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Immunogold localization of the cell-wall-matrix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways

Patricia J. Moore and L. Andrew Staehelin
Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347, USA

Abstract. We have localized two cell-wall-matrix polysaccharides, the main pectic polysaccharide, rhamnogalacturonan I (RG-I), and the hemicellulose, xyloglucan (XG), in root-tip and leaf tissues of red clover (*Trifolium pratense* L.) using immunoelectron microscopy. Our micrographs show that in both leaf and root tissues RG-I is restricted to the middle lamella, with 80–90% of the label associated with the expanded regions of the middle lamella at the corner junctions between cells. Xyloglucan, however, is nearly exclusively located in the cellulose-microfibril-containing region of the cell wall. Thus, these cell-wall-matrix polysaccharides are present in distinct and complementary regions of the cell wall. Our results further show that during cell expansion both RG-I and XG are present within Golgi cisternae and vesicles, thus confirming that the Golgi apparatus is the main site of synthesis of the non-cellulosic cell-wall polysaccharides. No label is seen over the endoplasmic reticulum, indicating that synthesis of these complex polysaccharides is restricted to the Golgi. The distribution of RG-I and XG in root-tip cells undergoing cell division was also examined, and it was found that while XG is present in the Golgi stacks and cell plate during cytokinesis, RG-I is virtually absent from the forming cell plate.

Key words: Cell wall polysaccharides – Cell plate – Hemicellulose – Immunolocalization – Golgi apparatus – Pectin – *Trifolium*.

Introduction

Plant cell expansion, and ultimately plant growth, requires the synthesis of new cell-wall material. Plant cell walls consist of cellulose microfibrils embedded in a matrix of polysaccharide and protein. Cellulose, the most abundant component of the cell wall, is thought to be synthesized at the cell surface by cellulose-synthesizing complexes which deposit the cellulose fibrils while moving laterally through the plasma membrane, thereby encircling the cell and creating a microfibrillar cocoon (Brown and Willison 1977; Giddings et al. 1980; Herth 1985). In contrast, cell-wall-matrix polysaccharides appear to be synthesized in the Golgi apparatus and move to the plasma membrane via Golgi-derived secretory vesicles. They are then released into the cell wall by fusion of the vesicles with the plasma membrane (see Robinson 1985; Chapter 10).

Early evidence for the involvement of the Golgi apparatus in the production of polysaccharides in plant cells includes cytochemical observations in which the cisternae and the secretory vesicles were shown to be rich in carbohydrate (Pickett-Heaps 1968; Van der Woude et al. 1971; Conrad et al. 1982), and autoradiographic studies involving the use of radioactive sugars that become incorporated into the cell wall (Pickett-Heaps 1966; Fowke and Pickett-Heaps 1972). Subsequently it was shown that when root-tip cells of corn entering division were incubated with [^{3}H]galactose, the radioactive precursor was first incorporated into the Golgi apparatus and then transferred to the cell plate (Dauwalder and Whaley 1974).

Biochemical evidence for the role of the Golgi apparatus in the synthesis and secretion of cell-wall polysaccharides has also been obtained. Isolated secretory vesicles from germinating pollen tubes contain a non-cellulosic glucan as well as polysaccharides rich in galacturonic acid which are similar to the pectic polysaccharides of the pollen-tube cell wall (Van der Woude et al. 1971). Other fractiona-

Abbreviations: ER = endoplasmic reticulum; RG-I = rhamnogalacturonan I; XG = xyloglucan

tion studies confirm that the carbohydrate content of the Golgi apparatus resembles the carbohydrate content of the matrix polysaccharides of the cell wall (Harris and Northcote 1971; Ray et al. 1976; Robinson et al. 1976). Enzymes capable of producing the polysaccharide-containing cell-wall components such as arabinosyl- (Gardiner and Chrispeels 1975), glucosyl- (Ray et al. 1969), xylosyl- (Ray 1980) and fucosyl transferases (Green and Northcote 1978; James and Jones 1979) have been shown to reside largely in the Golgi apparatus. Indeed, in the pea stem all the enzymes necessary to synthesize a fragment of one cell-wall polymer, xyloglucan, have been shown to exist in the Golgi apparatus (Camirand and MacLachlan 1986; Camirand et al. 1987). Taken together, these studies support the hypothesis that the Golgi apparatus is the main, if not exclusive, site of synthesis of non-cellulosic cell-wall polysaccharide.

We have studied the secretory pathway of two cell-wall-matrix polysaccharides, rhamnogalacturonan I (RG-I), the major pectic polysaccharide, and xyloglucan (XG), a hemicellulose. Using immunogold labeling we demonstrate the distribution of these molecules in dividing and expanding cortical cells in the root tips of red clover (*Trifolium pratense*). To our knowledge, this is the first immunocytochemical study documenting the site of synthesis and the intracellular transport of specific wall polysaccharides. Our results confirm that the Golgi apparatus is both the initial organelle in the secretory pathway of these molecules, and the source of the secretory vesicles involved in transporting these products to the cell surface.

Material and methods

Plant material. Red-clover (*Trifolium pratense* L.) seeds were obtained from a local food market, and were germinated on moist filter paper for 48 h. Leaf tissue was obtained from clover grown in vermiculite for one month under Philips 30-W cool-white fluorescent lemps (Conserve-a-watt Lighting, Denver, Colo., USA) at 300 μmol photons·m^{-2}·s^{-1} at 27° C. The plants were watered every other day with a 0.01% solution of Miracle-Gro plant food (Stern, Port Washington, N.Y., USA).

