Domain-specific and Cell Type–specific Localization of Two Types of Cell Wall Matrix Polysaccharides in the Clover Root Tip

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Abstract. Using immunocytochemical techniques and antibodies that specifically recognize xyloglucan (anti-XG), polygalacturonic acid/rhamnogalacturonan I (anti-PGA/RG-I), and methylesterified pectins (JIM 7), we have shown that these polysaccharides are differentially synthesized and localized during cell development and differentiation in the clover root tip. In cortical cells XG epitopes are present at a threefold greater density in the newly formed cross walls than in the older longitudinal walls, and PGA/RG-I epitopes are detected solely in the expanded middle lamella of cortical cell corners, even after pretreatment of sections with pectinmethylesterase to uncover masked epitopes. These results suggest that in cortical cells XG and PGA/RG-I are differentially localized not only to particular wall domains, but also to particular cell walls. In contrast to their nonoverlapping distribution in cortical cells, XG epitopes and PGA/RG-I epitopes largely colocalize in the epidermal cell walls. The results also demonstrate that the middle lamella of the longitudinal walls shared by epidermal cells and by epidermal and cortical cells constitutes a barrier to the diffusion of cell wall and mucilage molecules. Synthesis of XG and PGA/RG-I epitope-containing polysaccharides also varies during cellular differentiation in the root cap. The differentiation of gravitropic columella cells into mucilage-secreting peripheral cells is marked by a dramatic increase in the synthesis and secretion of molecules containing XG and PGA/RG-I epitopes. In contrast, JIM 7 epitopes are present at abundant levels in columella cell walls, but are not detectable in peripheral cell walls or in secreted mucilage. There were also changes in the cisternal labeling of the Golgi stacks during cellular differentiation in the root tip. Whereas PGA/RG-I epitopes are detected primarily in *cis*- and medial Golgi cisternae in cortical cells (Moore, P. J., K. M. M. Swords, M. A. Lynch, and L. A. Staehelin. 1991. *J. Cell Biol.* 112:589–602), they are localized predominantly in the *trans*-Golgi cisternae and the *trans*-Golgi network in epidermal and peripheral root cap cells. These observations suggest that during cellular differentiation the plant Golgi apparatus can be both structurally and functionally reorganized.

THE plant cell wall is a multilayered network of complex polysaccharides and glycoproteins linked to each other and to the plasma membrane (Roberts, 1989). The numerous functions of the primary cell wall include mechanical support; molecular sieving and ion exchange; regulation of cell shape and growth; and protection against physical damage and pathogens. In dicotyledonous plants, the primary cell wall contains four major classes of components: (*1*) cellulose, a β-(1→4)-linked polymer of D-glycosyl residues; (*2*) hemicelluloses, polymers that contain a cellulosic backbone to which various side chains are attached; (*3*) pectic polysaccharides, a structurally complex and heterogeneous class of matrix polymers typically rich in galacturonic acid; and (*4*) glycoproteins, which contain N-linked and/or O-linked oligosaccharide side chains.

The biosynthetic transport pathways of cell wall polysaccharides have been only broadly outlined. While cellulose is synthesized by plasma membrane-bound enzyme complexes, matrix polysaccharides are synthesized and processed in the Golgi apparatus and packaged into secretory vesicles for transport to the cell wall (Delmer and Stone, 1988). Unlike that in animal cells, secretion from the Golgi apparatus in plants continues during cell division. Indeed, during this stage of the cell cycle the Golgi apparatus produces the vesicles of the cell plate, which fuse with one another to produce the daughter cell wall.

Xyloglucan (XG)[1] and polygalacturonic acid/rhamnogalacturonan I (PGA/RG-I) are among the most studied complex polysaccharides of the cell wall matrix. XG, the major hemicellulose in dicotyledonous plants, consists of a back-

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1. *Abbreviations used in this paper*: PBST, PBS + Tween 20; PGA, polygalacturonic acid; PME, pectinmethylesterase; RG-I, rhamnogalacturonan I; SEP, sycamore extracellularpolysaccharide; TAM, tamarind seeds; TGN, *trans*-Golgi network; XG, xyloglucan.

bone of from 300 to 3,000 β-(1→4)-linked D-glucosyl residues of which ~60–75% are substituted at carbon six with side chains consisting of either a single xylosyl residue or with a trisaccharide of xylose, galactosyl, and fucosyl residues (Fry, 1989). These side chains are distributed in a highly regular pattern along the backbone. Functionally, XG not only contributes to the tensile strength of the cell wall, but also is one of the effectors and regulators of cell expansion (Fry, 1988, 1989; Hayashi, 1989). PGA/RG-I is a large (up to several hundred kilodaltons) acidic pectic polysaccharide that consists of two major covalently linked structural domains, PGA and RG-I (Bacic et al., 1988). The PGA domain consists of blocks of α-(1→4)-linked D-galactosyluronic acid residues interspersed periodically with an α-(1→2)-linked rhamnosyl residue (Powell et al., 1982; Konno et al., 1986). Each monosaccharide residue in a block of uninterrupted galactosyluronic acid residues can be methylesterified at carbon six (Kauss et al., 1967). It is generally believed that PGA is synthesized and secreted into the wall in a highly methylesterified form (Jarvis, 1984) where it is acted upon by pectinmethylesterase (PME; Yamaoka and Chiba, 1983; Pressey, 1984), an enzyme which deesterifies blocks of consecutively linked galactosyluronic acid residues (Pilnik and Rombouts, 1978). Adjacent blocks of the deesterified, polyanionic galactosyluronic acid can bind inter- or intramolecularly through Ca^{++} bridging and form stable pectin gels that contribute to the overall strength of cell walls (for review see Jarvis, 1984). Methylesterification may therefore function to keep PGA in a soluble form during its synthesis and transport to the cell wall. The RG-I domain of PGA/RG-I has been characterized extensively in suspension cultured sycamore cells (for review see McNeil et al., 1984). RG-I consists of a backbone of alternating rhamnosyl and galactosyluronic acid residues to which side chains rich in arabinosyl and galactosyl residues are attached. Pectic polysaccharides containing galacturonic acid, such as PGA/RG-I, are the primary determinants of pore size and sieving properties of the cell wall (Baron-Epel et al., 1988) and in their unesterified, Ca^{++}-crossbridged form contribute to the general strength of the wall (Jarvis, 1984). In addition, oligogalacturonan fragments of unesterified PGA have been shown to not only induce the synthesis of antimicrobial molecules crucial for the plant defense responses against pathogens (Ryan and Farmer, 1991), but also to regulate tissue morphogenesis in thin cell layer explants in tobacco (Trahn Thahn Van et al., 1985; Eberhard et al., 1989).

Plant root tips provide an excellent model system for investigating the synthesis and assembly as well as the tissue-specific expression of functionally important cell wall components such as XG and PGA/RG-I (Fig. 1). All of the cells are derived by cell divisions in the apical meristem(s). As new cells arise in the meristem, the older ones are displaced both towards the root tip apex and the root base, while simultaneously enlarging in size and differentiating. Developmental changes in specific types of cells can therefore be easily followed by tracing the fate of files of cells originating from the apical meristem(s).

In this paper we report on the characterization of polyclonal antibodies raised against highly purified XG and deesterified PGA/RG-I, and on the use of these antibodies and a mAb, JIM 7, which recognizes highly methylesterified pectins (Knox et al., 1990) to localize these polysaccharides into the cell walls and Golgi stacks of the clover root tip.

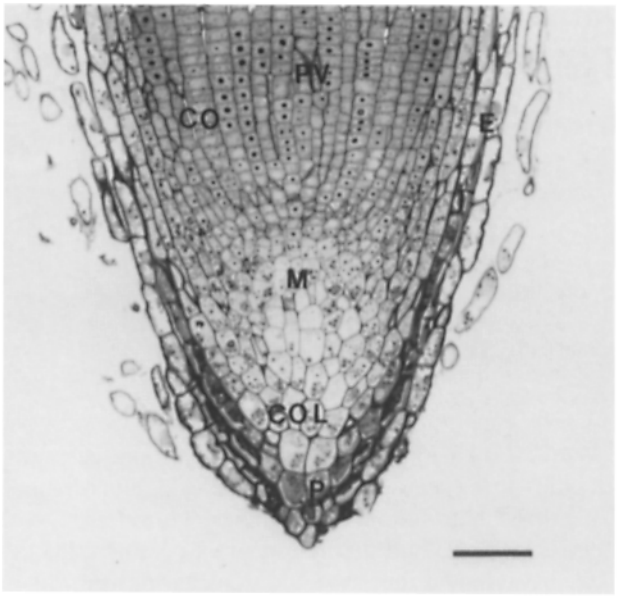

Figure 1. Longitudinal section of a clover root tip, stained with 0.5% Toluidine blue, 0.5% Na borate. *M*, meristem; *PV*, provascular cylinder; *CO*, cortex; *E*, epidermis; *COL*, columella cells; *P*, peripheral cells. A layer of darkly staining mucilage is secreted by, and surrounds, the root cap and epidermis. Bar, 50 μm.

Materials and Methods

Plant Material

Seeds of red clover (*Trifolium pratense L.*) were obtained from a local retailer. Seeds were germinated on moist filter paper in the dark for 48 h.

