Immunogold Localization of Xyloglucan and Rhamnogalacturonan I in the Cell Walls of Suspension-Cultured Sycamore Cells

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ABSTRACT
Plant cell walls serve several functions: they impart rigidity to the plant, provide a physical and chemical barrier between the cell and its environment, and regulate the size and shape of each cell. Chemical studies have provided information on the biochemical composition of the plant cell walls as well as detailed knowledge of individual cell wall molecules. In contrast, very little is known about the distribution of specific cell wall components around individual cells and throughout tissues. To address this problem, we have produced polyclonal antibodies against two cell wall matrix components: rhamnogalacturonan I (RG-I), a pectic polysaccharide, and xyloglucan (XG), a hemicellulose. By using the antibodies as specific markers we have been able to localize these polymers on thin sections of suspension-cultured sycamore cells (Acer pseudoplatanus). Our results reveal that each molecule has a unique distribution. XG is localized throughout the entire wall and middle lamella. RG-I is restricted to the middle lamella and is especially evident in the junctions between cells. These observations indicate that plant cell walls may have more distinct chemical (and functional?) domains than previously envisaged.

The functional properties of cell walls are determined both by their chemical composition and by the three-dimensional arrangement of these components. Thus, knowledge of cell wall structure is essential to understanding cell wall function. The primary cell wall of higher plants consists of a framework of cellulose microfibrils embedded in an amorphous matrix. The two major classes of molecules in the matrix are hemicelluloses and pectic polysaccharides (29). Adjacent cell walls are separated by an intercellular layer, often referred to as the middle lamella, which appears to be continuous with the matrix of the primary cell wall. In recent years significant progress has been made in studies of the constituents of plant cell walls. Advances have been made in the fractionation, purification, and characterization of fragments of the hemicelluloses and pectic polysaccharides, and in the elucidation of the detailed molecular structures of portions of these molecules (19). XG is a well-characterized hemicellulose with a β-1,4-linked glucosyl backbone. XG hydrogen-bonds to cellulose microfibrils (30), and probably limits self association between cellulose microfibrils, and provides sites through which cellulose microfibrils can be anchored into the covalently cross-linked meshwork of matrix molecules (19). RG-I, a highly branched pectic polysaccharide, contains a backbone of L-rhamnosyl and D-galactosy luronic acid residues (19).

Although structural information on individual molecules is essential, it provides only a partial picture of the plant cell wall. A complete understanding of the structure of cell walls also requires knowledge of how the polymers are attached to one another and how the polymers are physically arranged throughout the wall. The latter has been studied in the past by a variety of histochemical techniques, for example: (a) by selectively staining for classes of polysaccharides (2), (b) by selectively extracting components from the cell walls using chemicals or enzymes and studying what remains (7, 25, 26), and (c) by localizing the substrates of wall-degrading enzymes using enzyme-colloidal gold markers (27).

Pectic polysaccharides were first localized in cell walls of onions by means of chemical reactions which convert pectins to electron dense complexes (2). Reaction product was concentrated in the middle lamellae, some was near the surface of young primary walls, and the lowest density within the rest of the cell wall. Whether the lowest density of iron deposits was due to a lower wall concentration of pectins or the presence of pectic polysaccharides with a low proportion of pectin methyl esters could not be determined.

Roland et al. (26) reported that rhyozone (a 'hemicellulose') extraction of pea root and mung bean hypocotyl cell walls produces a general extraction of primary cell walls and a concomitant disorganization of cellulose microfibrils, but has no effect on the intercellular layer, suggesting that hemicelluloses are involved in stabilizing the organization of the cell wall microfibrils but are absent from the middle lamella. Deschande (7) found that pectinase treatment of petiole collenchyma caused cells to separate at the intercellular layer and layers of microfibrils to separate within the cell walls, consistent with the notion that pectins serve as 'glue' between adjacent cells as well as cell wall layers. The results of these experiments must be interpreted cautiously, however, since neither of the enzyme preparations were well characterized, and in neither study were the reaction products analyzed.