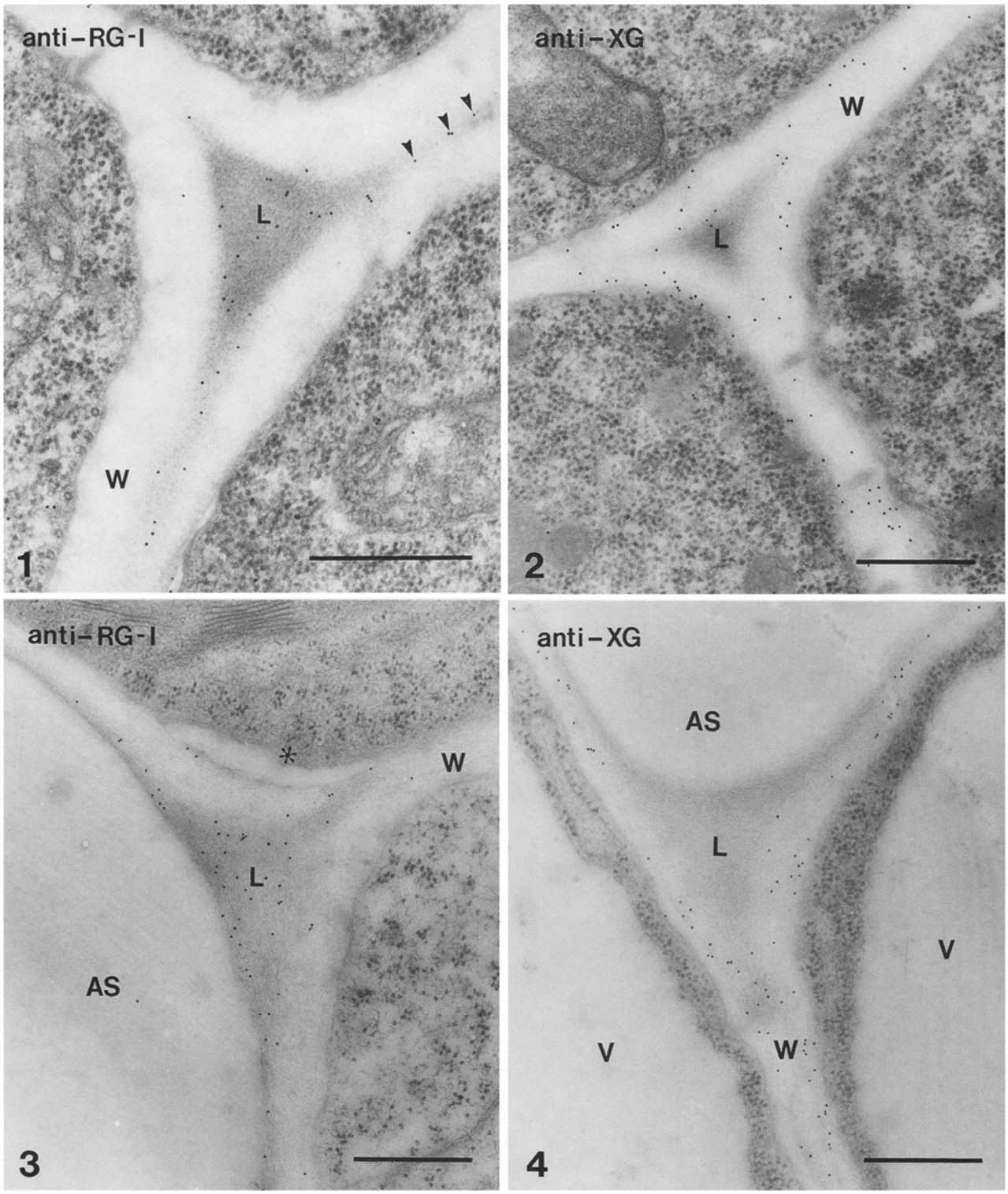
Electron microscopy. Root tips were fixed in 2.5% glutaraldehyde in 10 mM sodium-phosphate, buffer (pH 7.2) for 2 h at room temperature. After rinsing with the same buffer the root tips were postfixed in 1% osmium tetroxide in distilled H_2O for 1 h at room temperature, then washed three times with distilled H_2O and dehydrated in an ethanol series (30, 50, 70, 90, 100%; 10 min each step; room temperature). The root tips were infiltrated in 2:1 (v/v) ethanol: LR White resin (Polysciences, Warrington, Penn., USA) for 1 h, 1:2 (v/v) ethanol: LR White for 2 h, and 100% LR White overnight at 4° C (all other steps were carried out at room temperature). The infiltrated samples were embedded in gelatin capsules and allowed to polymerize overnight at 50° C.

Expanding leaves of clover were harvested and fixed in 2% paraformaldehyde plus 0.1% glutaraldehyde in 100 mM sodium-phosphate buffer, pH 7.2, for 2 h at room temperature, then washed in buffer, and postfixed in 1% OsO_4 in distilled H_2O for 1 h at room temperature. Dehydration, infiltration and embedding were done as described above. Silver to gold sections (70–100 nm) were cut on a Reichert Ultracut E (American Optical, Buffalo, N.Y., USA). Thin sections were picked up on formvar-carbon-coated nickel grids (300 mesh; Polysciences). Sections were examined, after antibody labeling, with a Hitachi (Tokyo, Japan) H600 electron microscope.

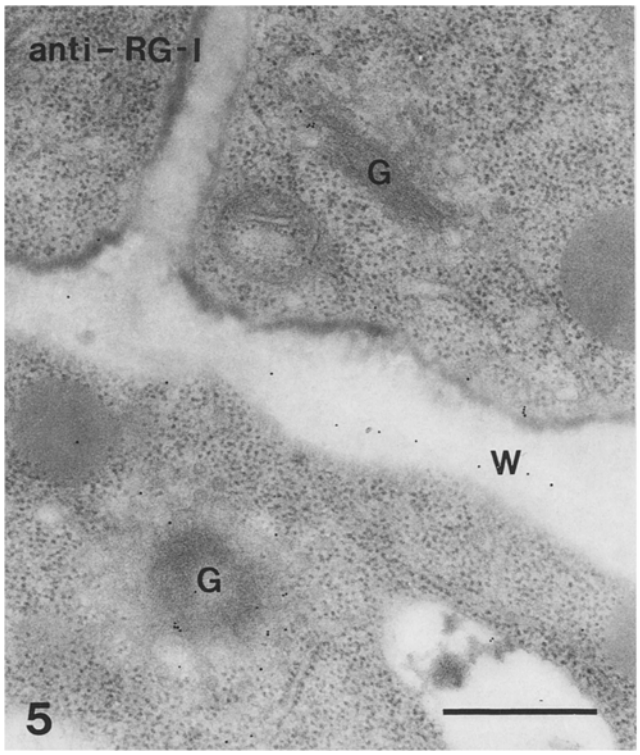
Antibody labeling. Antibodies were prepared and characterized as described in Moore et al. (1986). The grids were treated with 0.1 N HCl for 10 min to remove glutaraldehyde from the sections (Craig and Goodchild 1984), and then incubated in a blocking solution of 5% low fat dried milk (Carnation Co., Los Angeles, Cal., USA) in PBST (10 mM sodium phosphate, 500 mM NaCl, 0.1% Tween-20 (polyethylene sorbitan monolaurate); the blocking solution was made fresh daily) for 20 min. After the grids were blotted dry, they were incubated on primary antiserum diluted in PBST (anti-RG-I diluted 1:4, anti-XG diluted 1:10) for 30 min. The grids were then washed in a continuous stream of PBS containing 0.5% Tween-20 for 30 s and transferred to protein A-colloidal gold (7.5 nm; prepared according to Slot and Geuze 1985) diluted in PBST, for 20 min. Excess protein A-gold was removed by rinsing with PBST containing 0.5% Tween-20, followed by a distilled-water wash. Following immunolabeling, the sections were stained with 2% aqueous uranyl acetate for 5 min and Reynolds lead stain (Reynolds 1963) for 10 s. All steps were carried out at room temperature.

