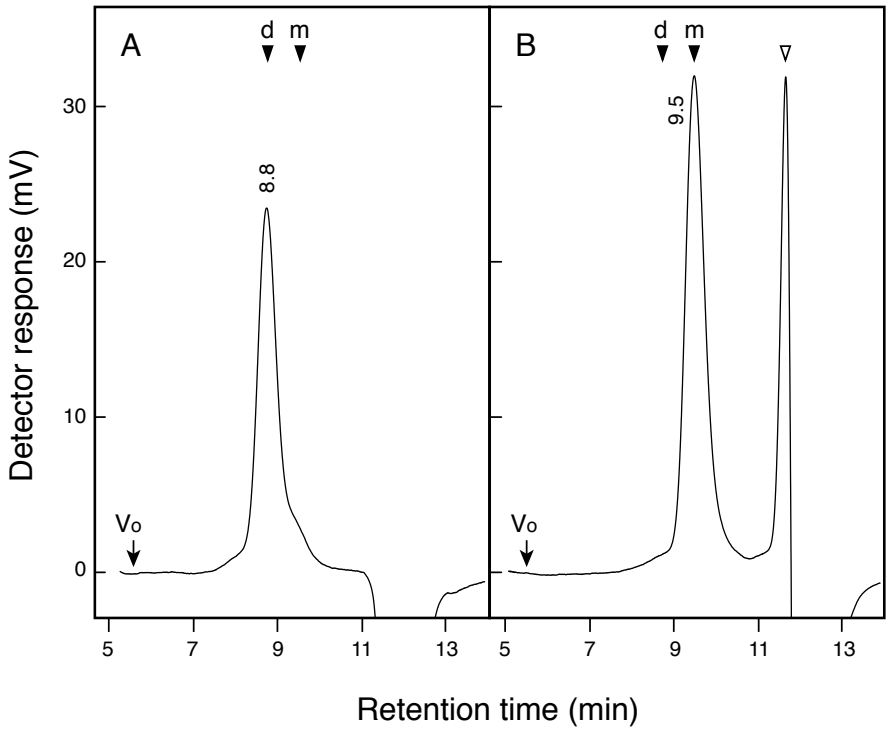

Figure 2.1 The purity of B-RG-II used for antibody production. Aliquot of 10 μL B-RG-II was subjected to size-exclusion chromatography (YMC-Pack Diol-120, 8×300 mm [YMC, Kyoto, Japan]) with a refractory index detector (TOSOH RI-8020). The column was equilibrated and eluted with 50 mM sodium acetate buffer (pH 5.2) containing 0.2 M sodium chloride at a flow rate of 1 mL/min. Panel A: the B-RG-II preparation. Panel B: B-RG-II preparation was hydrolyzed by 0.1 M HCl for 10 min at room temperature, neutralized with Tris, and then separated on the column. The void volume was indicated by arrows (Vo). Arrowheads indicate the elution position of RG-II dimer (d) and monomer (m). The peak with a white arrowhead shows the salts generated by acid treatment and subsequent neutralization.

2.3.2 Specificity of antibody

The reactivity of the antibody with B-RG-II was confirmed in the previous chapter with the results of a dot blot (Fig. 1.1) and ELISA (Fig. 1.3, 1.4, 1.5 and 1.7). Then, the specificity of the antibody was examined. Since the retention of polysaccharides onto solid surfaces may be different depending on their structures, a competitive ELISA was used for the assay. If the antibody in solution has affinity to the polysaccharide added to the solution as the competitor, the amount of antibody that binds to B-RG-II fixed on the plate will decrease, resulting in a weaker ELISA signal. It was observed that both B-RG-II and RG-II monomers in solution effectively weakened the ELISA signal (Fig. 2.2). The result indicated that the antibody recognized both dimeric RG-II cross-linked with B and monomeric RG-II, with slightly higher affinity for the dimer. The comparable reactivity with the dimer and monomer was similar to that observed with the polyclonal anti-RG-II antibody prepared previously (Matoh et al., 1998). The concentration of soluble B-RG-II required to reduce the signal by 50% was about 1 μg/mL. Cross-reactivity with HG was examined using two commercial pectin preparations with different degrees of esterification; PGA corresponding to unesterified HG, and esterified pectin in which more than 85% of GalA residues are methyl-esterified. However, neither preparation

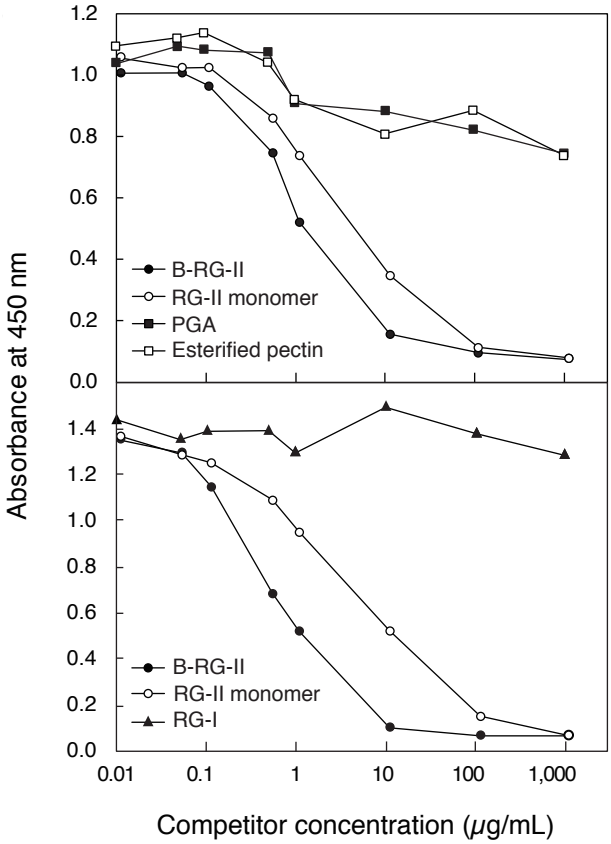

Figure 2.2 Specificity of anti-RG-II antibody. Competitive ELISA to examine reactivity of the antibody against various pectic polysaccharides. Antibody bound to plate wells was quantified using a peroxidase-conjugated secondary antibody and chemiluminescent reagent kit. Upper and lower panels show the representative results from independent experiments.

effectively reduced the ELISA signal (Fig. 2.2). Slight decline in the signal under higher concentration of these competitors might be caused by RG-II regions inside; the PGA and the esterified pectin preparation were estimated to contain RG-II at approximately 0.9 and 0.3% (w/w), respectively, based on the contents of RG-II-specific 2-keto-3-deoxysugars. This in turn suggests that HG, the main component of these preparations, was not recognized by the antibody. Rhamnogalacturonan I also did not work as competitor (Fig. 2.2). Based on all these results, it was concluded that the antibody recognized RG-II exclusively.

The antibody recognized both dimeric and monomeric RG-II (Fig. 2.2); hence, the recognized structure was not the one generated upon dimerization. The backbone was also unlikely to be the epitope, as the antibody did not recognize HG (Fig. 2.2). Thus, it is suggested that the antibody recognizes some structure involving RG-II side chains.

2.3.3 Immunofluorescent detection of RG-II epitope in dividing tobacco cells

Figure 2.3 shows the localization of the RG-II epitope in sections of cultured tobacco cells, as observed under a fluorescence microscope. To observe cell plates in dividing cells, cells in stationary phase were transferred to fresh medium and induced to resume proliferation, and then fixed after 23 h. Approximately 30% of the cells were dividing under these conditions, as judged by the morphology of DAPI-stained cell nuclei.

The fluorescence signal from the antibody was observed along the outer boundary of cells (Fig. 2.3A), as expected from the occurrence of RG-II in entire cell walls (Matoh et al., 1998). When IgG from unimmunized rabbit was used as the primary antibody, such a signal was not visible (Fig. 2.3D), verifying that the signal was due to the immunoreaction. Besides cell walls, the antibody stained a linear structure across the middle part of the cells, which seemed to be the forming cell plates in dividing cells. Callose, a (1→3)-β-D-glucan, is the marker polysaccharide of cell plates. Thus, the section was double labeled with anti-RG-II and anti-callose antibodies to determine whether the anti-RG-II antibody-reactive structure was indeed the cell plate. As shown in Fig. 2.3B, the anti-callose antibody also stained the linear structure inside the cell, and the signals from the two antibodies were found in juxtaposition (Fig. 2.3C). The results provided further evidence that the intracellular structure stained with the anti-RG-II antibody was the cell plate. The relative positions of this structure and microtubules were examined by double labeling with anti-RG-II and anti-tubulin antibodies. The cell plate initially forms as a disc between two clusters of microtubules in late anaphase during cell division, and then expands centrifugally during telophase. Thus, if the structure in question represents the cell plate, it should be found between two clusters of microtubules that are labeled with the anti-tubulin antibody. As shown in Fig. 2.4C, the intracellular signal from the anti-RG-II antibody was indeed located between two clusters of anti-tubulin signals, supporting that the structure was the forming cell plate. Taken together, these results strongly suggest that RG-II is present in the forming cell plates.