Roland and Vian (25) have used endopolygalacturonase digestion of mung beans hypocotyls to show that pectins are not evenly distributed throughout the cell wall. In contrast to the enzyme preparations used in earlier studies, endopolygalacturonase is a highly purified enzyme which digests oligogalacturonic acid domains in pectic polysaccharides (8). Roland and Vian

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(25) found an inner zone of the cell wall in their specimens to be resistant to digestion. This suggested that microfibrils are initially deposited in the cell wall with no pectic polysaccharides around them, or that pectins are initially esterified and are not recognized by endopolygalacturonase. Unexpectedly, the middle lamella was also found to be resistant to digestion by this enzyme. This insensitivity was again attributed to esterification of the pectins, since an earlier cytochemical study had shown a concentration of methyl ester sites in the intercellular layer (24). However, no analysis of released products was carried out.

A more direct method for studying the location of cell wall components is the use of enzyme-colloidal gold complexes to label thin sections. Ruel and Joseleau (27) employed xylanase-gold complexes to demonstrate that xylans are concentrated in the primary walls of parenchyma cells of the reed *Arundo donax* but are absent from the middle lamella and corners between cells.

The current study employs polyclonal antibodies raised against purified XG and RG-I in conjunction with colloidal gold-labeled secondary antibodies to localize these cell wall molecules in thin sections of suspension-cultured sycamore cells. Sycamore cells were selected for this study because our antibodies were produced against molecules derived from such cells.

**MATERIALS AND METHODS**

**Plant Material.** Suspension-cultured sycamore cells (*Acer pseudoplatanus*) were grown in M6PT medium (33) at 25°C and transferred to fresh medium once every 7 d. Cells were harvested for use 10 d after transfer.

**Preparation of Antibodies.** Antibodies were raised against purified RG-I, prepared according to McNeil et al. (18). Rabbits were immunized with RG-I by the method of Vreeeland (31) modified as follows: 0.084 mg RG-I and 0.084 mg methylated BSA (Sigma Chemical Co.) were mixed, emulsified in Freund’s adjuvant, and injected subcutaneously in six sites along the back. Rabbits were boosted with the same amount of material after 1 and 2 weeks and again at 9 weeks. Sera were tested for reactivity with antigen at the time of each injection. We detected antibodies 12 weeks following the initial injection.

Purified XG, prepared according to Bauer et al. (3), was coupled to a protein carrier, ovalbumin (Sigma Chemical Co.), by the periodate-lysine fixation method of McLean and Nakane (17). Excess fixative was removed by overnight dialysis against 50 mM Na-phosphate (pH 7.4). The XG-ovalbumin antigen was emulsified in Freund’s adjuvant and injected as above. Sera were tested at the time of each boost. We first detected antibodies 8 weeks after the initial injection.

Immunoglobulins were isolated from the sera by (NH₄)₂SO₄ precipitation (16).

Antisera from rabbits injected with either the RG-I-methylated BSA or the XG-ovalbumin complex contained antibodies against the protein carriers which had to be removed before use. To remove antibodies against methylated BSA, antisera from rabbits injected with RG-I-methylated BSA was preabsorbed against an equal volume of 0.5 mg/ml methylated BSA for 4 h, and the resultant immunoprecipitate was removed by centrifugation in a Beckman Microfuge. Antibodies against ovalbumin in the XG-ovalbumin antisera were removed by preabsorbing an equal volume of antisera and 10 mg/ml ovalbumin overnight before use.

**Screening of Antibodies.** Antibodies were screened using a Bio-Rad Immunoblot (goat anti-rabbit IgG horseradish peroxidase conjugate, GAR-HRP) Assay kit. Antigen (200 ng) in a 2 µl drop was dotted onto nitrocellulose paper (Amersham Corp.), Gelatin (3%) in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) was used to block nonspecific protein binding to the nitrocellulose paper. The paper was then incubated in immune serum (1:10 in 1% gelatin in TBS) for 2 h. After washing in TBS, blots were incubated in GAR-HRP (1:3000 in 1% gelatin) for 1 h. Following a TBS wash, antibody binding was detected by HRP substrate color development.