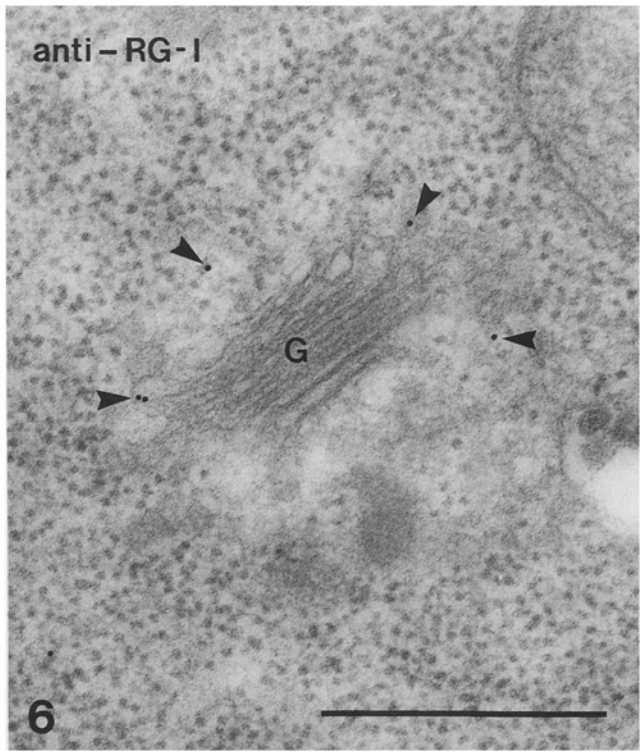
Quantitation of immunolabeling. The density of immunogold labeling was measured by counting the number of gold particles over a specified area on a photographic enlargement. Areas were measured on an IBM PC with a Jandel Scientific Digitizer Tablet (Jandel Corp., Sausalito, Cal., USA) using Sigma Scan, version 3.0 (Jandel Corp.). Values are reported as the mean ± one standard deviation (SD). Where assumptions for parametric tests of significance could be met, Student's t-test (Sokal and Rohlf 1981) was used to test for significant differences between means. The data were logarithm-transformed to meet assumptions of parametric statistics when necessary. Wilcoxon's signed-ranks was used to test for differences between paired observations in two groups (Sokal and Rohlf 1981).

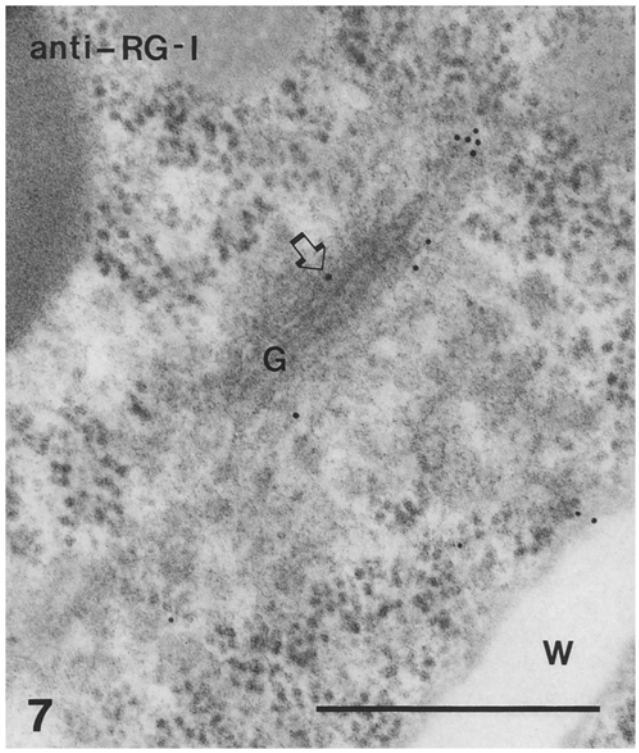
Cross-reactivity controls. To ensure that staining with the anti-XG antibodies was not a result of cross-reactivity with cellulose fibrils, the serum was preabsorbed overnight against sonicated ramie cellulose fibrils and then used in section labeling. Antibodies were also preabsorbed with specific sugars contained in the polymers. Equal volumes of antibody and 1 mg/ml sugar solution were mixed and the mixtures allowed to stand overnight at 4° C. Preabsorbed serum was then tested for reactivity against 200 ng of RG-I, XG, RG-II (a pectic polysaccharide; RG-I, XG, and RG-II were all generous gifts of Dr. P. Albersheim, University of Georgia, Athens, USA), extensin (a generous gift of Dr. J. Stafstrom, Yale University, New Haven, Conn., USA) or polygalacturonic acid using a Bio-Rad (Richmond, Cal., USA) Immuno-Blot (goat anti-rabbit IgG horseradish peroxidase conjugate) Assay kit. Anti-rhamnogalacturonan-I was preabsorbed with L-arabinose, D-galactose, polygalacturonic acid and L-rhamnose (Sigma Chemical Co., St. Louis Mo., USA). Anti-xyloglucan was preabsorbed with D-xylose and L-fucose (Sigma).



Figs. 1–4. Immunolabeling of root-tip cortical cells and leaf parenchyma cells of red clover with anti-cell wall matrix polysaccharide antibodies. Bars = 0.5 μm. **Fig. 1.** Root cortical cells labeled with anti-RG-I serum. Anti-RG-I antibodies label the expanded region of the middle lamella (*L*) as well as the closely appressed region of the middle lamella between cell walls (*arrows*). × 55000. **Fig. 2.** Root cortical cells labeled with anti-XG serum. Anti-XG antibodies label the cellulose microfibril-containing region of the cell wall (*W*) but not the expanded region of the middle lamella (*L*). × 40000. **Fig. 3.** Leaf parenchyma cells labeled with anti-RG-I serum. In these cells the anti-RG-I antibodies label the middle lamella (*L*) and the material lining the air spaces (*AS*), but not the rest of the cell wall (*W*). Label is occasionally seen at the inner surface of the cell wall in regions where the plasma membrane has pulled away from the cell wall (*star*) because of plasmolysis of the tissue during fixation. × 40000. **Fig. 4.** Leaf parenchyma cells labeled with anti-XG serum. As in the root cortical cells, anti-XG antibodies label the cell wall (*W*), but not the middle lamella (*L*). *AS*, Air space; *V*, vacuole. × 40000