Preparation and Characterization of Antibodies

Purified preparations of XG and of RG-I were kindly provided by members of the Complex Carbohydrate Research Center (Athens, GA). XG was isolated from the extracellular polysaccharide fraction of suspension cultured sycamore cells (SEPs) by the method of Bauer et al. (1973). RG-I was isolated from SEPs with the method of McNeil et al. (1980), which involves treatment of SEPs with endopolygalacturonase, an enzyme which catalyzes cleavage of unesterified PGA and thus releases RG-I and RG-II covalently linked to it. Polyclonal antibodies against XG coupled to ovalbumin and against RG-I coupled to methylated BSA were raised in rabbits and purified essentially as previously described (Moore et al., 1986). Specificities of the anti-XG and anti-PGA/RG-I antisera were determined by immunoblot analysis using the Immun-blot assay kit (Bio-rad Laboratories, Richmond, CA). Serial dilutions of potential antigens containing either 200, 100, 10, or 1 ng of antigen were spotted onto nitrocellulose, and probed with 1:20 dilutions of either anti-XG, anti-PGA/RG-I, or the corresponding preimmune serum. Binding of the primary antiserum was detected by incubation in a 1:3,000 dilution of goat anti-rabbit IgG (H+L) coupled to HRP followed by color development using 4-chloro-1-naphthol as substrate for HRP. Reactivity of antigens against the antisera was assessed immediately after developing the blot. Each antigen was tested a minimum of two times.

To characterize further the specificities of anti-XG and of anti-PGA/RG-I, antisera were preabsorbed with an excess of potential antigen and then used for immunolabeling. Equal volumes of 1:2 diluted antiserum and 1 mg/ml of potential antigen in dH_2O were coincubated at 4°C overnight. Samples were centrifuged at 15,000 rpm at 4°C for 30 min. The supernatant was removed, diluted to the appropriate final concentration in PBST (10 mM Na-phosphate, 500 mM NaCl, 0.1% Tween 20, 0.02% NaN_3, pH 7.2), and used for immunolabeling.

A mAb that recognizes methylesterified pectins (JIM 7; Knox et al., 1990) was kindly provided by J. Paul Knox (John Innes Institute, Norwich, UK).

Samples for dot blot characterizations were obtained from several sources. SEP XG, SEP RG-I, and SEP RG-II were obtained as gifts from the Complex Carbohydrate Research Center. β-(1→3)-(1→4)-D-glucan and

arabinogalactan protein were gifts from the laboratory of Dr. Nick Carpita, Purdue University (West Lafayette, IN). Ramie cellulose fibrils were obtained from Dr. Iain E. P. Taylor, the University of British Columbia, Vancouver, B.C., Canada. Extensin-1 was prepared by K. M. M. Swords, the University of Colorado, Boulder. All other samples were obtained from Sigma Chem. Co. (St. Louis, MO) or Hercules Inc. (Wilmington, DE).

Tissue Fixation and Microscopy

Root tips were fixed and embedded essentially as described in Moore et al., 1991. For immunofluorescence microscopy, 0.4-μm sections were cut on an Ultracut microtome (Reichert-Jung, Vienna, Austria) and immobilized onto polylysine-coated glass slides. Sections were incubated in 10% FCS (Gibco, BRL, Richmond, VA) in low salt PBS + Tween 20 (PBST; 10 mM Na phosphate, 50 mM NaCl, 0.05% Tween 20, 0.02% NaN_3, pH 7.2) for 20 min to block nonspecific binding sites. Sections were then incubated in primary antiserum diluted either 1:20 (anti-XG) or 1:16 (anti-PGA/RG-I) in PBST for 3 h, washed through five changes of low salt PBST, incubated for 2 h in secondary antibody (donkey anti-rabbit IgG [H+L] coupled to Texas red; Amersham Corp., Arlington Heights, IL) diluted 1:50 in low salt PBST, washed in low salt PBST, washed in dH_2O, and mounted in antifade (30% glycerol, 20% polyvinylpolypyrrolidone, 5% *N*-propylgallate, 45% PBS + 0.1% NaN_3). Sections were examined on an Axioskop equipped with epifluorescence illumination (Carl Zeiss, Inc., Thornwood, NY). Fluorescence was observed and photographed at 590 nm. For immunogold electron microscopy, silver-to-gold sections (80–100 nm) were cut using an Ultracut microtome (Reichert) and were collected onto carbon-coated, formvar-coated nickel grids. Grids were incubated in a blocking solution of 5% nonfat dry milk (Carnation Co., Los Angeles, CA) in PBST for 30 min. Excess blocking solution was wicked away and the grids were incubated for 2 h in primary antiserum diluted in PBST (1:10 anti-XG and 1:8 anti-PGA/RG-I). They were washed with a large volume of PBST + 0.5% Tween 20 and incubated for 30 min in a solution of 1:25 protein A-gold (Ted Pella Inc., Redding, CA) diluted in PBST. After washing with a large volume of PBST + 0.5% Tween-20 and then with a large volume of dH_2O, the grids were counterstained in 2% uranyl acetate in dH_2O for 5 min, and then in triple lead or lead citrate stain for 30 s. All incubations were at room temperature. For double-immunolabeling experiments, the procedure of Moore et al. (1991) was followed, except that the grids were incubated in the milk blocking solution before the second, as well as the first, primary antibody incubation. Sections were viewed on a CM 10 electron microscope (Philips Electronic Instrs. Co., Mahwah, NJ).

For the deesterification experiments, sections were pretreated for 30 minutes in a solution of PME (orange rind; EC3.1.1.1.11; Sigma Chem. Co.). Each grid was incubated in a drop of enzyme solution (1:110 in 40 mM Tris, 100 mM NaCl, pH 7.5), washed with buffer, and then immunolabeled as described above. Control grids were incubated in buffer alone and washed as above.

All anti-XG and anti-PGA/RG-I labeling experiments were done at least twice on two independently fixed and embedded groups of roots.

Quantitation of Immunolabeling Density/Statistics

The density of immunolabeling (number of gold particles/μm^2) was measured by counting the number of gold particles in an individual cell wall on an electron micrograph. Areas were measured on an IBM PC with a Scientific Digitizer Tablet (Jandel Corp., Sausalito, CA) and the Sigma Scan program 3.0 (Jandel Corp.). The density of labeling for each individual wall and the mean density of labeling for all walls of a given category were calculated. Nonspecific background binding was determined by calculating the density of immunolabeling in the mitochondria, which are not labeled specifically by either antibody. Density values are reported as the mean ± SEM. Density values are the mean of two separating immunolabeling experiments done using two independently fixed and embedded groups of root tips.

To quantitate the distribution of anti-XG and anti-PGA/RG-I immunogold label in secretory vesicles in double labeling experiments, all electron-lucent, uncoated, and roughly spherical secretory vesicles in every epidermal cell in every section were scored. Vesicles with ≤1 gold particle were categorized as unlabeled, and those with ≥2 gold particles assessed for presence of 10 nm gold only, 20 nm gold only, or both 10 and 20 nm gold.

Chemical Treatment of Polysaccharides

α-Cellulose (Sigma Chem. Co.) was solubilized in 4-methylmorpholine-*N*-oxide (Aldrich Chem. Co., Inc., Milwaukee, WI) essentially as described in Joseleau et al. (1981). PGA was chemically methylesterified by treating 1 g PGA in 36 ml 1 M H_2SO_4 in methanol at 4°C for 72 h with gentle agitation (Powell et al., 1982). A control sample of PGA was treated similarly in 100% methanol. Both samples were washed through three changes of 60% ethanol, dialyzed against dH_2O, and lyophilized to dryness.

Results

Characterization of Antibody Specificity

To determine the binding specificities of the polyclonal anti-XG and anti-PGA/RG-I antisera we subjected them to a large battery of immunoblot and preabsorption/immunolabeling tests. Immunoblot analysis (Table I) revealed that anti-XG reacts with as little as 1 ng of either xyloglucan isolated from suspension-cultured sycamore cells (SEP XG) or xyloglucan isolated from tamarind seeds (TAM XG). The antibodies also detected 1 ng of the major hemicellulose in graminaceous monocots, β-(1→3)-(1→4)-D-glucan, but do not bind to as much as 200 ng of β-(1→3)-D-glucan. While anti-XG does not bind to hydrogen-bonded native cellulose fibrils (Table I), it does specifically bind to cellulose solubilized in the powerful chaotropic agent, 4-methylmorpholine-*N*-oxide (Lynch, M., unpublished data). Anti-XG does not bind strongly to any other major cell wall components (Table I). Based on these results it was concluded that the major epitope recognized by the anti-XG antibodies contains β-(1→4)-linked glucosyl residues as found in the backbone of XG.