**Preparation for Microscopy.** Clumps of sycamore cells were fixed in 2.5% glutaraldehyde in M6PT medium for 15 h at 4°C. Cells were postfixed in 1% OsO₄ in 20 mM K-phosphate (pH 6.0) for 1 h on ice. After postfixation cells were washed in cold buffer and dehydrated through 30, 50, 70, 95, and 100% ethanol (each 10 min per step). The cells were then embedded in LR White resin (London Resin Co., Ltd.) and polymerized overnight at 60°C. Silver to gold sections were cut on a Reichert Ultracut, and after antibody labeling were viewed with a Hitachi H600 electron microscope.

**Antibody Labeling.** Thin sections were picked up on formvar/ carbon coated nickel grids (300 mesh). The sections were treated with 0.1 N HCl for 10 min to remove glutaraldehyde from the sections in order to expose antigenic sites (5). The grids were then incubated on a blocking solution of 10 mg/ml BSA (fatty acid free, Sigma Chemical Corp.). Next the sections were incubated in primary antisera diluted 1:10 in PBST (10 mM Na-phosphate, 500 mM NaCl, 0.1% Tween 20) for 30 min, washed with a continuous stream of PBST for 1 min, and incubated with protein A-gold (10 nm) diluted 1:20 in PBST for 20 min. Excess protein A-gold was removed from the grids by rinsing with PBST followed by a distilled H₂O wash. Following immunolabeling, sections were stained with 2% aqueous uranyl acetate for 5 min. All steps were carried out at room temperature.

**Immunocytochemical Controls.** To test the specificity of labeling, sections were labeled with antisera preabsorbed with their respective antigens. Equal volumes of anti-RG-I sera and 1 mg/ml RG-I or anti-XG and 1 mg/ml XG were incubated overnight before use in immunolabeling. To be certain that any loss of immunostaining was due to antigen binding, not the preabsorption treatment, both antisera were also preabsorbed against an equal volume of 1 mg/ml purified extensin, a cell wall glycoprotein. To determine whether staining was specific for carbohydrate moieties, some sections were treated with saturated sodium metaperiodate (NaO₂) for 10 min before immunostaining. Grids were also incubated with protein A-gold alone.

**RESULTS**

**Specificity of the Antibodies.** Sera from rabbits immunized with the RG-I-methylated BSA conjugate reacted with RG-I and methylated BSA. Complete removal of the antimethylated BSA was achieved by immunoprecipitation with methylated BSA (data not shown). Subsequent testing against the cell wall polysaccharides rhamnogalacturonan II (RG-II; another pectic polysaccharide) and XG revealed no cross-reactivity to these polymers (Fig. 1). A low level of reactivity with the cell wall glycoprotein extensin was observed in both the immune and preimmune sera (Fig. 1, A and B).

Rabbits immunized with XG-ovalbumin produced antibodies that recognized XG, but also showed a strong reactivity to ovalbumin. The ovalbumin reactivity was eliminated by preabsorbing the antisera against a large excess of ovalbumin (data not shown). The anti-XG antibodies were specific for XG when tested against cell wall components RG-I, RG-II, and extensin, except for minor cross-reactivity with extensin which was also present in the preimmune sera (Fig. 1, C and D).

**Immunostaining of Suspension-Cultured Sycamore Cells.** The antibodies specific for RG-I exclusively labeled the intercellular layer of sycamore cell walls. Gold was most apparent in the expanded region of the intercellular layer at corner junctions between cells (Fig. 2a), but was also evident between appressed cell walls and on the surface of walls exposed to the culture medium (Fig. 2, b and c). Staining with antisera that had not
had the antibodies against methylated BSA removed was identical (data not shown). There was no significant labeling of the cell walls with the preimmune serum (Fig. 2d).

Anti-XG antibodies preabsorbed with ovalbumin labeled both the intercellular layer and the cellulose-containing cell wall. This labeling was evident in junctions between cells, in closely appressed cell walls, and in walls exposed to the medium (Fig. 3, a–c). Sections treated with the anti-XG antibodies not pretreated with ovalbumin were heavily labeled over the entire cytoplasm and wall (data not shown). This was assumed to be nonspecific. No label was detected with preimmune serum (Fig. 3d); thus, the observed weak reactivity of this antisera with extensin in the dot blots (Fig. 1) did not lead to cell wall binding in our samples.