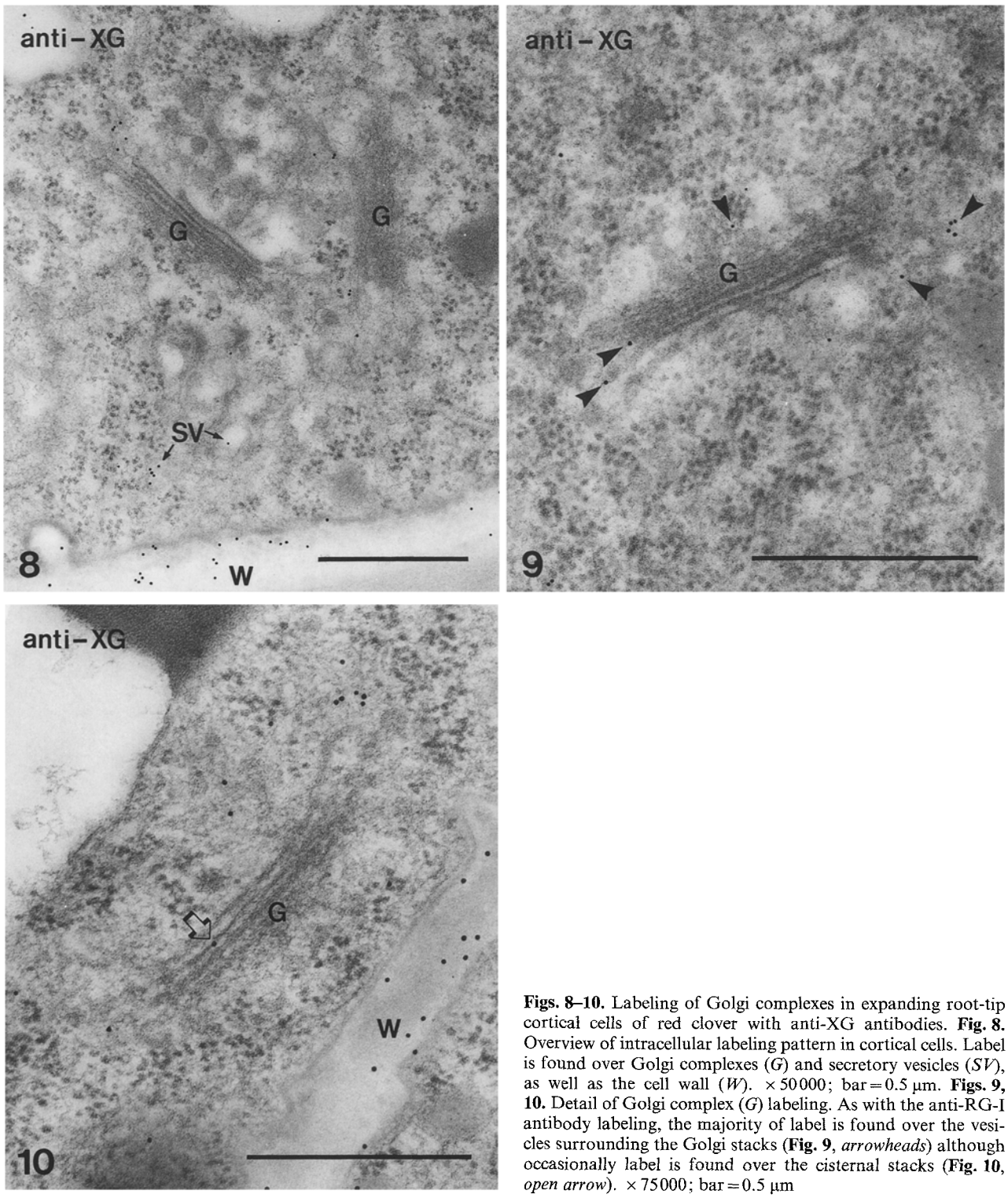




Figs. 5–7. Labeling of Golgi complexes in expanding root-tip cortical cells of red clover with anti-RG-I antibodies. **Fig. 5.** Overview of intracellular labeling pattern in cortical cells. Label is restricted to the Golgi complexes (*G*) and the cell wall (*W*). ×40000; bar = 0.5 μm **Figs. 6, 7.** Detail of Golgi complex (*G*) labeling. The majority of label is present over vesicles surrounding the Golgi stacks (**Fig. 6**, *arrowheads*) but label is also occasionally found over the cisternal stacks (**Fig. 7**, *open arrow*). ×75000; bar = 0.5 μm

Results

Localization of rhamnogalacturonan I and xyloglucan in cell walls. Expanding cortical cells of clover root tips and spongy-parenchyma cells of clover leaves were labeled with antibodies specific for RG-I. Labeling was most pronounced over the cell walls and appeared only over specific areas in these walls. In particular, dense labeling occurred over the expanded regions of the middle lamella at the



Figs. 8–10. Labeling of Golgi complexes in expanding root-tip cortical cells of red clover with anti-XG antibodies. **Fig. 8.** Overview of intracellular labeling pattern in cortical cells. Label is found over Golgi complexes (*G*) and secretory vesicles (*SV*), as well as the cell wall (*W*). ×50000; bar=0.5 μm. **Figs. 9, 10.** Detail of Golgi complex (*G*) labeling. As with the anti-RG-I antibody labeling, the majority of label is found over the vesicles surrounding the Golgi stacks (**Fig. 9**, *arrowheads*) although occasionally label is found over the cisternal stacks (**Fig. 10**, *open arrow*). ×75000; bar=0.5 μm

corner junctions between cells (Figs. 1, 3), and less dense labeling was seen over the middle lamella between closely appressed cell walls (Fig. 1, arrows). In leaf cells, a high density of label was also present over the edges of cell walls surrounding the air spaces (Fig. 3). The labeling at the inner surface of the cell wall seen in a small region of Fig. 3 (star) is an artifact that occurs when the plasma membrane is pulled back from the cell wall because of plasmolysis. Because most electron-mi-

croscope fixation, dehydration and plastic embedding schedules for plant tissues produce some plasmolysis, this kind of artifact is often difficult to circumvent. When the plasma membrane remains appressed against the cell wall, no such labeling of the inner cell-wall surface was observed (Fig. 3).

The same two cell types were also labeled with antibodies specific for XG. The walls surrounding the cells in both root-tip cortex and spongy-parenchyma cells of clover were heavily labeled (Figs. 2, 4). In contrast to the situation in the walls of suspension-cultured sycamore-maple (*Acer pseudoplatanus*) cells (Moore et al. 1986), gold label was found only in a very low level in the expanded regions of the middle lamella, and the label that was present was restricted the outer edges adjacent to the cell wall.

The localization of RG-I and XG was also examined in provascular tissue in root tips. In every case, the labeling patterns were identical to those found in the root cortical and leaf spongy-parenchyma cells.

Intracellular labeling of expanding cells in clover root tips. In addition to labeling the cell wall, anti-RG-I and anti-XG antibodies labeled a restricted set of intracellular compartments in expanding cortical cells in clover root tips. Both antibodies labeled Golgi complexes and associated vesicles (Figs. 5–10) but did not label the rough ER (rough endoplasmic reticulum; Fig. 11), mitochondria, nuclei or other organelles. The density of label over the Golgi apparatus (19.29 ± 10.53 particles/μm^2) was significantly higher than background (4.10 ± 2.88 particles/μm^2; $n=22$, $p<0.001$). As in cell walls, the density of the gold label over the Golgi complexes was higher in samples treated with anti-XG ($X=6.47 \pm 1.81$ particles/Golgi) than in samples treated with anti-RG-I ($X=3.47 \pm 1.66$ particles/Golgi; $n=44$, $p<0.001$). In both samples, the majority of the gold label was associated with vesicles surrounding the Golgi stacks, although label was also seen over the central, plate-like portion of the Golgi cisternae (Figs. 7, 10, open arrows; Table 1).