While anti-PGA/RG-I binds to as little as 1 ng of PGA, 10 ng of RG-I, and 10 ng of highly methylesterified pectin, it recognizes neither RG-II nor arabinogalactan, other major pectic polysaccharides (Table I). Since the antiserum was generated against a chemically demethylesterified form of RG-I, isolated by treatment of cell walls with endopolygalacturonase (see Materials and Methods), we hypothesized that anti-PGA/RG-I preferentially recognizes domains of deesterified PGA. To test this hypothesis, the reactivities of anti-PGA/RG-I against serial dilutions of chemically methylesterified PGA and of control PGA (PGAc; mock-treated in

Table I. Dot Blot Analysis of Reactivities of Anti-XG and Anti-PGA/RG-I Antibodies‡*

Antiserum	SEP XG§	TAM XG	β-1→3-β-1→4-glucan	β-1→3-glucan	PGA PGAc	PGA (methyl-ester)	RG-I	71.5% DE pectin	Cellulose fibrils	AG	AGP	Gum arabic	RG-II	EXT-1
Anti-XG	+++	+++	+++	−	−	ND	+	+	−	−	−	−	−	−
Anti-PGA/RG-I	−	−	−	−	+++	++	++	++	−	−	−	−	−	−

* +++, reaction with 1 ng antigen; ++, reaction with 10 ng antigen; +, reaction with 200 ng antigen; −, no reaction with 200 ng antigen.
‡ Neither the anti-XG preimmune serum nor the anti-PGA/RG-I preimmune serum reacted with 200 ng of any of the antigens tested.
§ PGAc, Polygalacturonic acid control; 71.5% DE pectin, 71.5% esterified pectin; AG, arabinogalactan; AGP, arabinogalactan protein; EXT-1, extensin-1.

100% methanol) were compared (Table I). Anti-PGA/RG-I recognizes a 10-fold lesser amount of both untreated unesterified PGA and of PGAc than of chemically methylesterified PGA. Taken together, these results strongly suggest that a major fraction of the anti-PGA/RG-I polyclonal antiserum recognizes unesterified domains of PGA. In contrast, JIM 7, a mAb that recognizes methylesterified pectins (Knox et al., 1990), recognized neither PGA, PGAc, nor RG-I in these studies, but recognized as little as 10 ng of methylesterified PGA (data not shown).

To further characterize anti-XG and anti-PGA/RG-I, the antisera were preabsorbed with a high concentration (1 mg/ml) of mono- and polysaccharides and the preabsorbed antisera used to immunolabel thin sections. The effect of the preabsorption on immunogold labeling of the cell walls was then assessed. As expected, preabsorption of anti-XG with either SEP XG or with TAM XG abolished labeling and preabsorption with a mixed linkage β-(1→3)-(1→4)-D-glucan greatly reduced labeling. Preabsorption with native cellulose fibrils had no effect on labeling, a result that suggests that there is no cross-reactivity of anti-XG with cellulose fibrils in the labeling experiments. Preabsorption with SEP RG-I also reduced labeling, a result consistent with the faint level of cross-reactivity observed in dot blot experiments. In contrast, preabsorption of anti-XG with 71.5% DE pectin, cellobiose, or cellulose fibrils had no effect on either the overall density or pattern of labeling. Similarly, preabsorption with the monosaccharide constituents of XG, i.e., D-glucose, D-xylose, or L-fucose, had no effect on immunolabeling.

Preabsorption of anti-PGA/RG-I with either SEP RG-I or PGA completely abolished labeling. In contrast, preabsorption with commercial 71.5% DE pectin produced variable effects for reasons we do not understand; in some experiments it reduced labeling and in others had no effect. Preabsorption with SEP XG, TAM XG, or mixed linkage β-(1→3)-β-(1→4)-D-glucan or preabsorption with the monosaccharide constituents of PGA/RG-I, i.e., D-galactose, D-galacturonic acid, L-rhamnose, L-arabinose, also had no effect on either the pattern or density of wall labeling.

Both preimmune sera were also tested for reactivity, and neither preimmune serum bound to any of the molecules tested in the immunoblot analysis or labeled any cell structure in immunogold labeling experiments (data not shown).

Distribution of XG Epitopes and PGA/RG-I Epitopes Is Tissue Specific

To determine if the pattern of distribution of XG epitopes and PGA/RG-I epitopes is the same in all cells of the root rip or if it differs between cells with different functions, anti-XG and anti-PGA/RG-I and the fluorochrome Texas red coupled to donkey anti-rabbit IgG were used to localize these epitopes in the different tissues of the root (Fig. 2). As shown in Fig. 2 *A*, XG epitopes are detected in all cell walls of all cell types, but are most abundant in walls surrounding epidermal and peripheral root cap cells. A high concentration of PGA/RG-I epitopes is also detected in epidermal and peripheral root cap cell walls; however, these epitopes are not detected in the walls surrounding cells of the meristem, provascular cylinder, or cortex (Fig. 2 *B*).

Although the use of the indirect immunofluorescence technique yields valuable information on the general localization of cell wall matrix components at the tissue level, it lacks the resolution and the sensitivity to detect subtle differences in the distribution of individual epitopes within an individual cell and cell wall. To detect such differences, immunogold labeling techniques were used to localize XG epitopes and PGA/RG-I epitopes at the subcellular level.

Distribution of XG Epitopes and PGA/RG-I Epitopes in Cortical Cells Is Spatially Restricted to Discrete Cell Wall Domains

In the region of the root in which cortical cells are both dividing and elongating, all of the cell files have been formed (Fig. 1). Cells in this region are thus no longer dividing in the longitudinal direction, but have commenced elongation. Cell division in this region occurs exclusively in the transverse direction. Undifferentiated three-way junctions, formed by

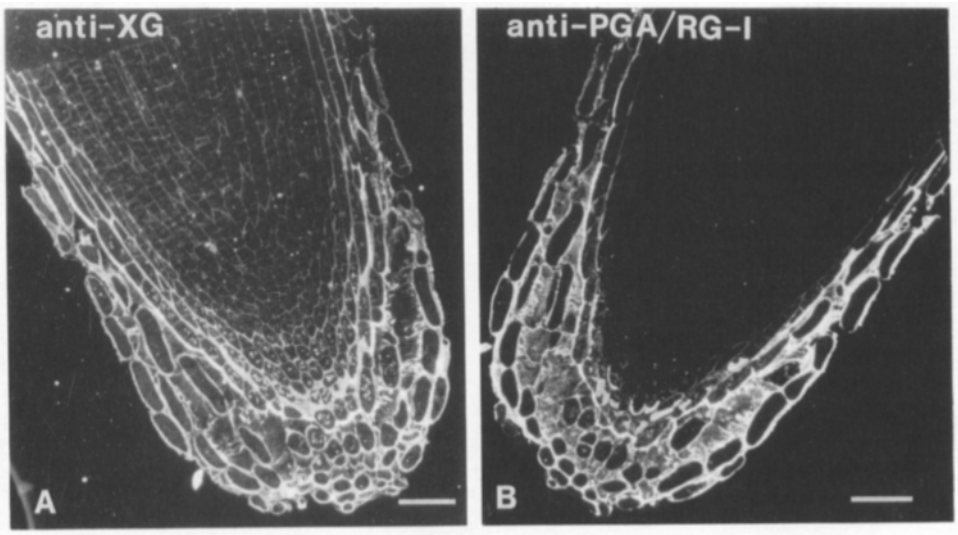

Figure 2. Immunofluorescence labeling of the clover root with anti-XG and anti-PGA/RG-I. Labeling with anti-XG (*A*) shows that XG epitopes are detected in all walls in all cell types, but are most abundant in walls of the epidermis and root cap, whereas labeling with anti-PGA/RG-I (*B*) shows that PGA/RG-I epitopes are detected in walls of the epidermal and peripheral root cap cells and in the extracellular mucilage layer. Unlike XG epitopes, PGA/RG-I epitopes are not detected with this technique in walls of columella cells, meristematic cells, cortical cells, or provascular cells. Bars, 50 μm.

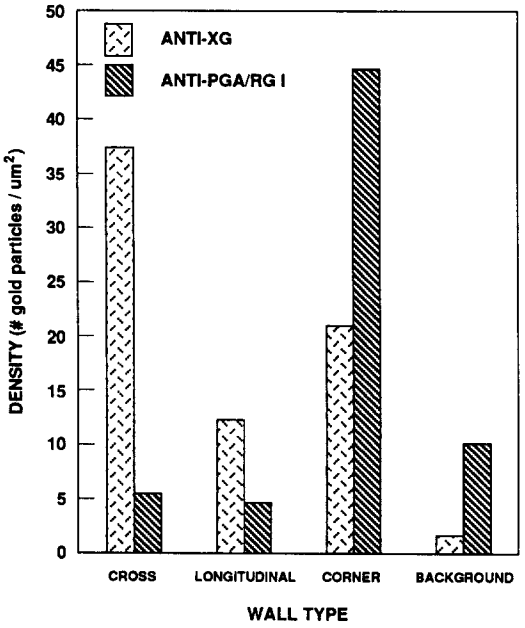

Figure 3. Histogram illustrating the density and distribution of anti-XG and anti-PGA/RG-I immunogold label in the different types of walls of cortical cells. The density of anti-XG labeling is 3.1-fold greater in cross walls than in longitudinal walls, and is 1.9-fold greater than in corners. In contrast, the density of anti-PGA/RG-I labeling is by far the greatest in the corners, and is not present above background levels in either the cross or the longitudinal walls. The density of gold particles was determined in cortical cell walls in the region of division and elongation. Only cell corners with an expanded, electron-dense middle lamella were scored. For quantitation of anti-XG labeling, the mean density of gold particles in 16–19 walls of each type was determined. For quantitation of anti-PGA/RG-I labeling, 12–18 walls of each type were scored to obtain the mean density of gold particles. Nonspecific background gold labeling was determined for both anti-XG and anti-PGA/RG-I by assessing the mean density of gold particles in mitochondria, to which neither antibody specifically binds. For each antibody, the mean density of gold particles in 85–100 cortical cell mitochondria was calculated.

fusion of a newly formed daughter cell wall with the mother longitudinal cell wall, undergo a well-defined sequence of development (Matar and Catesson, 1988). They first expand and develop a triangular, electron-dense middle lamellar region and then an airspace forms through the partial degradation in the central region of the corner.