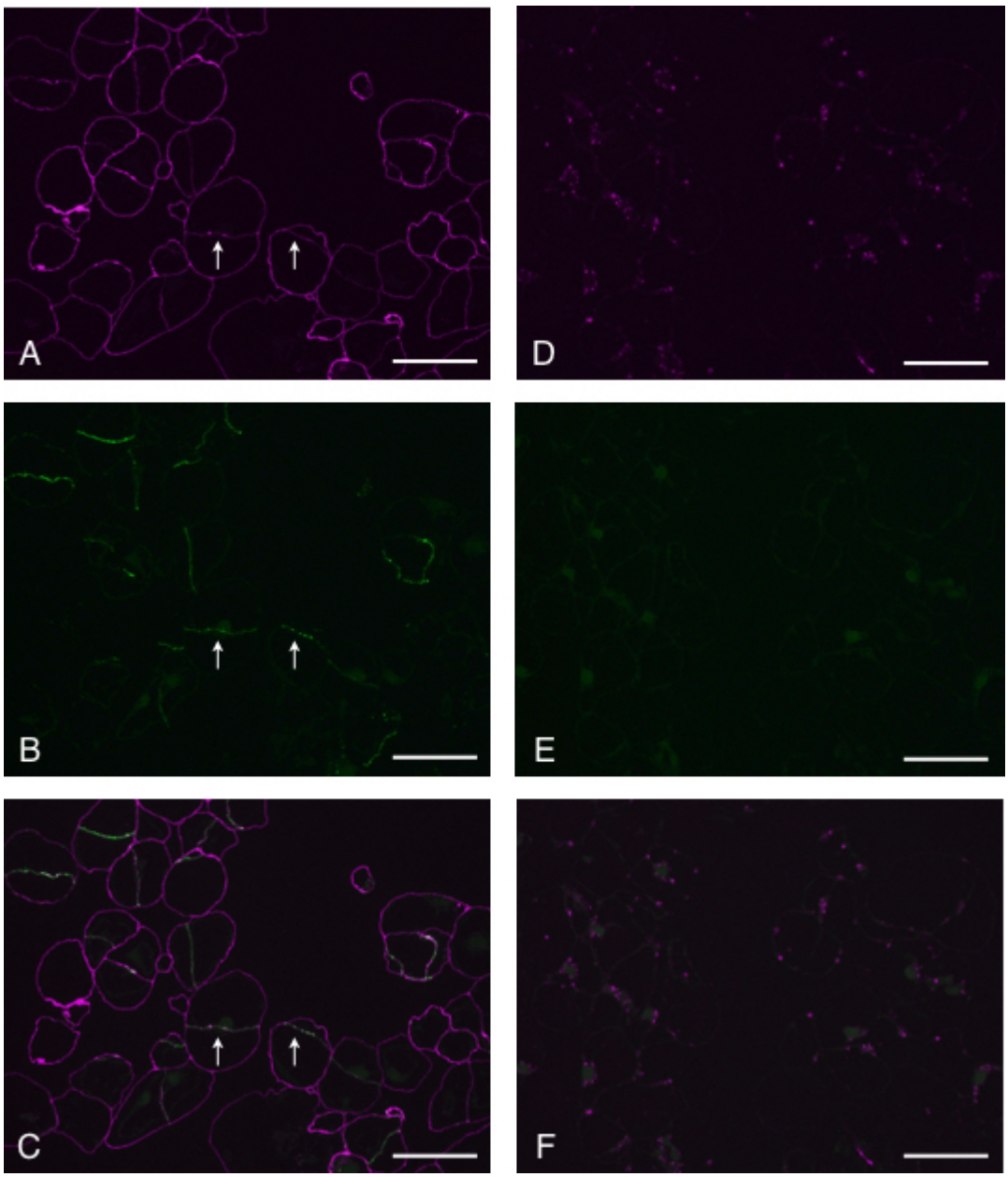

Figure 2.3 Immunofluorescent localization of RG-II and callose in dividing tobacco cells. Cells were double-labeled with anti-RG-II and anti-callose antibodies. A, Signal from anti-RG-II antibody; B, signal from anti-callose antibody; C, merged image of A and B. As negative control, IgG from unimmunized rabbit was used as primary antibody and signals from Alexa Fluor 568 (D) and 488 (E) were observed. F, Merged image of D and E. White arrows in A and C show representative intracellular structure stained with anti-RG-II antibody. Bar = 50 μm.

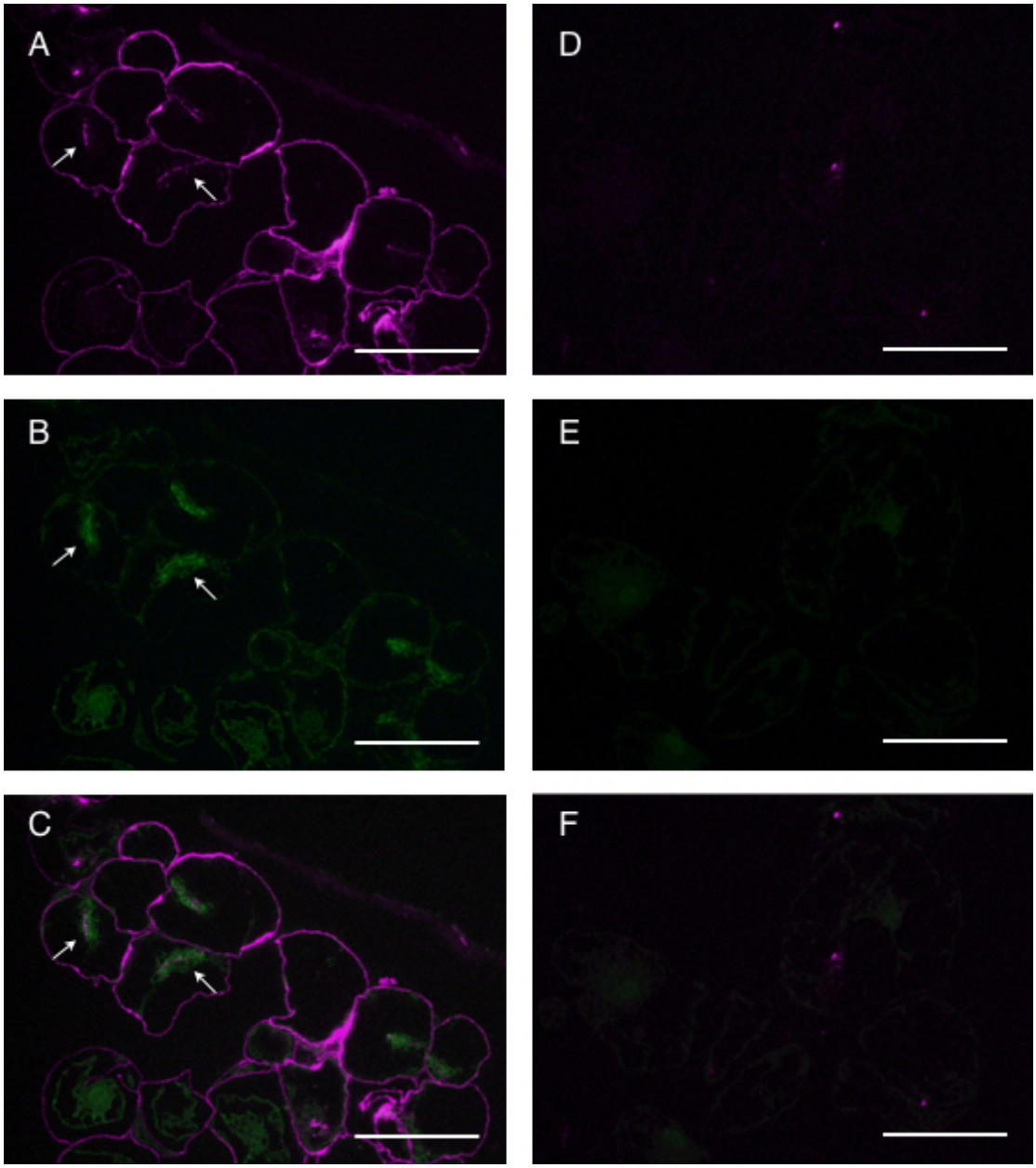

Figure 2.4 Immunofluorescent localization of RG-II and microtubules in dividing tobacco cells. Cells were double labeled with anti-RG-II and anti-β-tubulin antibodies. A, Signal from anti-RG-II antibody; B, signal from anti-β-tubulin antibody; C, merged image of A and B. As negative control, IgG from unimmunized rabbit was used as the primary antibody and signals from Alexa Fluor 568 (D) and 488 (E) were observed. F, Merged image of D and E. White arrows in A and C show representative intracellular structure stained with anti-RG-II antibody. Bar = 50 μm.