Slightly swollen vesicles could also occasionally be seen in the cytoplasm between the Golgi complex and the plasma membrane. Some of these vesicles appear to be involved in transporting matrix material to the plasma membrane since they were labeled with anti-RG-I (data not shown) and anti-XG (Fig. 8, arrow) antibodies.

Immunolabeling during cell-plate formation. Root-tip cortical cells undergoing cytokinesis exhibited

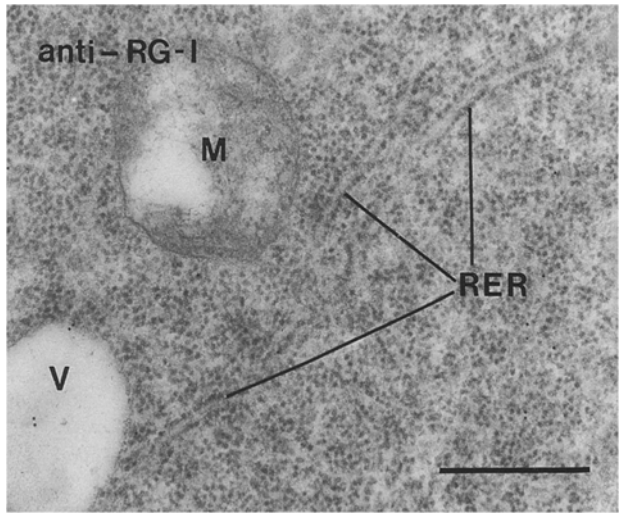


Fig. 11. Labeling of the rough endoplasmic reticulum (*RER*) in expanding root-tip cortical cells of red clover. Anti-RG-I antibodies do not label the RER. Very little label is found over other organelles such as the mitochondria (*M*) or vacuoles (*V*). The same is true for anti-XG antibodies (data not shown). ×40000; bar = 0.5 μm

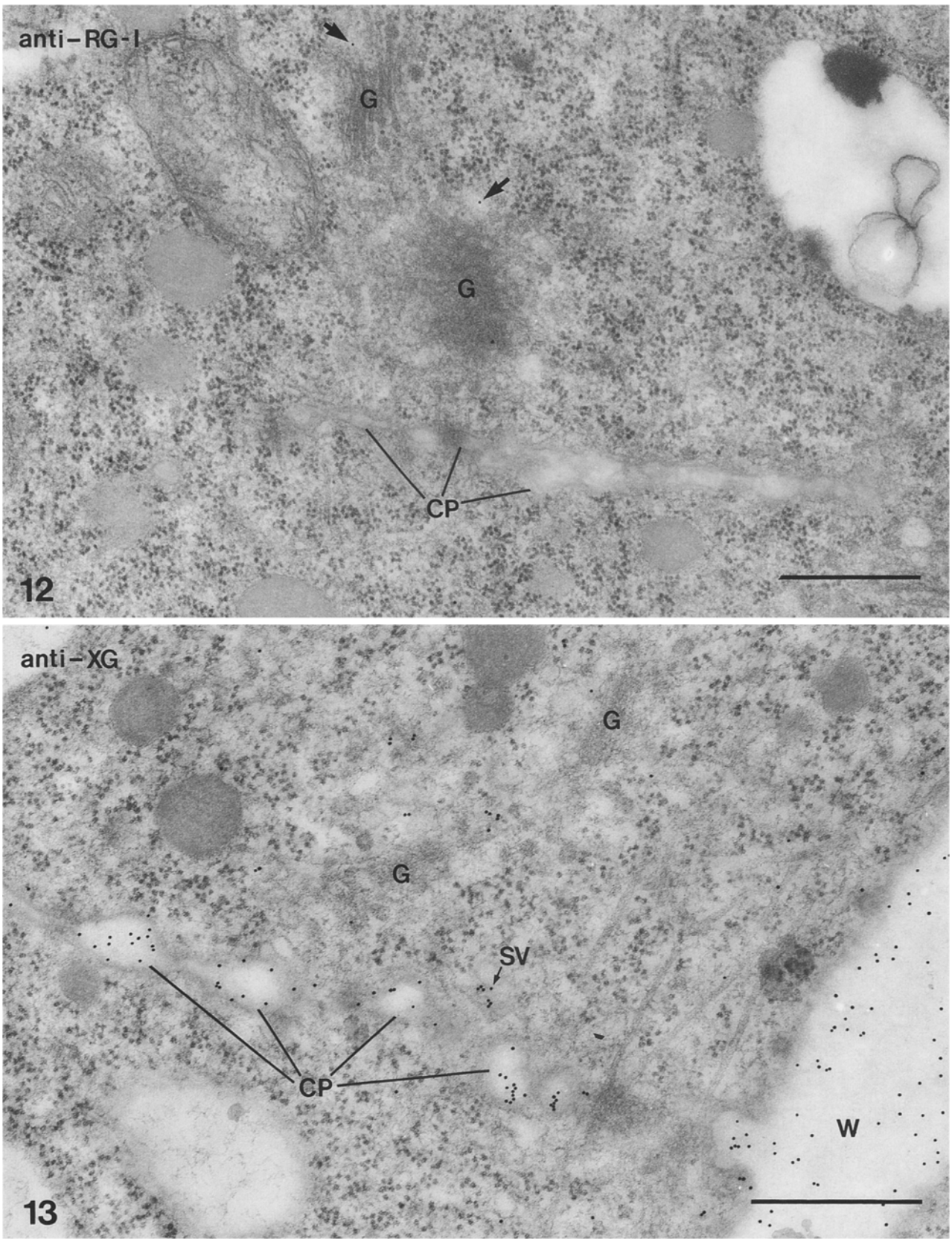
Table 1. Density[a] of immunogold labeling over Golgi vesicles versus cisternae in root-tip cortical cells of red clover. Densities were determined by counting the number of gold particles over measured areas of vesicles or cisternae of 20 Golgi complexes. The densities are reported as the mean ± 1 SD

	Vesicles	Cisternae	T[b]	*p*
α-RG-I	16.28 ± 10.87	5.00 ± 10.12	37	< 0.005
α-XG	24.03 ± 14.51	7.80 ± 9.56	20	< 0.005