At all stages of development of cortical cell walls, XG epitopes and PGA/RG-I epitopes are present in complementary and nonoverlapping domains (Moore and Staehelin, 1988). To precisely determine the distribution of XG epitopes and PGA/RG-I epitopes between cross walls, longitudinal walls, and young cell corner domains before the formation of airspaces, we quantitated the density of immunogold labeling by the anti-XG and anti-PGA/RG-I antibodies. As seen in Fig. 3, both XG epitopes and PGA/RG-I epitopes are differentially distributed between the three types of walls. The density of anti-XG labeling is 3.1-fold greater in the relatively younger cross walls than in the older, elongating longitudinal walls and is 1.8-fold greater in the young cross walls than in cell corners.

The distribution of anti-PGA/RG-I labeling contrasts markedly with that of anti-XG. Unlike anti-XG, anti-PGA/RG-I labels neither cross walls nor longitudinal cell walls. In fact, the only wall domain labeled by anti-PGA/RG-I is the middle lamella of cell corners. Quantitation of the density of anti-XG and anti-PGA/RG-I immunolabeling (Fig. 3) highlights the spatial restriction of XG epitopes and PGA/RG-I epitopes to discrete and nonoverlapping domains in cortical cell walls.

Both immunoblot (Table I) and preabsorption/immunogold labeling experiments show that anti-PGA/RG-I has a higher affinity to unesterified PGA than to methylesterified PGA. To ascertain if the expanded middle lamella of the cell corners contains additional PGA/RG-I epitopes, which are masked by methylesterification, we quantitated the effect of pretreatment of sections with PME on the anti-PGA/RG-I immunolabeling of cortical cell corners. Fig. 4 shows that PME pretreatment does indeed increase the density of anti-PGA/RG-I immunolabeling in the middle lamella of cell corners at both of the stages of corner development we examined. In the less mature corners, PME caused a 43–51% increase in labeling density compared to controls (Fig. 4, widely hatched bars). In the more mature cell corners with airspaces, PME-induced increase was 64–74% (Fig. 4, densely hatched bars). Notably, anti-PGA/ RG-I labeling of the cell wall proper (the area outside the middle lamella) remained low even after PME treatment (not shown), which suggests that there are no masked PGA/RG-I epitopes in the cell wall proper.

Expression of XG Epitope- and PGA/RG-I Epitope-containing Molecules Is Dramatically Increased in Mucilage-secreting Epidermal and Root Cap Cells

As illustrated in Fig. 2, there is a sharp transition in the expression of XG epitopes and PGA/RG-I epitopes between epidermal cells and cortical cells. This transition zone, shown in Fig. 5 *A*, was examined with greater resolution in electron micrographs of immunogold-labeled tissues. Fig. 5 *B* demonstrates that XG epitopes are present throughout the longitudinal wall that divides the epidermal cell layer from the cortical cell layer, but are more dense in the half of the wall bordering the epidermal cell. In contrast, the PGA/RG-I epitopes are found exclusively in the half of the wall bordering the epidermal cell (Fig. 5 *C*). In the longitudinal wall that divides the inner layer of epidermal cells from the outer layer of epidermal cells (Fig. 5 *A*), PGA/RG-I epitopes are present throughout most of the wall, but are strikingly absent from the central-most region of the wall (Fig. 5 *D*). Double im-

Table II. Double labeling of Secretory Vesicles in Epidermal Cells with Anti-XG and Anti-PGA/RG-I Antisera

Size of gold label	Vesicles labeled*	Vesicles
	n	%
10 nm (XG)	36	15
20 nm (PGA/RG-I)	49	20
Both	158	65

* With two or more gold particles.

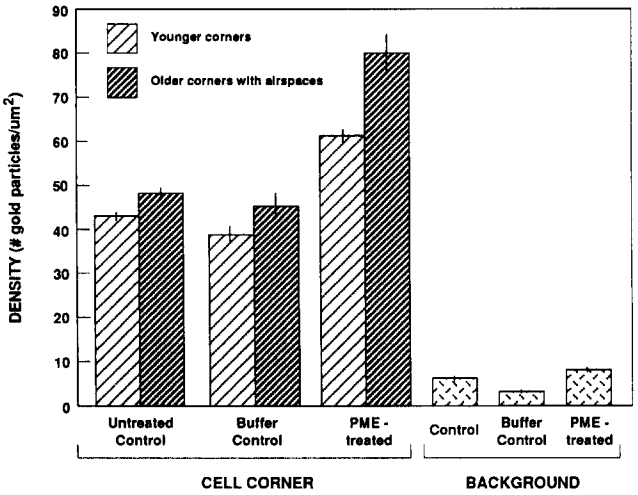

Figure 4. Histogram illustrating the effect of PME treatment on anti-PGA/RG-I immunolabeling of the expanded middle lamella of cortical cell corners. PME pretreatment significantly increases density of anti-PGA/RG-I labeling in both developmentally young corners with a solid electron-dense middle lamella and in more developmentally advanced corners in which airspace formation has begun. Error bars on histogram indicate the SEM. The density of nonspecific background binding of anti-PGA/RG-I was determined as described in the legend to Fig. 3. Between 60 and 75 cortical cell mitochondria were scored in each of the three types of pretreated sections.

munolabeling experiments demonstrate that in epidermal cells of the outer layer, XG epitopes and PGA/RG-I epitopes localize together both intracellularly in secretory vesicles and in the cell wall and in the overlying layer of secreted mucilage (Fig. 6). Quantitation of the distribution of small (anti-XG) and large (anti-PGA/RG-I) gold particles in secretory vesicles establishes that the antibodies label a largely, but not completely, overlapping population of secretory vesicles (Table II). Thus, unlike in cortical tissues, in which XG epitopes and PGA/RG-I epitopes are present in complementary and nonoverlapping domains of the wall (Fig. 3), these epitopes largely colocalize in epidermal cell walls (Fig. 6).

Like cells of the root tip proper, cells of the cap region are derived from cells in the meristematic region. As more cells are generated at the meristem, previously generated cells are displaced into the root cap. Cells first differentiate into columella cells, which function in graviperception (Moore and Evans, 1986). When these cells are further displaced into the outer region of the root cap, they differentiate into peripheral cells, whose chief function is to synthesize and secrete a soluble polysaccharide mucilage (for review see Rougier, 1981). As illustrated in Figs. 7 and 9, the synthesis and localization of both XG epitopes and PGA/RG-I epitopes varies distinctly with state of differentiation of the root cap cells. In the walls of columella cells, XG epitopes are present throughout the cell wall proper but are notably absent from the central most region of the cell corners (not shown). During subsequent differentiation into peripheral cells, XG epitope-containing molecules are found in all cell wall regions and accumulate to a high density in the mucilage layer that covers the outermost cell walls (not shown). The change in density and pattern of immunolabeling is even more pronounced for the anti-PGA/RG-I antibodies (Fig. 7). PGA/RG-I epitopes are rarely detected in columella cell walls (Fig. 7 *A*). However, as cells move outward and begin to differentiate into peripheral cells, there is a dramatic activation of PGA/RG-I epitope expression. This clearly marks the transition between inner and outer cap cells and is evidenced by the appearance of a narrow band of anti-PGA/RG-I labeling in the cell wall just outside the plasma membrane (Fig. 7 *B*). Older peripheral cells exhibit a high density of anti-PGA/RG-I label throughout the entire wall and the mucilage layer (Fig. 7 *C*). An opposite pattern of labeling is seen with JIM 7. While JIM 7 labels the walls of columella cells, it fails to label either the cell wall or the mucilage of peripheral cells (Fig. 8).

As shown in Fig. 9, *A–D*, these developmental changes in the patterns of labeling of root cap cells with the anti-PGA/RG-I and anti-XG antibodies are matched by equally striking changes in the labeling of the Golgi apparatus, the *trans*-Golgi network (TGN), and secretory vesicles of different cell types. In columella cells of the root cap, the anti-XG antibodies sparsely label the limited number of small secretory vesicles (Fig. 9 *B*). Consistent with the absence of any anti-PGA/RG-I epitopes in the cell walls of columella cells, neither the Golgi cisternae nor associated secretory vesicles show any anti-PGA/RG-I labeling (Fig. 9 *A*). In contrast, in mucilage-secreting peripheral cells, both anti-XG and anti-PGA/RG-I label large swollen secretory vesicles in close association with the TGN and the *trans* face of the Golgi apparatus (Fig. 9, *C* and *D*). These immunolabeling patterns imply that the conversion of the columella cells into mucilage-secreting peripheral cells involves the activation of synthesis and secretion of large quantities of XG epitope-containing and PGA/RG-I epitope-containing molecules.