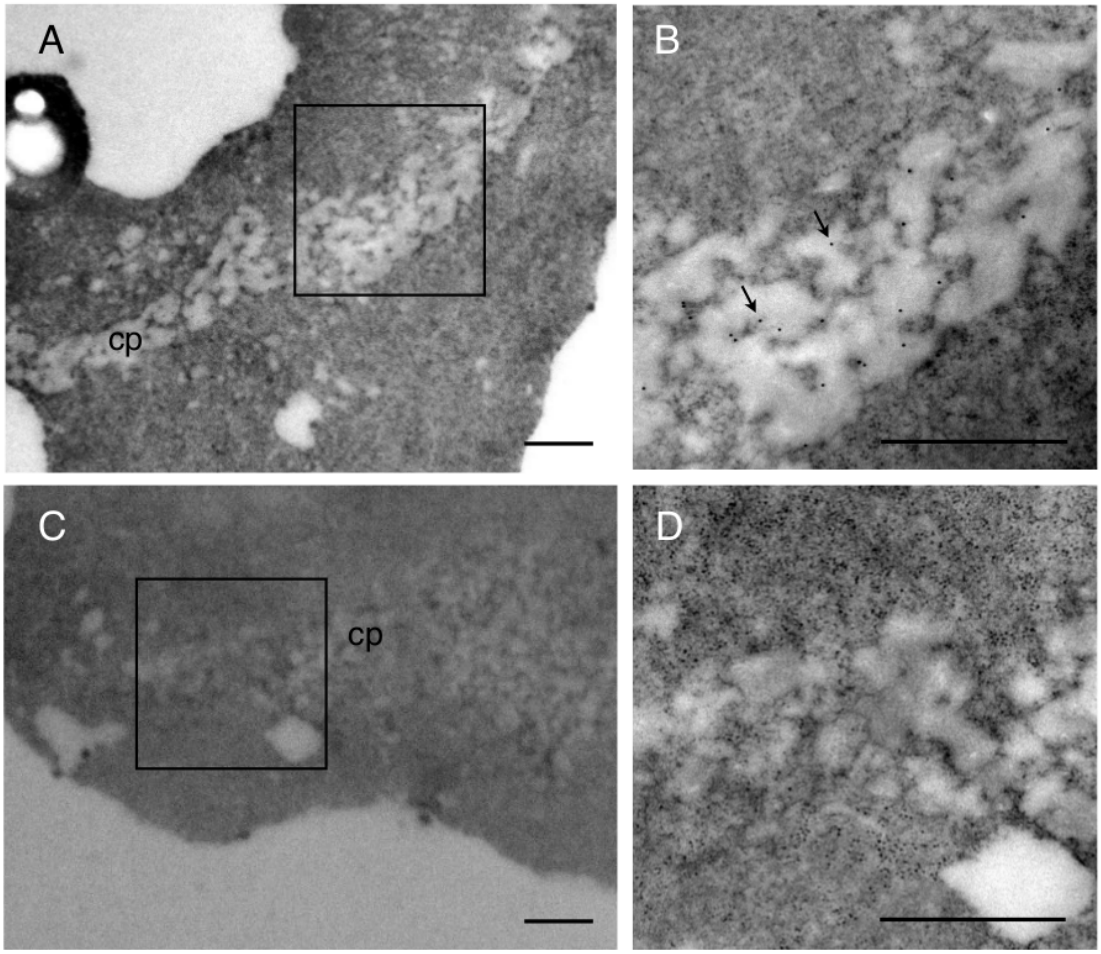

Figure 2.5 Immunogold labeling of RG-II in dividing tobacco cells. A and B, Section incubated with anti-RG-II antibody and gold-conjugated secondary antibody. C and D, Section incubated with normal rabbit immunoglobulin and gold-conjugated secondary antibody. B and D are higher magnifications of squares in A and C, respectively. Arrows in B show representative gold particle. Larger and less defined dots are ribosomes; cp, cell plates. Bar = 1 μm.

2.3.4 Immunoelectron microscopy detection of RG-II epitope in dividing tobacco cells

To analyze the location of signals generated by the antibody in more detail, ultrathin sections of tobacco cells were probed with the antibody and observed under TEM. Gold conjugated secondary antibodies were used to visualize the antibodies bound to the section. Figure 2.5 shows a dividing cell with the cell plate at the tubular network stage (Samuels et al., 1995). The gold particles were found within the tubular network in the section probed with the anti-RG-II-antibody (Fig. 2.5A and B), whereas such signals were hardly detectable in sections treated with normal rabbit IgG (Fig. 2.5C and D). The result further supported that RG-II is present in forming cell plates.

Discussion

In this chapter, evidence has been presented that RG-II is present in forming cell plates. In immunofluorescence analyses, the RG-II epitope was detected within two clusters of microtubules (Fig. 2.4C). This morphology of microtubules indicated that the cells were at early telophase, which is soon after the initiation of cell plate formation. Immunofluorescent labeling of a cell plate-like structure by anti-RG-II antibody in Arabidopsis roots has been reported previously (Dhonukshe et al., 2006), but detailed characterization of the putative RG-II in cell plate was not performed in the report. Thus, antibody-labeled structure was further analyzed by immunoelectron microscopy, with the aim of understanding the timing of appearance of RG-II during the cell division. The cell plates in Fig. 2.5 were electron-lucent with a discontinuous structure, suggesting that the cell plate was at its initial stage. These results suggested that RG-II is present in the cell plate from the early stage of its assembly. The cell plate may become the middle lamella after the new cell walls are laid down and cell division completes, although this transition has not been fully confirmed. Matoh et al. (1998) previously found that RG-II is not present in the middle lamella. That result appears to contradict those of the present study demonstrating that the cell plate, a putative precursor of the middle lamella, contains RG-II. This may be because the middle lamella undergoes remodeling as the cell wall matures, as suggested previously (Matoh, et al., 1998).

Because the antibody used in this study recognized both B-RG-II and RG-II monomers, it remains unknown whether the RG-II in forming cell plates is cross-linked with B. A previous report claimed that RG-II detected in the Arabidopsis cell plate was a dimer cross-linked with B (Dhonukshe et al., 2006). However, as the antibody used in that report also did not distinguish between B-RG-II and RG-II monomer (Matoh et al., 1998), the data did not support the argument. Given that B-RG-II can form spontaneously upon mixing boric acid and the RG-II monomer (O'Neill et al., 1996; Kobayashi et al., 1997), one may expect that the presence of RG-II indicates the formation of B-RG-II linkages. However, it is unclear whether boric acid is present in cell plates at a concentration capable of inducing RG-II dimer formation. The cytoplasmic B concentration is estimated to be very low, or nil (Matoh et al., 1992; Hu et al., 1994). Thus, if the RG-II on cell plates is the dimer cross-linked with B, there may be some mechanism by which B is transported into the compartment in which the incipient pectin will be cross-linked. In addition, RG-II built in a macromolecular pectin may not necessarily form the dimer even in the presence of B. A part of RG-II isolated from the extracellular polysaccharides of tobacco BY-2 cells was monomeric, despite the presence of B at a level sufficient to support the normal cell growth (Kobayashi et al., 1997). Chormova et al. (2016) presented the possibility that extensins could work as the chaperone helping RG-II to dimerize. The authors argue that the extensins may catalyze the dimer formation in either the cell wall or Golgi cistern or vesicles, but they did not discuss on the possibility of the borate cross-linking of RG-II in the cell plate. To clarify whether or not the RG-II in cell plates are cross-linked with B, any antibody that specifically recognizes either the RG-II dimer or monomer is expected.

As the polyclonal antibody, the anti-RG-II antibody used in this chapter may contain fractions recognizing different epitopes. Both B-RG-II and monomeric RG-II are recognized by the antibody, but the affinity was slightly higher with B-RG-II than with the monomer. The result suggests that the major fraction(s) of the antibody may recognize the structure common to the RG-II dimer and monomer, whereas a part of the immunoglobulins may recognize some

dimer-specific structures. Thus, removing the fractions recognizing both the dimer and monomer may give a dimer-specific fractions. Such a separation may be achieved by an affinity chromatography using a column carrying monomeric RG-II as the ligand; the fractions recognizing both dimer and monomer would be bound to the column, whereas those specific to the dimer would be eluted without binding to the column. The column with immobilized monomeric RG-II should be prepared by carbodiimide-mediated coupling of RG-II to the resin carrying amino groups, as in the case of coupling with amino-modified microtiter plates. Clarifying whether or not the pectin in cell plates is cross-linked with B, or in other words if B-RG-II serves to form higher-order structure of glycans in the very early stage of cell wall formation, would enhance our understanding of the physiological significance of B-RG-II complex.

Chapter 3

Generation of monoclonal antibody against rhamnogalacturonan II and subcellular localization analysis of rhamnogalacturonan II in Arabidopsis roots using the antibody

Introduction

In the previous chapter, RG-II was demonstrated to be present on the forming cell plates in cultured tobacco cells using rabbit polyclonal antibody. However, one caveat of polyclonal antibody is that it is not renewable. On the contrary, monoclonal antibody could be a renewable resource, once the desired hybridoma has been generated (Lipman et al., 2005). Another disadvantage of the polyclonal antibody is that it may recognize multiple epitopes within the antigen, possibly making it difficult to interpret the results. Thus, in this chapter, I describe a generation and characterization of novel monoclonal antibody against B-RG-II, and the immunocytochemical analyses of subcellular localization of RG-II in Arabidopsis roots using the antibody.

Materials and Methods

3.2.1 Plant material

Seeds of *Arabidopsis thaliana* L. ecotype Columbia-0 (Col-0) were planted on half-strength Murashige and Skoog (MS) medium containing 29 mM sucrose and 0.8% (w/v) gellan gum. At 2 and 4 d after planting, the seedlings were transferred to new plates containing 50 and 100 mM sucrose, respectively, and then used for cryofixation at 24 h after transfer to the 100 mM-sucrose medium.