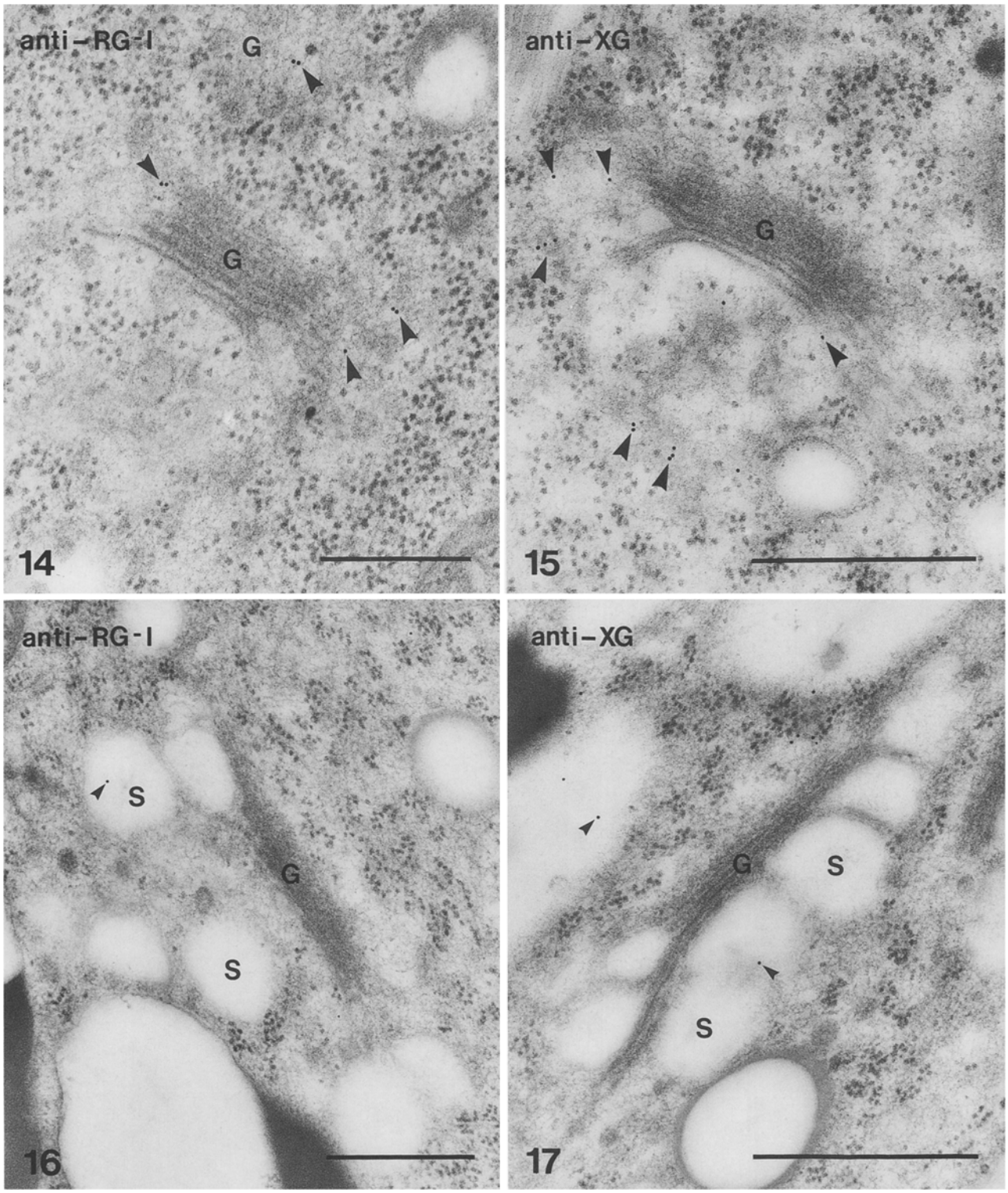
[a] Gold particles/μm^2
[b] Wilcoxon's signed-ranks test for n = 20

labeling patterns with the anti-RG-I and anti-XG antibodies that were very different from those of expanding cells. Dividing cells appear to produce very little RG-I. The level of labeling with RG-I antibodies was extremely low. Although Golgi vesicles occasionally had anti-RG-I labeling in these cells (Fig. 12, arrows), the forming cell plate often showed no label at all (Fig. 12). Anti-xyloglucan antibodies, on the other hand, labeled the cell plate and coalescing vesicles heavily (Fig. 13). Vesicles associated with adjacent Golgi stacks also were labeled heavily with these antibodies (Fig. 13).

Immunolabeling of root-cap cells. We have also used our anti-RG-I and anti-XG antibodies to examine what happens to the secretion of matrix polysaccharides as root-cap cells differentiate. The central cells in the root cap are fairly young cells, formed by division of the root-cap initials (see



Figs. 12, 13. Labeling of the forming cell plate in dividing root-tip cortical cells of red clover with anti-cell-matrix-polysaccharide antibodies. **Fig. 12.** Anti-RG-I antibodies do not label the forming cell plate (*CP*) in cells undergoing cytokinesis. Occasionally label is found over Golgi (*G*) vesicles (*arrows*). ×50000; bar=0.5 μm. **Fig. 13.** Anti-XG antibodies label the forming cell plate (*CP*) heavily. In addition, the Golgi (*G*) vesicles and secretory vesicles (*SV*) are heavily labeled. ×60000; bar=0.5 μm



Figs. 14–17. Labeling of Golgi complexes in root-cap cells of red clover with anti-cell-wall-matrix-polysaccharide antibodies. **Figs. 14, 15.** Inner root-cap cells labeled with anti-RG-I antibodies (**Fig. 14**) or anti-XG antibodies (**Fig. 15**). The Golgi complexes (*G*) in these cells are similar in morphology to the Golgi complexes in the root-tip cortical cells and the labeling pattern with the anti-cell-wall-matrix-polysaccharide antibodies is the same (*arrowheads*). Much of the gold label in the Golgi vesicles is clustered in groups of two to three particles. **Fig. 14** ×50000; **Fig. 15** ×72000; bar=0.5 μm. **Figs. 16, 17.** Outer root-cap cells labeled with anti-RG-I antibodies (**Fig. 16**) or anti-XG antibodies (**Fig. 17**). The Golgi complexes (*G*) of these cells have large swollen vesicles, presumably filled with slime polysaccharides (*S*). Very low amounts of anti-cell-wall-matrix antibody label is found in these vesicles (*arrowheads*) and the labeling always consists of single particles. **Fig. 16** ×50000; **Fig. 17** ×72000; bar=0.5 μm

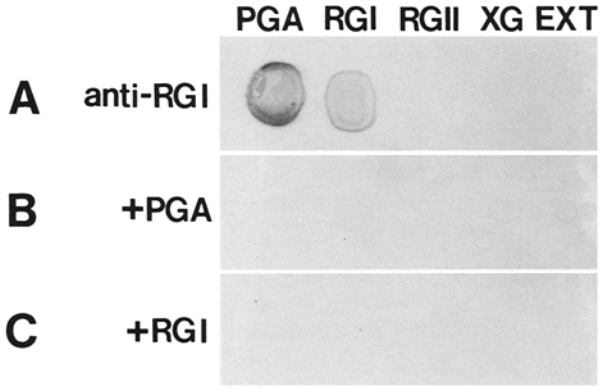


Fig. 18. Dot blot showing cross-reactivity of anti-RG-I to polygalacturonic acid (*PGA*). Droplets of 2 µl containing 200 ng of PGA, RG-I, RG-II, XG or extensin (*EXT*) were placed on nitrocellulose paper. Anti-RG-I serum reacts strongly with PGA and RG-I (*A*). Anti-RG-I serum preabsorbed with PGA (*B*) or RG-I (*C*) did not react with either PGA or RG-I