Discussion

Characterization of the Epitopes Recognized by the Anti-XG and Anti-PGA/RG-I Antibodies

In this study, we used antibodies raised against the hemicellulose XG and the pectin PGA/RG-I to map the distribution of epitopes recognized by these antibodies in the developing root tip of clover. The anti-XG serum was raised in rabbits against a highly purified fraction of SEP XG. As shown in Table I, the only major cell wall components that anti-XG recognizes in small (≈1 ng) quantities are SEP XG (which contains a terminal fucosyl residue), TAM XG (which contains no terminally linked fucose), and β-(1→3)-(1→4)-D-glucan. Moreover, anti-XG labeling of cortical cell walls is abolished by preabsorption with either SEP XG or TAM XG and greatly reduced with β-(1→3)-(1→4)-D-glucan. Since dot blots show that anti-XG recognizes both types of xyloglucans, β-(1→3)-(1→4)-D-glucan, and solubilized cellulose (Table I and Results), and since anti-XG labeling of cortical cell walls is abolished by preabsorption with either SEP XG or TAM XG, and is greatly reduced by preabsorption with β-(1→3)-(1→4)-D-glucan, we hypothesize that the major component of the anti-XG antiserum recognizes the β-(1→4)-linked D-glucosyl backbone, which is the only structural domain common to all of these molecules. The anti-PGA/RG-I antibodies react strongly with PGA, RG-I, and commercially

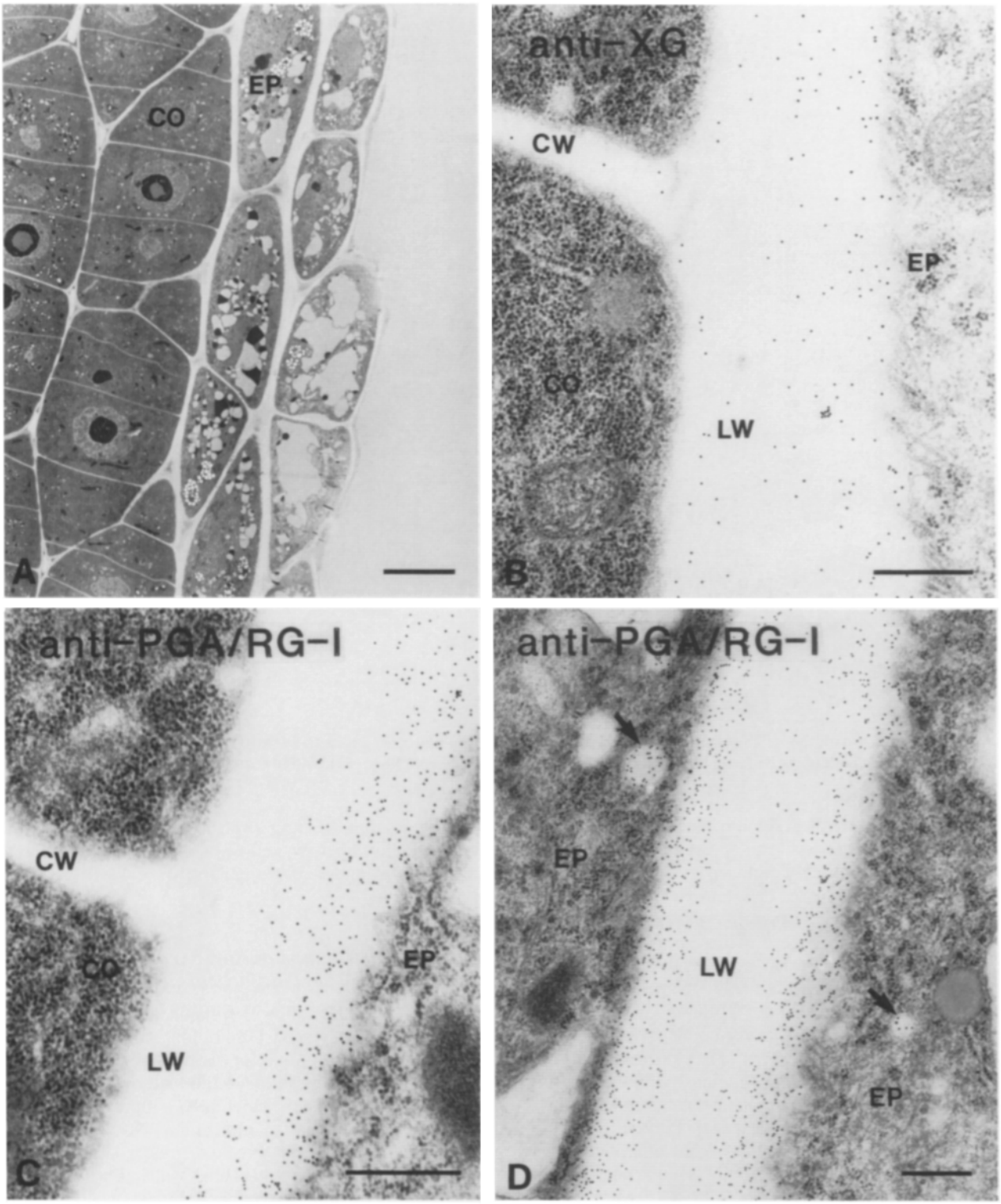

Figure 5. Immunolabeling of epidermal cells. (*A*) Overview of the longitudinal wall shared by epidermal cells (*EP*) and cortical cells (*CO*). (*B*) Anti-XG labels all domains of the longitudinal wall, albeit more densely the half of the wall nearest the epidermal cell. The cross wall (*CW*) between cortical cells is also labeled. (*C*) Anti-PGA/RG-I labels only the half of the longitudinal wall nearest the epidermal cell. No label is seen over the half of the longitudinal wall nearest the cortical cell or in the cross wall between cortical cells. (*D*) Anti-PGA/RG-I labeling of the longitudinal wall shared by an inner epidermal cell and an outer epidermal cell. Note the conspicuous absence of label in the central most region of the wall. Bars: (*A*) 10 μm; (*B–D*) 0.5 μm.

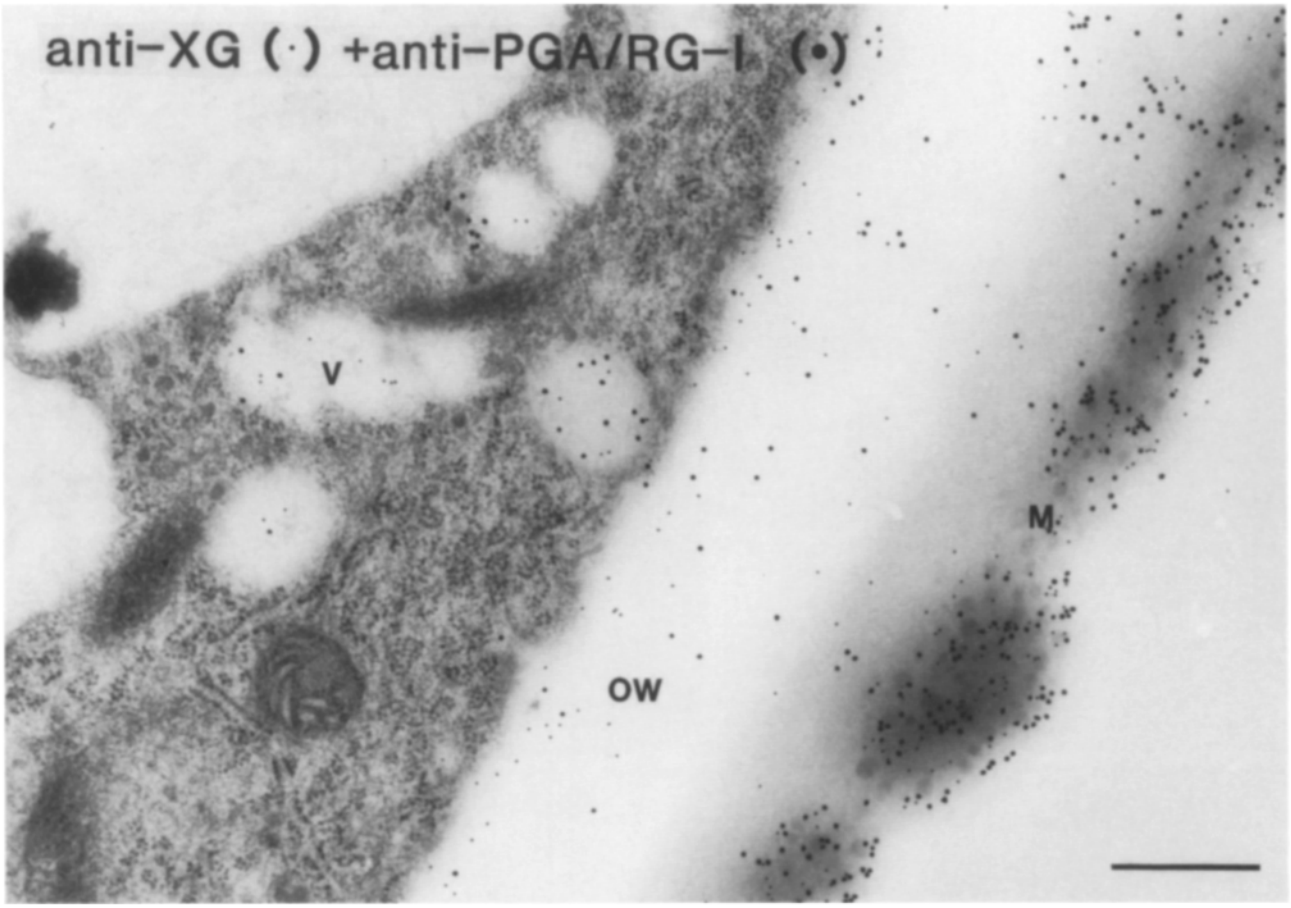

Figure 6. Double immunolabeling of an outer epidermal cell with anti-XG (10-nm gold) and anti-PGA/RG-I (20-nm gold) shows that the pattern of labeling is completely overlapping both in the outer cell wall (*OW*), the mucilage (*M*), and in secretory vesicles (*V*). Bar, 0.5 μm.