Controls for Immunolabeling. To ensure that the antisera recognized only the specific molecules in the cell wall, the sera were preabsorbed against their respective antigens. With both the anti-RG-I and the anti-XG antisera, allowing the antibodies to sit overnight in the presence of purified polysaccharides RG-I and XG, respectively, prevented labeling of the cell walls (Fig. 4, a and b). Preabsorption with extensin had no effect on binding of these two antisera (Fig. 4, c and d).

Sodium metaperiodate, which oxidizes selected carbohydrate moieties (17), can be used to destroy the antigenicity of certain types of carbohydrate antigens. Pretreatment of sections with NaIO₄ eliminated binding of the anti-RG-I antibodies (Fig. 5). This control could not be done with the anti-XG antibodies since the antibodies were raised against periodate treated XG and recognized periodate treated RG-I as well as periodate treated XG (Fig. 6).

Sections treated with protein-A gold without prior exposure to primary antibodies showed no significant labeling (data not shown).

**DISCUSSION**

We can learn more about the mechanical and regulatory functions of specific polysaccharides in plant cell walls by pinpointing their time of synthesis and secretion and their location in growing and mature walls with antibody probes. To this end, we have used polyclonal antibodies raised against purified RG-I and XG to map their distribution within the walls of suspension-cultured sycamore cells. The localization of these molecules can intimate possible mechanisms for cell wall biogenesis as well as suggest potential mechanical and physiological functions.

**Localization of XG and RG-I.** Our studies on the localization of the hemicellulose XG in suspension-cultured sycamore cell walls with antibodies against XG have yielded results that differ significantly from those obtained in earlier work on the localization of hemicelluloses. Both extraction with hemicellulase (26) and localization with a xylanase–gold complex (27) indicated that hemicelluloses are present in the cellulose-containing region of the cell wall, but not in the middle lamella or in the corner junctions between cells. Our micrographs clearly reveal that, in suspension-cultured sycamore cells, the hemicellulose XG is present throughout the wall, including the middle lamella.

The immunolocalization of RG-I provides information on the distribution in the cell wall of a specific component instead of a whole class of molecules as has been the case in previous studies. Thus, the exclusive localization of RG-I in the intercellular layer is not inconsistent with these studies. Immunocytochemical techniques such as the one used in this study, open up the possibility of obtaining information on the differential distribution of chemically distinct pectic polysaccharides and learning more about their functional properties in cell walls. In this context, we are currently raising antibodies against RG-II, a pectic polysaccharide of approximately 60 residues which is structurally quite different than RG-I (19). RG-II is a very complex polysaccharide containing unusual glycosyl residues and glycosyl interconnections (6).

**How Localization may Relate to Cell Wall Biogenesis.** Relatively little is known about the mechanism of cell wall biogenesis. It is clear that cellulose microfibrils are assembled at the plasma membrane by synthesizing complexes (4, 10, 12). As successive layers of microfibrils are deposited each cell becomes surrounded by a ‘cocoon’ of microfibrils. The matrix in which the microfibrils are embedded appears to extend beyond this cocoon and into the intercellular layer of the cell wall. Autoradiographic and cellular fractionation studies have indicated that matrix components are synthesized in the Golgi apparatus (11, 23) and secreted via Golgi-derived vesicles (9, 20, 22). Virtually nothing is known, however, about when, where, and how these molecules are assembled in the cell wall.

The presence of RG-I only in the intercellular layer may be a consequence of the mode of biogenesis of the wall. The synthesis and secretion of RG-I may be restricted to early stages of cell plate formation and RG-I may become trapped between the two cellulose regions of the cell wall that are subsequently laid down. In contrast to the discrete localization of RG-I, XG appears to be distributed fairly evenly throughout the entire cell wall, including the middle lamella. The wide distribution of XG may indicate that it is being continuously secreted, during both cell plate formation and cell wall expansion.

**How Localization May Relate to Cell Wall Structure and Function.** Plant cell walls appear to serve both as static mechanical structures and as the source of signaling molecules. In particular, fragments of cell wall carbohydrates can act as regulatory molecules (1). However, at present the type of polysaccharides from which these fragments are derived, as well as the location of these polysaccharides, is poorly understood. RG-I is a potential source of one such signaling molecule since fragments derived from RG-I have been shown to induce the synthesis and accumulation of two protease inhibitors which are normally induced by wounding or insect attack (28). The location of RG-I in the intercellular layer and around the air spaces seems ideal for signaling mechanical damage to surrounding cells.