3.2.2 Preparation of antibody

For preparation of the antigen, B-RG-II was isolated from radish root cell walls as described in Chapter 1, and conjugated with mBSA as previously described (Matoh et al., 1998). Immunization of rabbits and the production of monoclonal antibodies were carried out by Abcam (Tokyo, Japan). Three rabbits were immunized with 0.5 mg B-RG-II conjugated with mBSA (B-RG-II-mBSA) and 4 additional injections with 0.25 mg of B-RG-II-mBSA at 3, 5, 7, and 9 weeks after the first injection. Titers of the rabbit sera were assayed with ELISA using a conjugate of B-RG-II and PLL as the immobilized antigen as described in 1.2.2. The spleen was taken from the rabbit whose serum showed the highest titer with the assay and then used for the cell fusion of lymphocytes and partner cells. Positive clones were screened from the pool of hybridomas by assessing the binding affinity of IgGs secreted in the culture

supernatants. One clone secreting IgG with the highest affinity to B-RG-II was selected and subjected to recombinant antibody production.

3.2.3 Competitive ELISA

B-RG-II was covalently linked to the amino-group modified surface of a microtiter plate via carbodiimide coupling as described in 1.2.4. Competitive ELISA was performed as described in 2.2.3, except that 0.5 ng/mL antibody 42-6 in PBST was used as the primary antibody. RG-I was prepared from radish cell wall as described previously (Darvill et al., 1978), except that DEAE-Sepharose FF and HiLoad Superdex columns (GE Healthcare) were used as the media for ion exchange and gel-permeation chromatography, respectively. PGA and esterified pectin, both were prepared from citrus, were obtained from Nacalai Tesque and Sigma-Aldrich, respectively.

3.2.4 Dot-blot analysis

B-RG-II was immobilized on the membrane as described in 1.2.6. The blot was probed with either the rabbit monoclonal or polyclonal anti-RG-II antibody. As a negative control, probing with normal rabbit IgG was also included in the analysis.

3.2.5 High pressure frozen/freeze substitution of Arabidopsis root tips

The following stock solutions were prepared at least 2 days before carrying out the high pressure frozen/freeze substitution experiment: stock solution A, 2% (v/v) GLU dissolved in acetone (stored at –20°C); stock solution B, 32% (v/v) 2,2-dimethoxypropane dissolved in acetone (stored at –20°C). Stock solutions A, B, and acetone were mixed at a ratio of 1:1:2 (v/v) to make the freeze substitution solution.

Roots less than 3 mm in length were cut from seedlings and immersed in 0.2 M sucrose in a petri dish. The roots were transferred onto an aluminum specimen carrier and frozen in a HPM-010 high pressure freezer (Bal-Tec AG, Balzers, Lichtenstein). The frozen samples were immediately transferred into liquid-nitrogen. They were removed from the carrier in liquid nitrogen and transferred to cryovials held in liquid nitrogen.

Two mL of the freeze substitution solution was transferred into 10-mL glass vials, and the vials were transferred to a polystyrene foam container filled with liquid nitrogen. The specimens in liquid nitrogen were poured onto the frozen surface of the freeze substitution solution. The glass vials were stored at –80°C for 4 d to allow for the substitution to proceed. The vials were then gradually warmed at –20°C for 2 h, 4°C for 2 h, and room temperature for 2 h. The specimens were washed for 15 min in acetone 4 times, and then dehydrated in 99.5% ethanol for 15 min 3 times.

The dehydrated specimens were gradually infiltrated with 33% LR White resin (Electron Microscopy Sciences, Fort Washington, PA, USA) in ethanol at room temperature overnight, followed by 50% LR White resin in ethanol at room temperature for 12 h, 75% LR White resin in ethanol at room temperature overnight, and 100% LR White resin at room temperature for 12 h. After transferring the specimens to gelatin capsules, the LR White resin was allowed to polymerize at 60°C overnight.

3.2.6 Immunoelectron microscopy

Ultrathin sections (90–100 nm thick) were cut from the embedded blocks by an Ultracut E microtome (Reichert-Jung, Vienna, Austria). The sections were mounted on nickel grids (Nisshin EM Co., Ltd. Tokyo, Japan; 100 mesh). In some experiments, the grids were covered with a supporting film (0.67% [w/v] formvar, 67% [v/v] chloroform, and 33% [v/v] acetone) before mounting the sections. The sections were blocked by soaking in 50 µL blocking buffer containing 1% (v/v) normal goat serum (Dako, Glostrup, Denmark) and 0.1% (w/v) sodium azide in TBS for 30 min at room temperature. The sections were soaked in 50 µL of antibody 42-6 (0.5–0.7 ng/mL) in blocking buffer for 2 d at 4°C. For the control, IgG from an unimmunized rabbit (Sigma-Aldrich) was used at 3.6 ng/mL instead of the antibody. Labeled sections were washed with blocking buffer for 15 min 3 times. The sections were then incubated at 35°C for 2 h with goat anti-rabbit secondary antibody conjugated to 15-nm colloidal gold (British Biocell International, Cardiff, UK) diluted 100 times in blocking buffer. The sections were washed with blocking buffer for 15 min 6 times, and then once with deionized water for 5 min. Washed sections were stained with 2% uranyl acetate for 10 min and washed thoroughly with deionized water. A JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) operated at 100 kV was used for observations.

3.2.7 Assay methods

Total carbohydrates, 2-keto-3-deoxysugars, and uronic acids were assayed by the phenol-sulfuric acid method (Dubois et al., 1956), the modified thiobarbituric acid method (York et al., 1985), and the *m*-hydroxybiphenyl method (Blumenkrantz et al., 1973), respectively. Identification of neutral sugars were performed by the alditol acetate method as previously described (Kobayashi et al., 1996), using a Shimadzu GC-14A gas-liquid chromatograph equipped with a SP-2330 capillary column (15 m×0.25 mm, SUPELCO). Uronic acids were identified using thin-layer chromatography. Polysaccharide samples were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 1 h, evaporated under an air stream, dissolved in a small amount of water, then spotted on a silica gel plate along with standard monosaccharides. The plate was developed using a mixture of acetonitrile and water (85:15, v/v) as the mobile phase, and the sugar was detected on the plate by spraying 0.5% 1-naphthol in 5% (v/v) sulfuric acid followed by heating.

Results and discussion

3.3.1 Production of rabbit monoclonal antibody against B-RG-II

As a relatively small hydrophilic polysaccharide fragment, RG-II is not an efficient immunogen. Hence, a conjugate of B-RG-II with mBSA was prepared as previously described (Matoh et al., 1998) and immunized three rabbits with the conjugate. Rabbits were selected as host animal as their immune system shows better response to diverse small molecules than those of mice and other rodents. Antisera recognizing B-RG-II were indeed obtained from multiple individuals, as were the cases in previous polyclonal antibody production in our laboratory (Matoh et al., 1998 and Chapter 2), while trials to start from mice was failed because the titer was not increased after immunization (data not presented). Lymphocytes taken from a

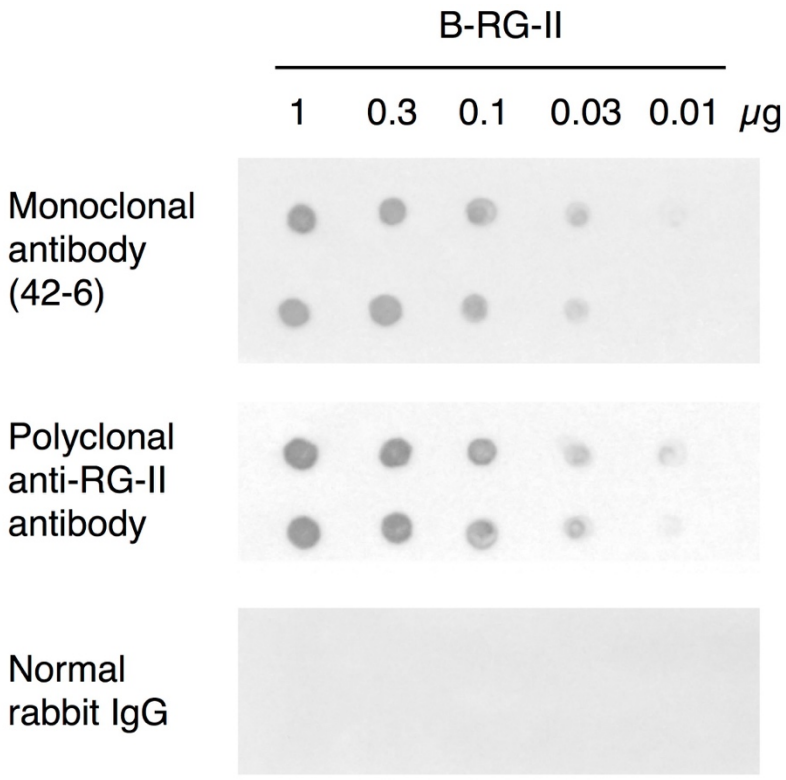

Figure 3.1 Reactivity of monoclonal antibody with B-RG-II. A positively charged nylon membrane was spotted with 1 µL of 20 mM Tris-HCl (pH 8.0) containing various amounts of B-RG-II in duplicate, and then subjected to chemiluminescent immunodetection with anti-RG-II antibodies. Monoclonal antibody (42-6), the recombinant rabbit monoclonal antibody used at 1 ng/mL; polyclonal rabbit antibody used at 2 µg/mL; normal rabbit IgG, immunoglobulin G from unimmunized rabbit used at 2 µg/mL.

rabbit individual whose serum showed the highest reactivity against B-RG-II were used for hybridoma production. Screening of the hybridoma pool identified at least three clones secreting B-RG-II-reactive immunoglobulins into their culture supernatants, and the one with the highest reactivity, named 42-6, was selected for the recombinant antibody production.