available pectin, but not with arabinogalactan, nor RG-II, or any of the other plant cell wall molecules tested (Table I). These antibodies were raised in rabbits against a highly purified SEP RG-I fraction prepared by extensive endopolygalacturonase digestion and deesterification of a pectic polysaccharide fraction of SEPs, a procedure designed to remove all covalently linked PGA (Materials and Methods). However, as documented in Table I, the RG-I fraction injected into the rabbit gave rise to antideesterified PGA antibodies, suggesting that at least some of the purified RG-1 molecules contained short but antigenic PGA tails. This is not surprising given that at least three consecutively linked unesterified α-(1→4)-D-galacturosyluronic acid residues must be present for endopolygalacturonase to catalyze cleavage of these linkages (York et al., 1985). Since the anti-PGA/RG-I antibodies bind strongly to both PGA and to RG-I (Table I), and can be completely preabsorbed by either PGA or with RG-I, we propose that the most likely epitope recognized by the anti-PGA/RG-I antibodies is the PGA-α-(1→2)-L-rhamnosyl transition region of PGA/RG-I, a structural domain which is also present in the rhamnose-interrupted linear PGA polymers (Bacic et al., 1988). Interestingly, the JIM 5 antibodies, which also recognize unesterified PGA, do not recognize the RG-I molecules used to produce our anti-PGA/RG-I antibodies (Knox et al., 1990) and thus appear to recognize the unesterified galactosyluronic acid backbone of PGA. The idea that anti-PGA/RG-I and JIM 5 recognize different epitopes is supported by immunolabeling experiments which show that these antibodies label the cell walls of root tips in different patterns (Moore and Staehelin, 1988; Knox et al., 1990; and this paper).

Differential Distribution of Anti-XG and Anti-PGA/RG-I Label in Cortical Cell Walls

In cortical cells in the region of division and elongation, cell division occurs transversely but not longitudinally. A newly formed cross wall is thus developmentally younger and more closely related in composition to cell plate vesicles than the flanking longitudinal wall, which is at a more developmentally advanced stage, that of elongation. Notably, XG epitopes are detected at over threefold greater density in the cross walls than in the longitudinal walls (Fig. 3). These results could be attributed to either a greater accessibility of XG epitopes in cross versus longitudinal cell walls, to a dilution of the XG epitopes during wall expansion, or to preferential degradation of XG epitopes in longitudinal cell walls. We favor, however, a fourth interpretation, i.e., that the Golgi apparatus in cortical cells produces at least two different populations of secretory vesicles: vesicles that give rise to the cell plate and contain a relatively large proportion of XG relative to other matrix molecules, and vesicles destined for elongating longitudinal cell walls, which contain a lesser proportion of XG, and thus a greater proportion of

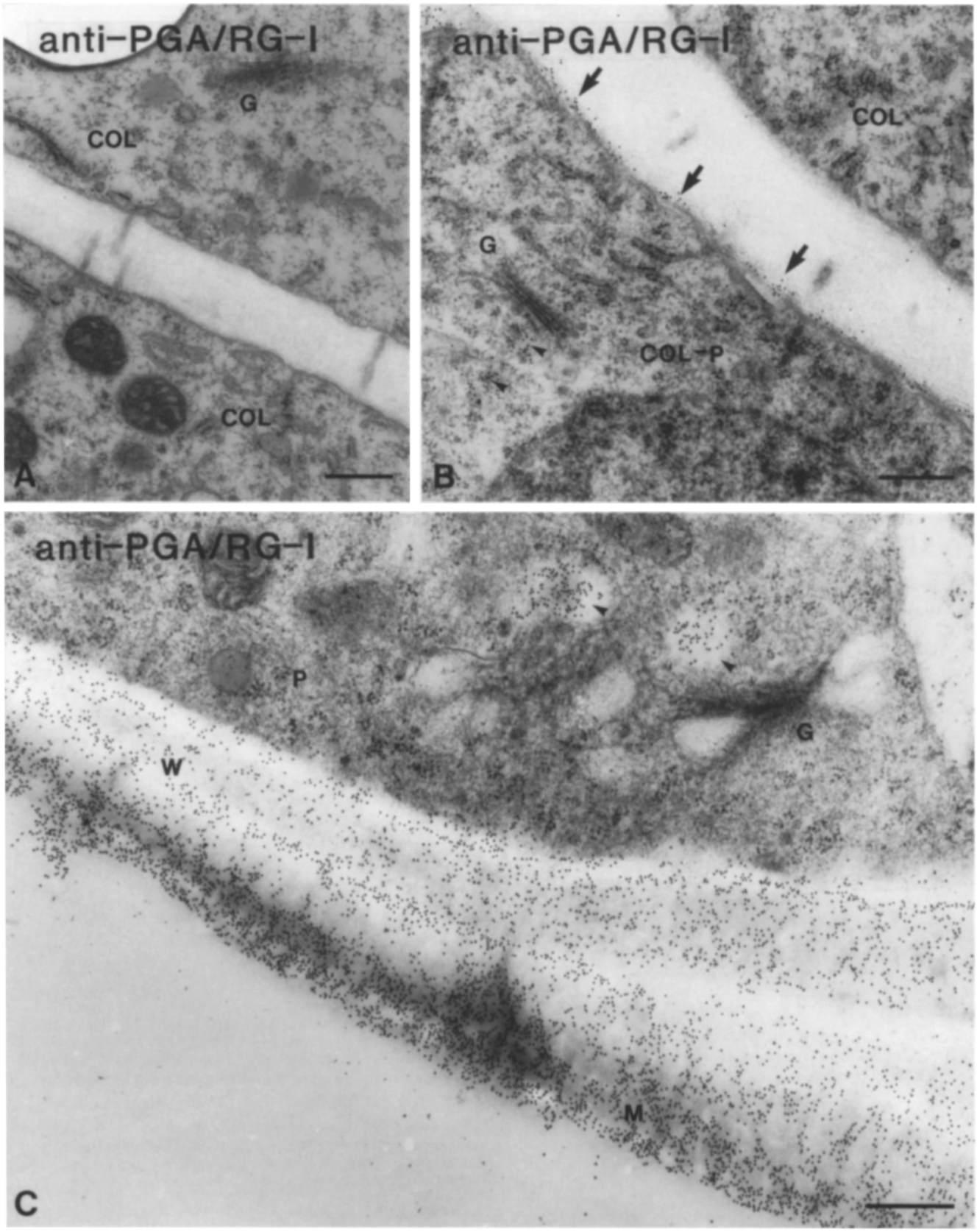

Figure 7. Anti-PGA/RG-I labeling of columella cells and peripheral cells of the root cap. (*A*) Anti-PGA/RG-I does not significantly label the walls of columella cells (*COL*). Few secretory vesicles are seen in these cells, and when they are present, they are unlabeled. *G*, Golgi apparatus. (*B*) In columella cells beginning to differentiate into peripheral cells (*COL-P*), anti-PGA/RG-I labels secretory vesicles (*arrowheads*) and the region of the cell wall just outside the plasma membrane (*arrows*). (*C*) In peripheral mucilage-secreting cells, anti-PGA/RG-I abundantly labels the cell wall (*W*), overlying mucilage layer (*M*), and secretory vesicles (*arrowheads*).