The location and chemical properties of RG-I also suggest that it functions as ‘glue’ between the cellulose-containing regions of adjacent cell walls. It has long been known that plant cells can
Fig. 2. Electron micrographs of suspension-cultured sycamore cells immunolabeled for RG-I. Anti-RG-I serum labels the intercellular layer (IL) between the cells (a and b) as well as a thin layer of material on the free surface of cells exposed to the culture medium (M; c). The anti-RG-I antiserum did not bind to cell walls. No labeling is seen on cells treated with preimmune serum (d). ×30,000; bar = 0.5 μm.
FIG. 3. Electron micrographs of suspension-cultured sycamore cells immunolabeled for XG. In contrast to the anti-RG-I labeling seen in Figure 2, the anti-XG serum labels the intercellular layer (IL) as well as the cell wall (CW; [a-c]). Cells treated with preimmune serum are not labeled (d). M, culture medium. ×30,000; bar = 0.5 μm.
Fig. 4. Immunocytochemical controls for antibody labeling of suspension-cultured sycamore cells. Cells immunolabeled with anti-RGI serum preabsorbed with purified RG-I (a). Cells immunolabeled with anti-XG serum preabsorbed with purified XG (b). In both cases, preabsorption with purified antigen blocks staining. Cells immunolabeled with anti-RGI and anti-XG (c and d, respectively) preabsorbed with purified extensin. The binding of the antisera is not affected. (a, b, and c), ×30,000; (d), ×40,000; bar = 0.5 μm.
XG probably also has both mechanical and regulatory functions. XG has been shown to hydrogen-bond tightly to cellulose fibrils and probably helps to keep cellulose fibrils anchored into the cell wall matrix (30). Its presence in the cellulosic region of the cell wall is therefore not surprising. The reason for its accumulation in the intercellular layer is less clear and may be an artifact of the atypical cell type used in this study. There may be, however, specific interactions between XG and other cell wall polymers in the intercellular layer. We are currently testing whether XG is present in the intercellular layer of mature plant tissues.

Since small amounts of XG are released from the cell during auxin-induced growth (14), XG may play a role in cell wall elongation (13). If the XG around the cellulose microfibrils is solubilized during auxin-induced growth, the microfibrils may become less tightly bound into the matrix, allowing the fibrils to slip past one another under the force of turgor pressure (13). York et al. (32) have found that elongation of pea epicotyls can be inhibited by adding a fragment of XG.

In conclusion, we have demonstrated distinct localizations for cell wall matrix polysaccharides. The spatial organization of the plant cell wall matrix appears to be more complex than previously imagined and suggests that localization studies may contribute in a significant manner to the elucidation of functional parameters of individual matrix polysaccharides. We hope to localize RGI and XG in the cell walls of both developing and differentiated tissues, and to address questions relating to the synthesis and assembly of cell wall matrix components.

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LITERATURE CITED


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Fig. 5. Electron micrograph of suspension-cultured sycamore cells immunolabeled with anti-RG-I serum following treatment of the sections with saturated sodium metaperiodate. The periodate abolished staining with this antibody. IL, intracellular layer; CW, cell wall. ×25,000; bar = 0.5 μm.

Fig. 6. Dot blot showing reactivity of anti-XG serum (1:10 dilution; not preabsorbed with ovalbumin) with purified XG, ovalbumin, XG treated with periodate-lysine fixative and RGI treated with periodate-lysine fixative (1 μl drops containing 100 ng antigen). The antiserum reacted with the XG as well as the periodate-lysine fixed XG. The antiserum also recognized periodate-lysine fixed RGI, but not unfixed RGI (see Fig. 1). be separated by the divalent cation chelating agent EDTA with little damage to individual cells (15). Pectins can be crosslinked in vitro to form gels by the addition of Ca²⁺ ions (21). These calcium pectate gels appear to be a strong and cohesive network. Similar interactions involving RGI may be the basis for the adhesion between cells in vivo.