The reactivity of 42-6 with B-RG-II was confirmed by dot-blot analysis. As shown in Figure 3.1, the 42-6 antibody recognized B-RG-II with a sensitivity comparable to that of the rabbit polyclonal anti-RG-II antibody. However, the amount of antibody required to get signals were much less than that of our conventional rabbit polyclonal antibody. The result of ELISA shown as Figure 3.2 indicates that strong signals could be obtained with less than 1 ng antibody/mL of solution added to the wells, which is much lower than the concentration used in the case of the polyclonal antibody (Fig. 1.3). The higher efficiency might be due in part to the uniform composition of the monoclonal antibody, which is different from complicated composition of the polyclonal antibody containing the fractions recognizing mBSA moiety of the conjugate rather than B-RG-II.

The reactivity of the 42-6 antibody with several pectic polysaccharides was examined using competitive ELISA. B-RG-II was covalently cross-linked via carbodiimide coupling to the

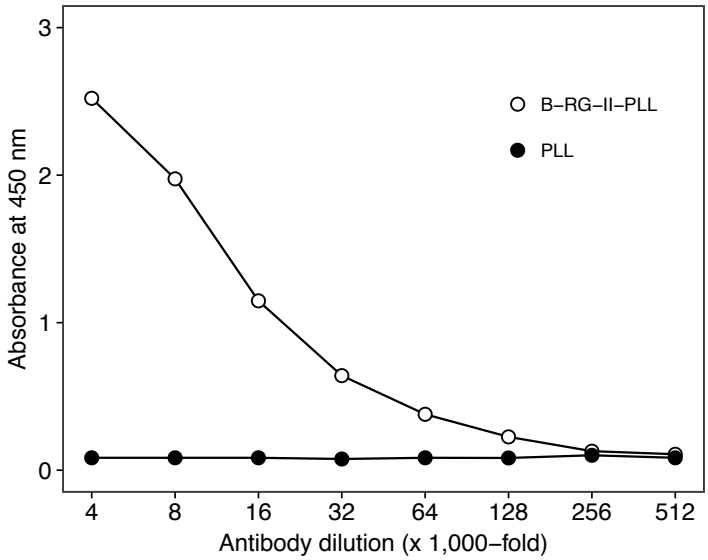

Figure 3.2 Titration of rabbit monoclonal anti-RG-II antibody. Microtiter plates were coated with either B-RG-II-PLL conjugate (open circles) or PLL alone (closed circles). The amount of antigen used for immobilization was 6.25 µg total B-RG-II-PLL (including both PLL-bound and unbound form) or 6.25 µg PLL per well. The wells were applied with two-fold dilution series of the rabbit monoclonal anti-RG-II antibody, prepared from the stock solution of 1.78 µg IgG/mL. The antibody bound to the wells were quantified using peroxidase-mediated color reaction.

plate modified with amino groups, and the 42-6 antibody was then allowed to bind to the immobilized B-RG-II in the presence of competitor. Figure 3.3 shows a representative result of the assay. Both B-RG-II and RG-II monomer not cross-linked with a borate diester linkage decreased the ELISA signal in a dose-dependent manner. This result indicated that the 42-6 antibody recognized both dimeric RG-II cross-linked with B and RG-II monomers, with slightly higher affinity to the dimer. This selectivity was similar to those observed with the rabbit polyclonal anti-RG-II antibodies previously raised in our laboratory. The amount of B-RG-II required to reduce the signal to the half of maximum level was around 1 µg/mL, confirming the result of Figure 3.1 that the sensitivity of the 42-6 was comparable to that of our conventional rabbit polyclonal anti-RG-II antibody (Fig. 2.2). Neither PGA nor esterified pectin worked as effective competitors. PGA corresponds to polygalacturonic acid with a low degree of methyl esterification in their carboxylic acid groups, whereas more than 85% of carboxylic acid groups are methyl-esterified in the esterified pectin preparation used in this assay. The ELISA signal was slightly decreased in the presence of 1 mg/mL pectin. This may be due to the interaction of the antibody with the RG-II region contained within the pectin macromolecule. However, such a decrease in signal by pectin was not very reproducible; therefore, at present, the possibility that, the change was due to some non-specific interference of antibody binding to the plate caused by the high concentration of the polysaccharide, could not be excluded. Another pectic polysaccharide, RG-I, also did not decrease the ELISA signal. These results suggest that the 42-6 antibody specifically recognizes RG-II.

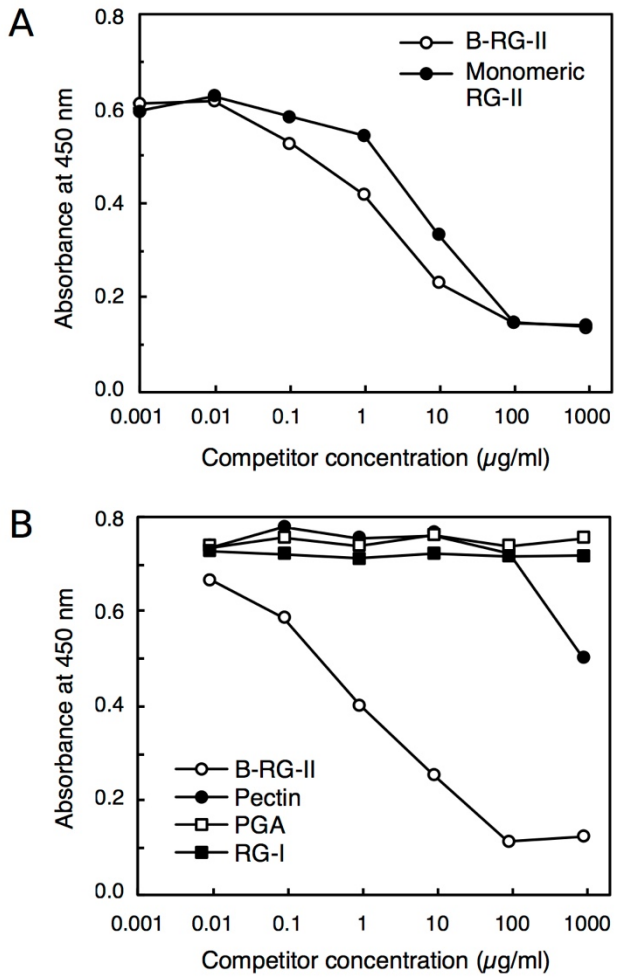

Figure 3.3 Specificity of rabbit monoclonal antibody. Competitive ELISA to examine the reactivity of the 42-6 antibody against various pectic polysaccharides. Antibody bound to plate wells was quantified using a peroxidase-conjugated secondary antibody and TMB reagent kit. Shown are representative results from at least four reproducible, independent experiments. A, comparison of the reactivity toward B-RG-II and RG-II monomer. B, examination of reactivity with various pectic polysaccharides.

The reactivity of the 42-6 antibody was further examined against various pectic fragments generated by pectinase digestion of cell walls. Figure 3.4 shows the separation of a pectinase digest of radish cell walls using a DEAE-Sepharose column, through which B-RG-II was purified. Fractions were assayed for total sugars and 2-keto-3-deoxysugars as a diagnostic sugar of RG-II, and the fractions containing major peaks were subjected to a competitive ELISA analysis. It has been reported previously that separation of pectinase digest of cell walls on DEAE-Sepharose resulted in three peaks that were positive in the 2-keto-3-deoxysugar assay (Kobayashi et al., 1997); peaks I, II, and III eluted at approximately 0.05, 0.1, and 0.15 M Cl^-, respectively. Peaks II and III contained the RG-II monomer and B-RG-II, respectively. Peak I contained oligosaccharides, but its identity as the 2-keto-3-deoxysugar-containing side chains of RG-II has not been established. Then, from the chromatogram shown in Fig. 3.4A, fractions 62, 72, and 83 were taken as those corresponding to peak I, II, and III, respectively. Fractions