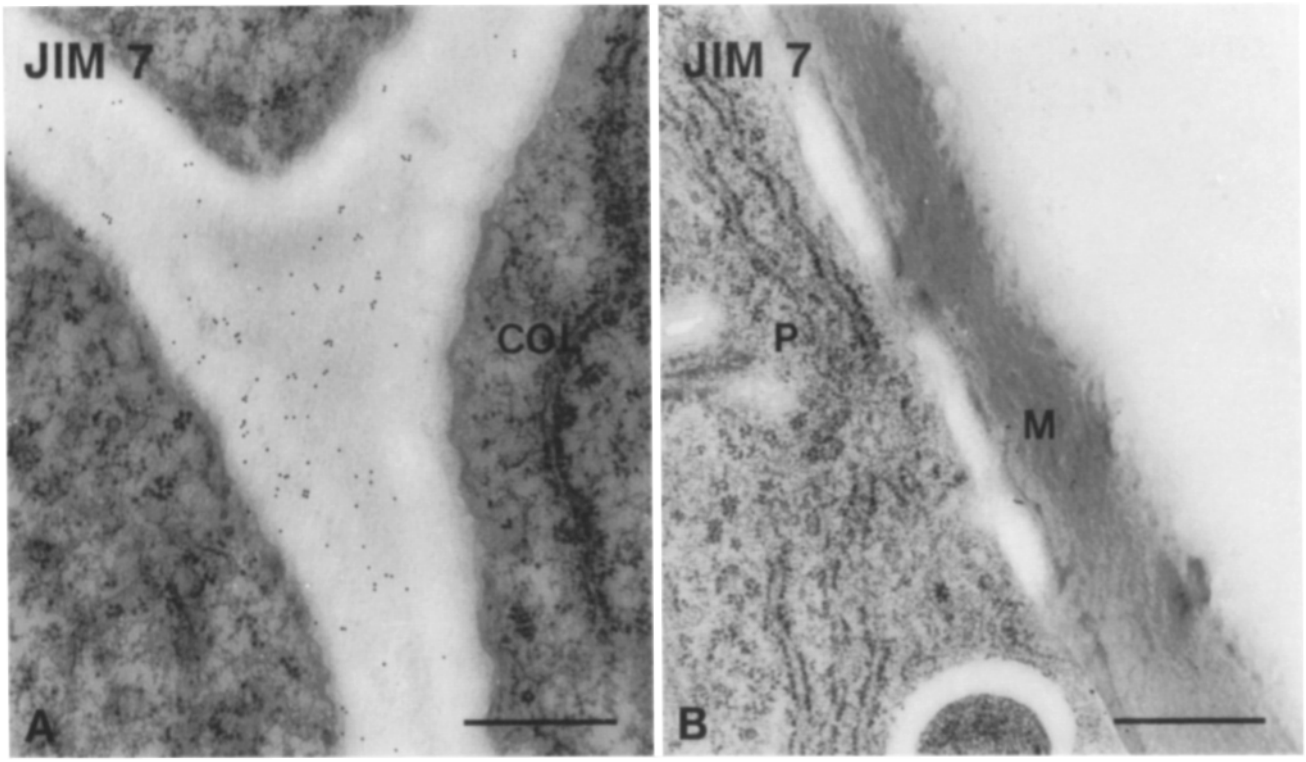

Figure 8. JIM 7 labeling of the cell wall and mucilage (*M*) in columella cells (*A*) and peripheral root cap cells (*B*). Whereas JIM 7 epitopes are abundant in cell walls of columella cells (*A*), they are present at extremely low levels in the wall and mucilage (*M*) of peripheral root cap cells (*B*). Bars, 0.5 μm.

other matrix molecules than the vesicles destined for the cell plate. This interpretation predicts that given subpopulations of secretory vesicles are specifically targeted to either the region of the future cross cell wall or to the elongating longitudinal cell wall, respectively. This view is supported by not only the results presented in Fig. 3, but also by a number of other lines of evidence. Previous results from our laboratory showed that cell plate vesicles do indeed contain XG epitopes (Moore and Staehelin, 1988). Moreover, the close association between cell plate vesicles and microtubules during cell division suggests that these vesicles may be targeted to the plane of the future daughter cell wall by a microtubule-mediated targeting mechanism (Gunning and Steer, 1975). In addition, the fact that longitudinal cell walls of parenchymous cells elongate to up to 10 times their original length (without cell wall thinning), while the cross cell walls elongate to only 1.7 times their original length (Hayashi, 1989), implies that secretory vesicles containing cell wall precursors may be preferentially targeted to the longitudinal wall in elongating cortical cells in the root tip.

While XG epitopes are localized in all but the middle lamella of cortical cell walls, PGA/RG-I epitopes are localized exclusively to this region both before and after airspace formation (Moore and Staehelin, 1988). In addition to PGA/RG-I, other molecules, such as pectins, β-(1→3)-glucanase, and hydroxyproline-rich glycoproteins have been localized by immunogold labeling to the middle lamella of the cell corners (Roberts, 1990; Swords and Staehelin, 1989). A common feature of these cell corner components is that they all appear to be involved in plant defense responses against stress or pathogens (Mauch and Staehelin, 1989; Ryan and Farmer, 1991). By localizing these defense-related molecules to regions where an initial plant–pathogen interaction is likely to occur, e.g., to the middle lamella material that coats the airspaces in cell corners, the plant might rapidly mobilize its defenses. Our previous finding (Moore et al., 1991) that in root cortical parenchyma, XG epitopes and PGA/RG-I epitopes are found not only in separate cell wall domains, but also in separate populations of secretory vesicles, is consistent with the interpretation that there is targeting of different populations of secretory vesicles to particular cell wall domains.

Structural and Functional Aspects of Methylesterification of Pectic Polysaccharides

To confirm our findings showing that anti-PGA/RG-I specifically recognizes the unesterified PGA/RG-I epitopes, and to test the hypothesis that the middle lamella contains both methylesterified and unesterified forms of PGA/RG-I, we pretreated thin sections of clover root tip with PME to deesterify pectins in situ and then immunolabeled them with anti-PGA/RG-I. As seen in Fig. 4, PME significantly increased the density of labeling in the middle lamella of cell corners compared with controls. The magnitude of increase in labeling density is similar to that observed by Vreeland et al. (1989), who, using a fluorescently labeled polygalacturonate hybridization probe to detect unesterified pectins in the cell wall, found that chemical deesterification of pectins increased the intensity of labeling 1.1–1.9-fold in leaf tissue of the dicotyledon *Dubautia*. Since other immunolabeling studies done with JIM 7 and JIM 5 implied that methyl-

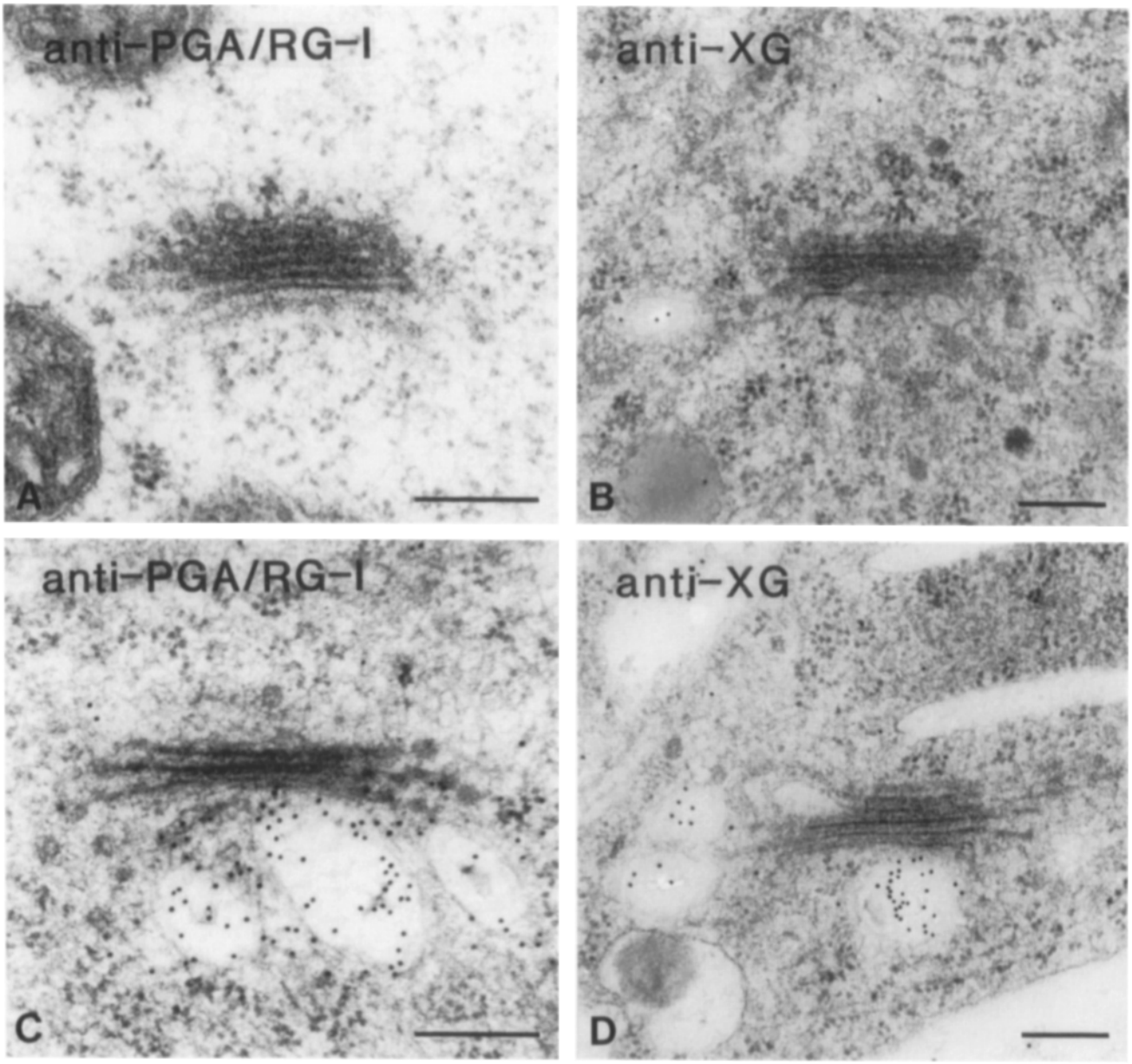

Figure 9. Anti-XG and anti-PGA/RG-I labeling of the Golgi apparatus and secretory vesicles in peripheral root cap cells. In columella cells, there is little, if any, labeling of secretory vesicles by anti-PGA/RG-I (*A*), whereas anti-XG labels secretory vesicles nearby the Golgi apparatus (*B*). The secretory vesicles themselves are small and sparse. (*C* and *D*) In peripheral cells, secretory vesicles are larger and more numerous. Both anti-PGA/RG-I (*C*) and anti-XG (*D*) label such secretory vesicles. Bars, 0.25 μm.

esterified pectins appear to be relatively abundant in the cell wall proper, we were surprised that anti-PGA/RG-I labeling of this region still remained extremely low even after PME treatment. These seemingly contradictory results can be reconciled by the hypothesis that polysaccharides containing both unesterified and methylesterified PGA/RG-I epitopes are targeted specifically to the middle lamella, whereas those containing JIM 7 epitopes and masked methylesterified JIM 5 epitopes are localized throughout the wall.