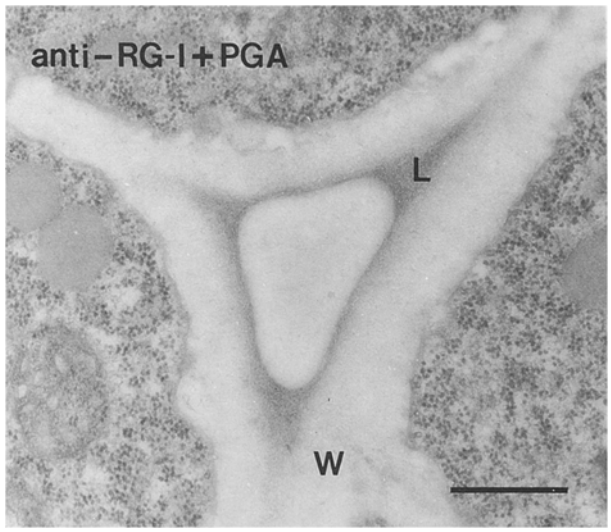


Fig. 19. Labeling of root tip cortical cells of red clover with anti-RG-I antibodies preabsorbed overnight against polygalacturonic acid. The preabsorbtion completely eliminates antibody staining of the middle lamella (*L*). ×30000; bar = 0.5 µm

Fahn 1982, p. 254), and are surrounded by thin cell walls which contain RG-I and XG. The Golgi stacks in these cells are morphologically similar to those in the root cortical cells and were labeled with anti RG-I and anti-XG antibodies (Figs. 14, 15), indicating that these cells are producing cell-wall-matrix polysaccharides. As the cells mature to become outer cap cells which synthesize and secrete large amounts of slime polysaccharides, the Golgi complexes become increasingly hypertrophied. These Golgi complexes do not appear to be synthesizing much RG-I or XG since they could not be labeled substantially with either anti-RG-I or anti XG antibodies (Figs. 16, 17).

Immunocytochemical controls. The RG-I and XG labeling we have observed appears to be very specific (Moore et al. 1986). The anti-XG antibodies did not appear to crossreact with cellulose, which also contains β-1,4-linked glucose residues like the backbone of XG, since labeling patterns did not change when the anti-XG antiserum was preabsorbed with ramie cellulose fibrils overnight before staining the sections (data not shown). Our antibodies also did not seem to cross-react with the slime polysaccharide since the hypertrophied slime-secreting Golgi of the outer root-cap cells were not substantially labeled with either anti-XG or anti-RG-I antibodies (Figs. 16, 17). Preimmune serum did not label either the cell wall or the Golgi complexes (data not shown). Preabsorption of anti-RG-I with arabinose, galactose or rhamnose did not affect antibody binding either to dot blots or clover root-tip cell walls. Polygalacturonic acid, however, did cross-react with the anti-RG-I serum (Fig. 18A) and preabsorption with commercial polygalacturonic acid abolished antibody reactivity against RG-I both on dot blots (Fig. 18B) and sections (Fig. 19). Likewise, preabsorption of the anti-RG-I serum with purified RG-I blocked staining of the polygalacturonic acid on dot blots (Fig. 18C) and cell walls (data not shown). Preabsorption of anti-XG with xylose or fucose did not affect antibody binding to dot blots or cell walls (data not shown).

Discussion

Characterization of the anti-RG-I and anti-XG antibodies. In this paper we have examined the cell-wall and intracellular location of two well-characterized cell-wall polymers, RG-I and XG, by immunogold localization. Rhamnogalacturonan-I is a pectic polysaccharide with a degree of polymerization of about 2000 (McNeil et al. 1980, 1984). It contains a backbone of alternating 2-linked L-rhamnose residues and 4-linked D-galacturonic-acid residues, and indicated by its mode of isolation, small flanking domains of polygalacturonic acid. About half of the rhamnosyl residues are branched, and the molecule contains as many as 30 different side chains containing D-galactose, L-arabinose and small amounts of L-fucose (McNeil et al. 1984). Xyloglucan, a hemicellulose, has a backbone of β-4-linked D-glucosyl residues. Some of the glucosyl residues contain xylose α-linked at 0–6. Some

of the xylose chains are extended by the addition of D-galactose, L-fucose and D-galactose (Bauer et al. 1973; McNeil et al. 1984).

The validity of any antibody study is heavily dependent on the specificity of the antibodies used. As previously shown (Moore et al. 1986) our anti-RG-I and anti-XG antibodies appear to be specific for RG-I and XG, respectively, as judged by the following criteria. The anti-RG-I antibodies do not cross-react with XG, RG-II, a pectic polysaccharide that is structurally very different from RG-I (McNeil et al. 1984), or the cell-wall glycoprotein extensin, and the anti-XG antibodies do not cross-react with RG-I, RG-II, extensin or cellulose. Preabsorption of either antibody with extensin, or anti-XG with cellulose, does not alter the labeling patterns. On the other hand, preabsorption of either antibody with its specific antigen completely abolished section labeling. Our anti-RG-I antibody appears to be recognizing the stretches of polygalacturonic acid on the ends of the RG-I molecule, based on its cross-reactivity with commercially available polygalacturonic acid (Figs. 18, 19). This opens up the possibility that our anti-RG-I antibody also recognizes other pectic polysaccharides with long regions of repeating galacturonic acid residues, such as homogalacturonan.

X-ray crystallographic studies of specific antibody-antigen complexes have shown that the size of the contact region between the antibody and its antigen is sufficiently large ($2.0 \cdot 2.5\ nm^2$) to cover a domain of up to several amino acids or sugar residues (Amit et al. 1985). Thus antibodies against large polysaccharides like RG-I and XG are probably not recognizing single sugar residues, but a set of residues in a specific spatial arrangement. Based on current knowledge of the specific linkage groups of sugar oligomers derived from enzymatically degraded complex cell-wall polysaccharides such as RG-I and XG (see McNeil et al. 1984), the spatial arrangement of sets of sugar residues should be unique for each cell-wall polymer. The lack of cross-reactivity between our anti-RG-I and anti-XG antibodies and other cell-wall polymers, and the inability of monosaccharides to inhibit binding of these antibodies to the cell wall are thus consistent with the notion that our antibodies are indeed highly specific probes for the two cell-wall-matrix polysaccharides.

Immunolocalization of cell-wall-matrix polysaccharides. Our immunocytochemical studies confirm our earlier result with suspension-cultured sycamore-maple cells (Moore et al. 1986) that a pectic polysaccharide, RG-I, is present in the middle lamella between cells. The presence of pectic polysaccharides in the middle lamella has been demonstrated previously using cytochemical (Albersheim et al. 1960) and enzymatic digestion (Deshpande 1976; Roland and Vian 1981) techniques. However, in quantitative terms, the amount of RG-I associated with the middle lamella (10–20%) is dwarfed by the amount of RG-I located in corner junctions and the lining of air spaces (80–90%).