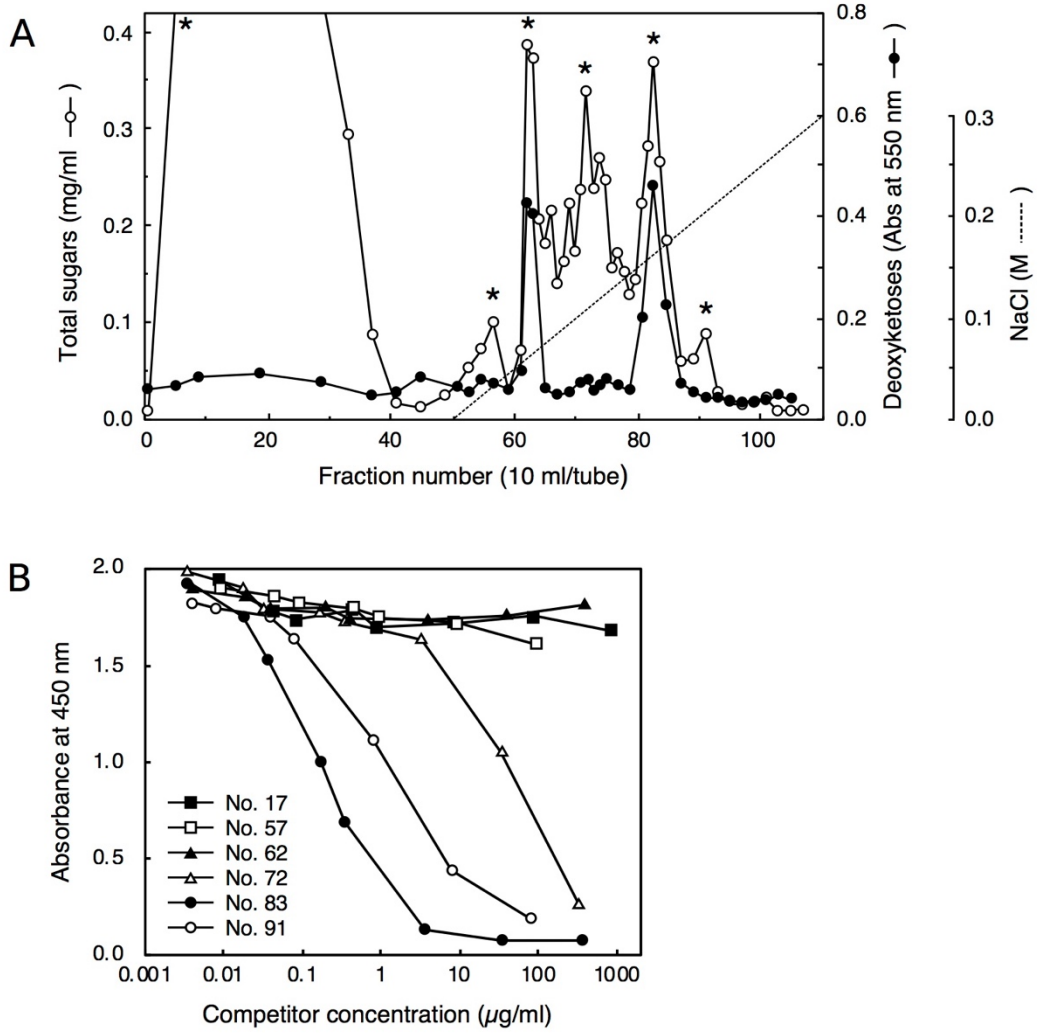

Figure 3.4 Examination of antibody reactivity toward pectic fragments generated by pectinase digestion of radish cell walls. Radish cell walls were digested with pectinase and the digest was applied to a column of DEAE-Sepharose (Cl^- form, 16×60 mm) equilibrated with column buffer (20 mM Tris-HCl, pH 8.0). After the unbound material was washed with the column buffer, the bound material was eluted using a linear gradient of 0–0.5 M NaCl (0.5+0.5 L) in the column buffer. Fractions were assayed for total sugars and 2-keto-3-deoxysugars to generate a chromatogram (A), and fractions containing major peaks (indicated with asterisks) were subjected to competitive ELISA analysis (B).

72 and 83, respectively, containing the RG-II monomer and B-RG-II, reduced the ELISA signal as expected (Fig. 3.4B). Fraction 62 did not act as a competitor, which may be because the 2-keto-3-deoxysugar-containing side chain of RG-II, if contained in the fraction, is not the epitope recognized by the 42-6 antibody. Besides these fractions, fractions 17 and 57 without detectable amounts of 2-keto-3-deoxysugars were not reactive with the 42-6 antibody. On the other hand, fraction 91, which did not contain 2-keto-3-deoxysugars, showed activity to reduce the ELISA signal. To examine the possibility that the 42-6 antibody-reactive substance is a part of RG-II devoid of 2-keto-3-deoxysugars, fraction 91 was analyzed for its glycosyl residue composition. As summarized in Table 3.1, the fraction contained rhamnose, xylose, fucose, and uronic acids

Table 3.1 Glycosyl residue composition of polysaccharide in fraction 91

Monosaccharide	molar ratio[a]
	%
Uronic acid[b]	40
Rhamnose	33
Xylose	15
Fucose	7
Galactose	2
Glucose	2
Arabinose	1

[a] % of sugars detected

[b] determined as galacturonic acid equivalent

as the major component monosaccharides. At least a part of the uronic acids was galacturonic acid, as revealed by a thin-layer chromatography analysis (Figure 3.5). The 42-6 antibody-reactive substance was unlikely to be an oligosaccharide, because the reactivity was recovered in a high-molecular weight fraction upon fractionation on a desalting column (data not presented). RG-II contains rhamnose, fucose, and galacturonic acid, but not xylose (O'Neill et al., 2004). In addition, if the substance is a partially degraded RG-II lacking several residues or side chains, it should still contain apiose as the residue connecting the backbone and rhamnose- or fucose-containing side chains (O'Neill et al., 2004). However, apiose was not detected in fraction 91. Hence, the 42-6-reactive substance in fraction 91 was not considered to be derived from RG-II.

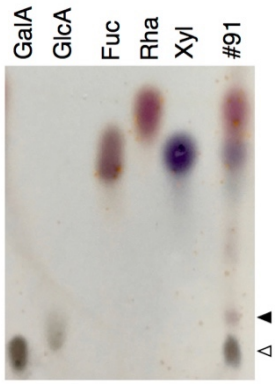

Figure 3.5 Thin-layer chromatography separation of acid hydrolysate of polysaccharide in fraction 91. Lanes GalA, GlcA, Fuc, Rha, and Xyl were spotted with authentic D-galacturonic acid, D-glucuronic acid, L-fucose, t-rhamnose, and D-xylose, respectively. Lane #91 was spotted with acid hydrolysate of the polysaccharide contained in fraction 91 after separation on ion exchange column (Fig. 3.4). White and black arrowheads denote the spot of D-galacturonic acid and an unidentified substance, respectively.

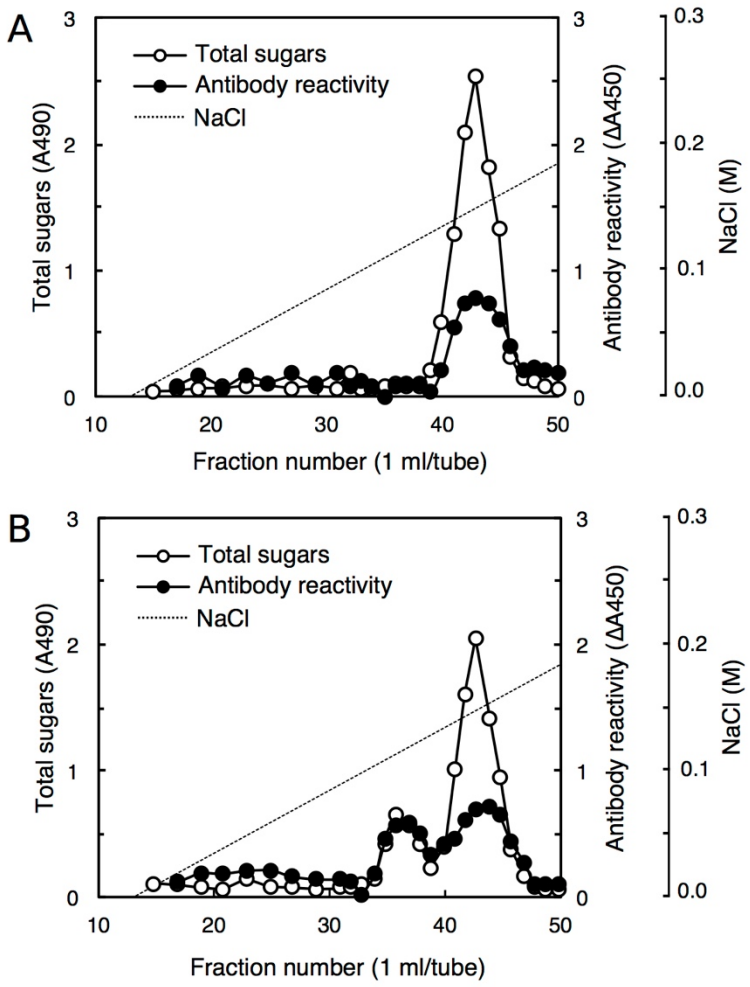

Figure 3.6 Peak shifts for rhamnogalacturonan II (RG-II) and antibody reactivity. Borate-RG-II complex without (A) or with (B) acid treatment was separated on a DEAE-Sepharose column (10×100 mm), and the fractions were subjected to total sugar analysis for RG-II and competitive ELISA for antibody reactivity. For the antibody reactivity, the decrease in absorbance at 450 nm (ΔA450) caused by the addition of each fraction was plotted.

As fraction 91 was eluted immediately after B-RG-II, it cannot be excluded that the B-RG-II preparation was contaminated with the 42-6 antibody-reactive substance in fraction 91. This notion raises the concern that the apparent reactivity with RG-II (Fig. 3.1-3.3) might be due to the substance that peaked at fraction 91 but not to B-RG-II itself. To address the issue, I assessed whether the treatment resulting in the RG-II peak migration also induced the shift in the antibody-active fractions. Firstly, DEAE-Sepharose column chromatography of the B-RG-II preparation was performed, and the 42-6 antibody reactivity of each fraction was assessed using competitive ELISA. Peaks around fraction 43 exhibited a positive reaction with the 42-6 antibody as expected (Figure 3.6A). The B-RG-II preparation was treated with 0.1 M HCl for 10 min at room temperature and separated the sample on the same DEAE-Sepharose column. The acid treatment hydrolyzed borate diester linkages between two RG-II chains, resulting in the generation of RG-II monomers that could be eluted earlier than B-RG-II (Kobayashi et al., 1997) as shown in Figure 3.6B. The newly generated peak around fraction 35 was also reactive with the 42-6 antibody (Figure 3.6B). As the acid treatment used here was mild and unlikely to

break glycosidic linkages or other polysaccharide modifications including methyl-esterification or acetylation, contaminating polysaccharides in the preparation, if any, would not change its elution volume as B-RG-II did. Thus, the shift due to the 42-6 antibody reactivity observed here indicates that the 42-6 antibody indeed recognizes RG-II, even if it may not be reactive to this polysaccharide only.