Functional Implications of the Differential Localization of XG Epitopes and PGA/RG-I Epitopes

Figs. 3, 5, and 6 show that the distribution of XG epitopes and PGA/RG-I epitopes differs between cell types; i.e., between epidermal cells and cortical cells of the clover root. As mentioned above, the PGA/RG-I epitopes are limited to the expanded middle lamella region of the cell corners of cortical cells, whereas XG epitopes are excluded from it. These same epitopes are present throughout the outer walls of epidermal cells and are especially abundant in the secreted mucilage. The localization patterns of PGA/RG-I epitopes in longitudinal walls shared by a cortical cell and an epidermal cell (Fig. 5 *C*) and those shared by adjacent layers of epidermal cells (Fig. 5 *D*) are especially intriguing. In the former, PGA/RG-I epitopes are present only in the half of the wall adjacent to the epidermal cell, and in the latter, are strikingly excluded from the central-most middle lamella region of the

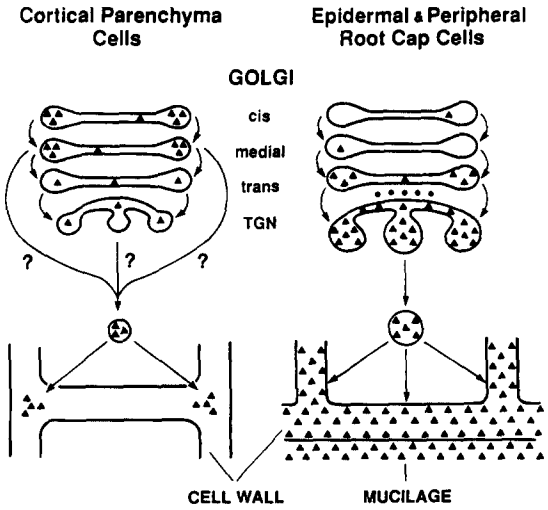

Figure 10. Diagrammatic model illustrating the distribution of PGA/RG-I epitopes in different cell types in the clover root tip and possible trafficking patterns of complex polysaccharides of the cell wall and mucilage in the Golgi apparatus of plant cells. We postulate that these differential patterns reflect a functional specialization of the Golgi in a cell type-specific manner.

wall. These observations are consistent with the hypothesis that localization of wall polysaccharides is spatially restricted to particular domains in the cell wall by the middle lamella, which acts as a barrier to prevent free diffusion of wall molecules throughout the entire width of the wall.

We hypothesize that the greater abundance of PGA/RG-I epitopes in epidermal cells than in cortical cells reflects the functional differences between these two cell types. Epidermal cells function in water and mineral absorption and act as a first line of defense against pathogens. It is therefore not surprising that PGA/RG-I, which has water-binding, molecular sieving, and ion-exchange capabilities (for review see Bacic et al., 1988), and which serves as a source of PGA fragments that elicit plant defense responses against pathogens (Ryan and Farmer, 1991), would be present in all of the types of walls surrounding epidermal cells. In contrast, cortical cells function primarily in storage, so there is not the same need for PGA/RG-I to be distributed throughout the cell walls. Instead, it is present only in the middle lamella of cell corners. The formation of airspaces through degradation of the center of the middle lamella creates a path not only for gas exchange but also for pathogen invasion. The PGA/RG-I in the middle lamella bordering airspaces would therefore serve as a ready source of PGA fragments for elicitation of plant defense responses as postulated by Mauch and Staehelin (1989).

Synthesis of Hemicellulosic and Pectic Polysaccharides Is Highly Regulated during Cellular Differentiation in the Root Cap

Concomitant with the differentiation of the graviperceptive columella cells of the inner root cap into the mucilage-secreting peripheral cells, there is a dramatic increase in anti-XG and anti-PGA/RG-I labeling of intracellular secretory vesicles and of the cell wall and the mucilage layer (Figs. 7 and 9). These results demonstrate that mucilage contains molecules structurally related to cell wall matrix polysaccharides. Consistent with this interpretation are the findings that a cellulase-gold complex that binds to β-(1→4)-D-glucans labeled the root cap mucilage of *Calluna vulgaris* (Peretto et al., 1990) and that mAbs that recognize pectic polysaccharides labeled the epidermal mucilage of carrot (Knox et al., 1990) and the root cap mucilage of *Calluna vulgaris* (Peretto et al., 1990). Biochemical studies of mucilage (Wright and Northcote, 1974, 1976) established that maize root mucilage contained a β-(1→4)-linked D-glucosyl backbone linked to pectic-like polysaccharides rich in galacturonic acid, arabinose, galactose, and fucose. The finding that clover root mucilage is labeled by the anti-XG antibodies, which recognize the β-(1→4)-linked D-glucosyl backbone of XG, supports these biochemical studies. Similarly, the presence of significant amounts of galacturonic acid in mucilage (Wright and Northcote, 1974) correlates with the observation of mucilage labeling by anti-PGA/RG-I, which appears to react specifically with the transition region between the unesterified PGA domains and RG-I domains of PGA/RG-I.

As illustrated in Figs. 7 and 8, the degree of esterification of pectic polysaccharides is highly regulated during cellular differentiation in the root cap. As columella cells differentiate into peripheral cells—concomitant with the disappearance of methylesterified PGA (as detected with JIM 7)—a dramatic increase in both the synthesis and secretion of unesterified PGA/RG-I (as detected with anti-PGA/RG-I) occurs. The disappearance of JIM 7 epitopes implies that either methylesterified PGA is preferentially degraded in peripheral cells and/or it is deesterified by PME. Localization of PME activity would distinguish between these two possibilities. Conversely, both synthesis and secretion of unesterified PGA/RG-I is activated during differentiation of the peripheral root cap cells. Thus localized PME activity in the cell wall cannot be solely responsible for potential differences in esterification. The high concentrations of PGA/RG-I epitopes in peripheral cells may reflect the synthesis of copious amount of mucilage which contains large amounts of gel-forming unesterified pectins.

Structural and Functional Reorganization of the Golgi Apparatus Varies with Cell Type and Cell Function

As reported by Staehelin et al. (1990) the macromolecular organization of plant Golgi stacks varies in a cell type-specific manner in root tips of *Arabidopsis* and *Nicotiana*. This cell type-specific differentiation was postulated to reflect a tailoring of the Golgi stack and the TGN to meet the specific synthetic demands of each cell type. The Golgi stacks in the actively dividing meristematic cells, from which the columella and peripheral cells of the root cap differentiate, are small, compact structures of 5–6 cisternae lacking intercisternal elements and associated with a lightly staining TGN. In contrast, the Golgi stacks of peripheral root cap cells, in which large quantities of polysaccharide mucilage are being synthesized, are larger, contain easily recognizable intercisternal elements, and are associated with an extensive TGN filled with darkly staining polysaccharide mucilage. Thus, in plant (Staehelin et al., 1990) as well as in animal (Griffiths et al., 1985) cells, the structure and extent of the TGN also varies dramatically with the secretory state of the cell.

Fig. 10 diagrammatically illustrates the cell type–specific structural and functional variability of the Golgi apparatus observed in the developing root. It was previously demonstrated that at a precise stage of cortical cell elongation in root tips the PGA/RG-I epitopes are detected largely in *cis*- and medial Golgi cisternae, and the anti-XG epitopes largely in *trans*-Golgi cisternae and the TGN (Moore et al., 1991; Fig. 10). In this study it is shown that in mucilage-secreting epidermal and peripheral root cap cells the anti–PGA/RG-I epitopes predominantly colocalize with the anti-XG epitopes in swollen vesicles associated with the *trans*-Golgi cisternae and the TGN (Fig. 6 and Table II). What makes this shift in the functional organization of Golgi stacks even more intriguing is the fact that in the cortical parenchyma tissue the PGA/RG-I epitopes are detected exclusively in the expanded middle lamella of the cell corners, whereas in the epidermal and root cap peripheral tissues, the PGA/RG-I epitopes are found throughout the cell walls and the cell corners as well as in the mucilage layer (Fig. 10). These results are consistent with the hypothesis that the enzymes involved in synthesis of polysaccharides containing XG epitopes and PGA/RG-I epitopes are localized to different Golgi cisternae in functionally different cell types. Such cell type–specific variability in the functional organization of the Golgi stacks has also been observed in animal cells. Specifically, Roth et al. (1986) demonstrated that the subcompartmental localization of sialyltransferase in the Golgi apparatus differs in functionally different types of intestinal epithelial cells. Future studies will determine if there is a causal relationship between the putative subcompartmental sites of synthesis and sorting of polysaccharides in the plant Golgi stack and their targeting to and localization in the cell wall.

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