Rhamnogalacturonan-I is the major pectic polysaccharide of the primary cell wall of dicotyledons (Darvill et al. 1980). However, it is but one of several known types of pectic polysaccharides in the cell walls of dicotyledons. Thus, we can not draw a general conclusion about the location of all pectic polysaccharides. Indeed, the enzymatic-digestion studies of Deshpande (1976) and Roland and Vian (1981) indicate that pectins may also be present within the cell wall surrounding the cellulose microfibrils. However, the possibility of cross-reactivity of our anti-RG-I antibodies with homogalacturonan could indicate that those polymers are also located in the middle lamella, thus accounting for at least 40% of the pectic polysaccharides present in the primary cell wall. Further studies will be needed to determine if the other pectic polysaccharides such as arabinans and galactans (Darvill et al. 1980) are located exclusively in the middle lamella, in the corner junctions and along air spaces, similar to RG-I, or if they are present in the matrix around the microfibrils. As reported by Selvendran (1985) 10–50% of the total pectins are inextractable from the cell walls and appear to be cross-linked to other cell-wall polymers. Where these insoluble pectins are located in cell walls cannot be deduced from our study.

In contrast to the findings with the RG-I antibodies, the pattern of immunolabeling for XG in the walls of root-tip and mesophyll cells of clover is different from that observed in clumps of suspension-cultured sycamore-maple cells (Moore et al. 1986). In the present study, XG has been found to be exclusively associated with the cellulose-fibril-containing regions of the cell wall, whereas in the suspension-cultured sycamore-maple cells XG was found both in the cellulose-containing region of the cell wall and in the middle lamella. The presence of XG in the middle lamella of the suspension-cultured sycamore-maple cells may be an artifact of cell culture, in that copious amounts of XG may have to be constantly produced by these cells to compensate for the loss of XG into the growth medium. Indeed, XG is most easily isolated from the culture medium of these cells (Bauer et al. 1973). In the intact tissues of red clover we

studied XG appears to be absent from the middle lamella (Fig. 1). This is consistent with earlier studies localizing hemicelluloses in the cell-wall matrix surrounding the cellulose microfibrils but not in the middle lamella (Ruel and Joseleau 1984; Roland et al. 1977). Therefore, we suggest this to be the normal localization of XG in plant tissues. This distribution is in agreement with the proposed function of XG in the regulation of extensibility of the cell wall (Labavitch and Ray 1974; York et al. 1984).

It is interesting that the two molecules we studied appear to occupy complementary regions of the plant cell wall. In fact, the density of XG labeling reaches a minimum where RG-I labeling is highest, i.e. in the corner junctions and along air spaces. This finding is inconsistent with cell-wall models that show XG and RG-I forming the matrix between the cellulose fibrils together and thereby contributing to the integrity of the cell wall (Albersheim 1975). Why RG-I accummulates preferentially in the corner junctions and along air spaces is still unknown.

Immunolocalization of cell-wall-matrix polysaccharides in the Golgi apparatus. As explained in the *Introduction*, previous cytological and biochemical studies have produced evidence that the Golgi apparatus is the site of synthesis of cell-wall-matrix polysaccharides. However, our anti-RG-I and anti-XG antibodies have enabled us to examine the precise intercellular location of two specific cell-wall polysaccharides and to localize these molecules to specific regions of Golgi stacks. They permitted us to show that the concentration of XG and RG-I in the appressed central regions of the Golgi cisternae is always very low. Either synthesis occurs near the dilated edges of the Golgi cisternae or newly synthesized molecules are rapidly packaged into transfer and secretory vesicles such that the concentration of molecules in the process of being synthesized in the central regions of the cisternae is kept low.

No RG-I or XG can be detected in the ER, indicating that the ER is not involved in the synthesis of these molecules. We cannot rule out, however, the presence within the ER of a proteoglycan precursor such as those associated with the biosynthesis of slime (Green and Northcote 1978) and starch (Tandecarz et al. 1975) and which presumably would not react with antibodies raised against mature molecules. Biochemical studies with elongating pea stems labeled with [^{14}C]glucose do not seem to support this latter idea in that the first membrane fraction to be labeled is the Golgi apparatus, and that label in the ER fraction comes from secretory vesicles which co-purify with the ER (Ray et al. 1976; Robinson et al. 1976).

Immunolocalization of cell-wall-matrix polysaccharides during cell-plate formation. The cell plate is formed by the coalescence of small vesicles which accumulate in the plane of cell division. Based on the position of the Golgi apparatus and the staining characteristics of Golgi vesicles and of the cell plate, it has been proposed that these vesicles are derived from the Golgi apparatus (Whaley and Mollenhauer 1963; Frey-Wyssling et al. 1964; Whaley et al. 1966), even though this is a "plausible interpretation, not a proven fact" (O'Brien 1972). Our data, which demonstrate the presence of XG both in the forming cell plate of dividing cells and in adjacent Golgi stacks and vesicles, strengthen the argument that the Golgi apparatus is the source of cell-plate material.

Rhamnogalacturonan-I appears to be largely absent from the cell plate. This observation was unexpected since, historically, it has been believed that the cell plate is rich in acidic pectic polysaccharides (Frey-Wyssling et al. 1964). Jones and Payne (1977) point out, however, that the cell plate does not stain with toluidine blue, indicating either that the free carboxyl groups on the pectic polysaccharides are methylated, or that the acidic polysaccharides are deposited at later stages. One interesting question raised by the absence of RG-I from the cell plate is how RG-I is transported through the cellulosic cell wall to the middle lamella and corner junctions during cell expansion, and why RG-I accumulates there.

In conclusion, using immuno-electron microscopy we have been able to demonstrate that, in the primary cell walls of root-tip and leaf parenchyma cells of red clover, RG-I, the major pectic polysaccharide, is localized in the middle lamella, whereas XG, a hemicellulose, is localized in the cellulose-containing region of the cell wall. Contrary to expectations, our micrographs also showed the forming cell plate to be devoid of the major pectic polysaccharide RG-I, but highly enriched in XG. Thus, the two major cell-wall-matrix polysaccharides are not only localized in distinct, complementary regions of the cell wall, but also show different patterns of synthesis and secretion. This spatial organization is consistent with the notion that RG-I and XG serve different structural and physiological functions. We have also confirmed that the Golgi apparatus is the site of synthesis of these matrix polysaccharides both during cell expansion and cell-plate formation. However, based on the

altered labeling of the outer root-cap cells, it appears that during differentiation, the synthetic activities of Golgi complexes can be changed to meet different needs. A similar switch in Golgi function has been reported to occur in gibberellic-acid-stimulated barley aleurone cells (Johnson and Chrispeels 1973; Fernandez and Staehelin 1985). Further studies will focus on variations in the functional activities of the Golgi apparatus during the cell cycle and during tissue differentiation.

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