To further increase the usability of the 42-6 antibody, clarifying its epitope structure is mandatory. Therefore, it would be informative to compare the structures of RG-II and the 42-6-reactive polysaccharide in fraction 91 (Fig. 3.4). To my knowledge, the glycosyl residue composition of fraction 91 (Table 3.1) does not coincide with that of any plant cell wall polysaccharides known so far. For example, RG-I is rich in galacturonic acid and rhamnose, but it does not contain xylose as a major constituent (Ridley et al., 2001). The molecular entity of the substance in question is thus not clear at present, warranting further investigation on its origin and structure.

3.3.2 Immunoelectron microscopy using rabbit monoclonal anti-RG-II antibody

Having confirmed the recognition of RG-II by the 42-6 antibody, this antibody was employed for immunoelectron microscopy to analyze the subcellular localization of RG-II. In Chapter 2, it was found that the rabbit polyclonal anti-RG-II antibody bound to developing cell plates in suspension-cultured tobacco cells, suggesting that the RG-II domain of pectin was already present at an early stage of cell wall assembly. Then in this chapter, I investigated whether similar results could be obtained with the new rabbit monoclonal antibody, using root tips of Arabidopsis seedlings as the plant material. The delicate membrane networks that are produced via vesicle fusion during the cell plate formation are sensitive to chemical fixation techniques (Samuels et al., 1995). Gilkey et al. (1986) first introduced the high-pressure freezing substitution methods for preserving cells. This method could help to preserve the cellular ultrastructure, and it could capture and stabilize transient cell plate and phragmoplast structures (Seguí-Simarro et al., 2004). Thus, in this study, the structure of Arabidopsis root tip cells was fixed by high pressure freezing, then probed with 42-6 antibody to localize RG-II.

Ultrathin sections were excised from Arabidopsis roots that were fixed using a high pressure frozen/freeze substitution technique, probed with the 42-6 antibody, and observed under a transmission electron microscope. Gold-conjugated secondary antibodies were used to visualize the antibodies bound to the section. Figure 3.7 shows the cells in root tip of Arabidopsis seedlings. Observed in the central part of Figure 3.7A is a cell undergoing cytokinesis. Treated with the 42-6 antibody, gold particles were found in cell walls as shown in Figure 3.7B, whereas such a signal was not detectable in sections treated with preimmune serum (Figure 3.7D). The labeling with rabbit polyclonal anti-RG-II antibody was previously reported to be found preferentially at the membrane-proximal region of the cell wall (Matoh et al., 1998), whereas such a gradient distribution of signals across the wall was not obvious with the 42-6 antibody (Figure 3.7B). On the other hand, the average densities of labeling found in longitudinal walls were significantly higher ($p < 0.01$) than that in transverse walls (Figure 3.8). The difference suggests the presence of heterogeneity in the cell wall microstructure within a single cell. Alternatively, the density of RG-II in longitudinal walls may be transiently higher while the cells are young and longitudinally short, but it may decrease to a similar level to that

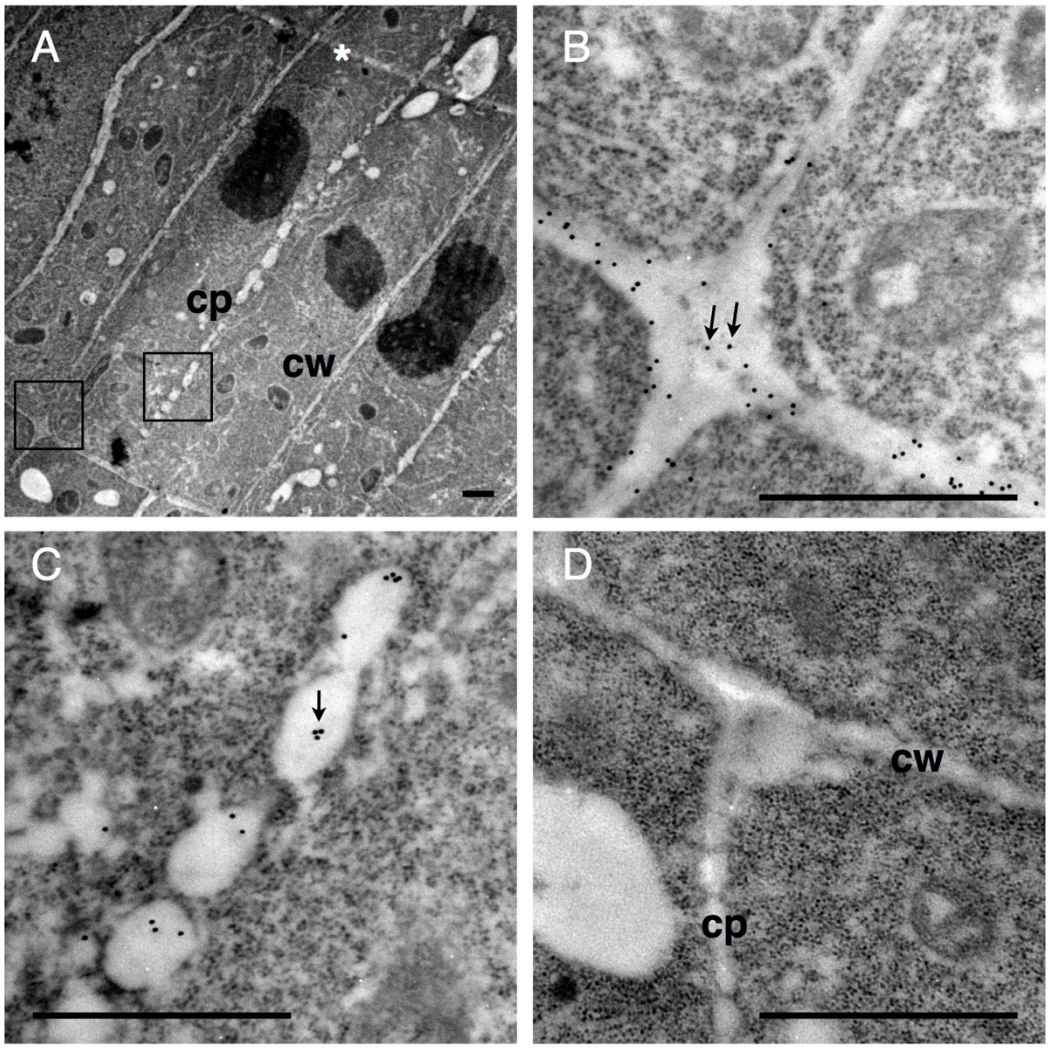

Figure 3.7 Immunogold labeling of RG-II in dividing cells in Arabidopsis roots. (A–C) Section incubated with the 42-6 primary and gold-conjugated secondary antibodies. (D) Section incubated with normal rabbit immunoglobulin and gold-conjugated secondary antibody. (B) and (C) are higher magnifications of squares drawn in the left and middle part in (A), respectively. Arrows in (B) and (C) show representative gold particles. Larger and less defined dots are ribosomes. A line in the upper-right corner in (A), as indicated by the asterisk, is an artificial scratch generated during the section preparation. cp, cell plates; cw, cell walls. Bar = 1 μm.

in transverse walls as the cells elongate. Further analysis of labeling density in the walls of elongated cells is necessary. The cell in Figure 3.7A has electron-lucent luminal structures aligned between two daughter nuclei. The structures are considered to be the cell plate at the fenestrated sheet stage, which corresponds to the later phase of cell plate formation (Samuels et al., 1995). In the sections treated with the 42-6 antibody, gold particles were found in these structures (Fig. 3.7C), whereas such labeling was not observed in the section treated with preimmune serum (Fig. 3.7D). The result indicates that RG-II is present in forming cell plate.

The labeling with the 42-6 antibody was also observed in cell plates at an earlier developmental stage. In Figure 3.9A, a developing cell plate could be observed in the center of the cell, with a cluster of vesicle-like structures at the edge. The edge was still distant from the

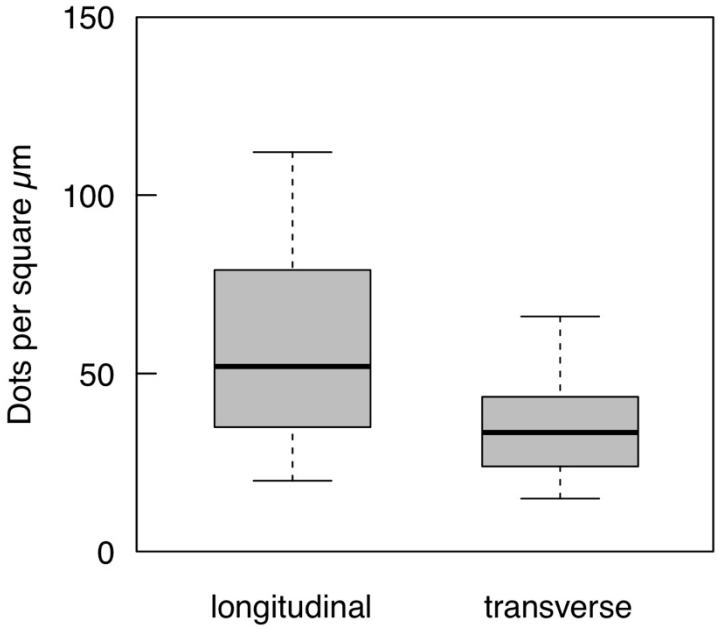

Figure 3.8 Quantification of the 42-6 antibody immunogold labeling density. Boxplot representing the number of gold particles per square micrometer after immunogold labeling of RG-II in longitudinal and transverse cell walls. Gold particles were counted in 20 micrograph images for each direction and divided by the area measured using the ImageJ software (Schneider et al., 2012). The micrographs used represent 17 independent cells from three seedlings. Box encompasses the 25–75 percentiles with the solid line indicating the median. Whiskers show the 10–90 percentiles.

walls of parental cells (Figure 3.9A). Based on these morphologies, the cell plate was considered at the tubular network stage. Gold labeling was found on this cell plate in both the vesicular structure in the edge region and the central luminal structures (Figure 3.9B). These results together suggest that RG-II appears in the cell plate by the tubular network stage, which corresponds to mid-telophase of cytokinesis (Samuels et al., 1995). The assumption is consistent with the conclusion obtained using rabbit polyclonal anti-RG-II antibody and suspension-cultured tobacco cells in Chapter 2.

This study has demonstrated that the developing cell plates contain RG-II. The finding raises the possibility that the pectin in cell plates is cross-linked with B at RG-II regions as in the case of pectin in primary cell walls. However, since the antibodies employed in this study do not distinguish the dimeric RG-II cross-linked with B and the monomeric RG-II, immunomicroscopic observation alone cannot tell whether or not the RG-II on cell plates are indeed cross-linked with B. Methods to isolate phragmoplasts from dividing cells has been established in tobacco BY-2 cells. Although direct structural analysis of RG-II in phragmoplasts would be difficult because of limitation of the amount available, combination of appropriate fractionation of the dimer and monomer and the sensitive detection of RG-II with 42-6 antibody would make it possible to judge whether or not the RG-II on developing cell plates are cross-linked with B. The fractionation of the two forms would be possible on either size-exclusion or anion exchange column as shown in Fig. 2.1 or Fig. 3.6.

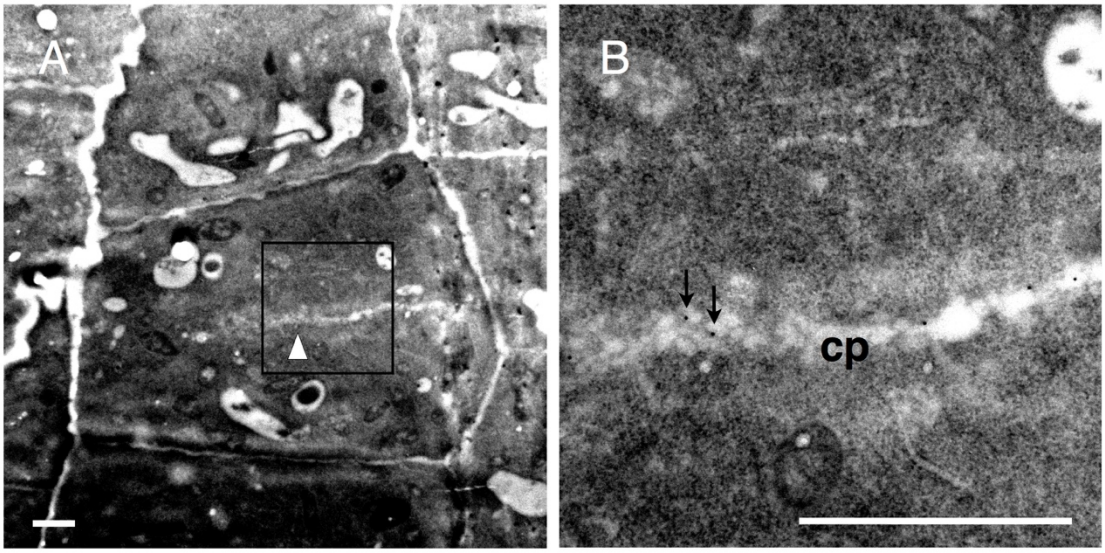

Figure 3.9 Immunogold labeling of rhamnogalacturonan II in root cells at an earlier stage of cell division. (A) A dividing cell with a developing cell plate, which is presumably at the tubular network stage (white arrowhead). (B) Higher magnifications of the square in (A). Arrows in (B) show representative gold particles. cp, cell plate. Bar=1 μm.

3.3.3 Merit of monoclonal antibody in RG-II research

The monoclonal antibodies are usually raised in mice or rats. However, in our hand, B-RG-II-KLH conjugate did not stimulate immune responses in mice, thus mouse monoclonal antibodies against B-RG-II could not be produced (data not presented). Techniques to produce monoclonal antibodies from rabbits were developed recently, then found to be useful to obtain antibodies recognizing antigens and epitopes which were not immunogenic in rodents (Spieker-Polet et al., 1995). The basic principle for preparing a rabbit monoclonal antibody is the same as that for mouse or rat monoclonal antibodies. Production of rabbit monoclonal antibodies was difficult since a fusion partner for plasmacytoma was not available (Spieker-Polet et al., 1995). Mouse-rabbit heterohybridomas were unstable and difficult to be cloned (Raybould and Takahashi, 1988; Verbanac et al., 1993). Spieker-Polet et al. (1995) developed a plasmacytoma fusion partner from a *myc/abl* double transgenic rabbit. By using this plasmacytoma fusion partner, they successfully established plasmacytoma cell lines, thus stable rabbit-rabbit hybridomas could be produced. By virtue of this technique, the production of monoclonal antibody against RG-II became available.

Monoclonal antibodies can label plant cell wall glycans with high sensitivity through the specific recognition of oligosaccharide structures, thus have been used to detect the structural changes in cell walls during the process of cellular and tissue development (Cornuault et al., 2015; McCartney et al., 2005; Meikle et al., 1994; Puhlmann et al., 1994; Ralet et al., 2010; Shinohara et al., 2015; Torode et al., 2015; Willats et al., 1998). Using multiple monoclonal antibodies against plant cell wall polysaccharides, a microarray to probe the presence of specific glycan was made, and it has been applied to the attempt of typing cell wall structures based on multivariate analysis approach (Leroux et al., 2015; Sousa et al., 2015). Until now, however, monoclonal antibody against RG-II has not been used in such researches. With the identification of epitope structure, the 42-6 antibody established in this study would be useful

in those studies to gain insights into the structure and physiological function of pectin in primary cell walls.

Another possible application of the 42-6 monoclonal antibody is to track the dynamic change in B-RG-II structure upon B deprivation. Previous studies in our laboratory show that the deprivation of B from the culture medium induces immediate responses and damages in plant cells (Kobayashi et al., 2017; Koshiba et al., 2010, 2011, 2013; Oiwa et al., 2013). In Arabidopsis roots, cells in root elongation zone are killed within 1 h of B deprivation, probably due to the overproduction and accumulation of reactive oxygen species. One can assume that the immediate response should be somehow related to the malformation of B-RG-II cross-linking. However, it is still unknown if the B deprivation for such a short term indeed affects the properties of RG-II, including the structure, secretion, distribution within the cell, or rate of cross-linking. Immunocytochemical inspection of B-deprived Arabidopsis roots using the 42-6 antibody will give us useful information on the behavior of RG-II associated with the immediate –B responses.

Conclusion

RG-II is a region of pectin present in the plant primary cell walls. RG-II serves as the site of cross-linking with borate diester. The cross-linking lets pectin form a hydrophilic gel with various putative physiological functions. The B-RG-II is therefore the structure critically important for the pectin function. However, still only limited information is available on the formation and distribution of the B-RG-II complex. Thus, in this study I performed an immunocytochemical analysis of the subcellular localization of RG-II, with special attention to the occurrence in cell plates as the precursor of primary cell walls.

In Chapter 1, as the method necessary in production and characterization of the antibody used in the subsequent chapters, protocols to immobilize B-RG-II on solid surfaces were established.

In Chapter 2, using a rabbit polyclonal anti-RG-II antibody previously obtained in our laboratory, subcellular localization of RG-II in suspension-cultured tobacco cells was investigated. The antibody labelled the cell walls and a cell plate-like intracellular structure of dividing cells. The identity of the labeled intracellular structure as the cell plate was confirmed by the colocalization with callose and microtubules. The labeling could be observed in the cell plates at the early stage of formation.

In Chapter 3, a new rabbit monoclonal antibody was produced, then using the antibody an immunoelectron microscope analysis was performed on the cells in Arabidopsis root tips. Consistent with the results in Chapter 2, the cell plates were labeled, even at the early stage of their formation.

Taken these results together, I conclude that RG-II occurs in the forming cell plates. The finding suggests that the pectin in cell plates can be cross-linked with B-RG-II complex as in the case of primary cell walls. A new rabbit monoclonal antibody prepared in this study would reveal the secret of RG-II, such as specific localization in the elongating cell walls.

Acknowledgements

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In this thesis, four years of my study were summarized.

I would like to express my sincere gratitude to Professor Toru Matoh, who always encourages me to fight with the difficulties in the research. He encouraged me to move on, and give me so much help in my study and my daily life, so I could not feel the difficulties as a foreigner.

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