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Developmental and Tissue-Specific Structural Alterations of the Cell-Wall Polysaccharides of *Arabidopsis thaliana* Roots[1]

Glenn Freshour, Ronald P. Clay, Melvin S. Fuller, Peter Albersheim, Alan G. Darvill, and Michael G. Hahn*

The University of Georgia, Complex Carbohydrate Research Center and Departments of Botany (G.F., R.P.C., M.S.F., M.G.H.) and Biochemistry and Molecular Biology (P.A., A.G.D.), 220 Riverbend Road, Athens, Georgia 30602–4712

The plant cell wall is a dynamic structure that plays important roles in growth and development and in the interactions of plants with their environment and other organisms. We have used monoclonal antibodies that recognize different carbohydrate epitopes present in plant cell-wall polysaccharides to locate these epitopes in roots of developing *Arabidopsis thaliana* seedlings. An epitope in the pectic polysaccharide rhamnogalacturonan I is observed in the walls of epidermal and cortical cells in mature parts of the root. This epitope is inserted into the walls in a developmentally regulated manner. Initially, the epitope is observed in atrichoblasts and later appears in trichoblasts and simultaneously in cortical cells. A terminal α-fucosyl-containing epitope is present in almost all of the cell walls in the root. An arabinosylated (1→6)-β-galactan epitope is also found in all of the cell walls of the root with the exception of lateral root-cap cell walls. It is striking that these three polysaccharide epitopes are not uniformly distributed (or accessible) within the walls of a given cell, nor are these epitopes distributed equally across the two walls laid down by adjacent cells. Our results further suggest that the biosynthesis and differentiation of primary cell walls in plants are precisely regulated in a temporal, spatial, and developmental manner.

The understanding of the role(s) of cell walls in plant biology has evolved considerably in recent years. Whereas the function of cell walls was initially viewed as primarily providing form and structure to plant cells, it has now become clear that the wall has a variety of other functions in plant growth and development (Roberts, 1990) and in the interactions of plants with their environment and other organisms (Hahn et al., 1989). It is therefore important to learn where the macromolecular components are located within the walls of individual cells and within tissues and how the structures of the wall polymers change as a function of plant growth and development and during active defense against disease and environmental stress.

Monoclonal antibodies have proven to be valuable tools for studies of the dynamics of cell surface glycoconjugates in animals (Hakomori, 1984; Feizi and Childs, 1987). Studies of the temporal, spatial, and developmental dynamics of plant cell-wall glycoconjugates have lagged behind corresponding animal studies because of the more limited collection of monoclonal antibodies against cell-wall carbohydrate epitopes that is available and because of the difficulty in characterizing the epitopes recognized by the available antibodies. Previous studies of plant cell-wall dynamics have focused on cell surface glycoproteins, since arrays of monoclonal antibodies have been generated against two types of such complex glycoconjugates, the arabinogalactan-proteins (Anderson et al., 1984; Brewin et al., 1985; Norman et al., 1986; Villanueva et al., 1986; Hahn et al., 1987; Knox et al., 1989, 1991; Pennell et al., 1989, 1991; Horsley et al., 1993; Puhlmann et al., 1994; Kreuger and Van Holst, 1995) and Hyp-rich glycoproteins (Smallwood et al., 1994, 1995; Knox et al., 1995). Immunohistochemical studies have documented subcellular (Herman and Lamb, 1992; Rae et al., 1992; Van Aelst and Van Went, 1992; Horsley et al., 1993; Sherrier and VandenBosch, 1994) and cell-type-specific (Knox et al., 1989, 1991; Stacey et al., 1990; Van Aelst and Van Went, 1992; Benfey et al., 1993; Schindler et al., 1995) distribution of arabinogalactan epitopes. Several studies have followed the temporal and spatial expression of these glycoprotein epitopes as a function of plant development (Stacey et al., 1990; Pennell et al., 1991, 1992; Smallwood et al., 1994) and during interactions with microorganisms (VandenBosch et al., 1989; Rae et al., 1992). The observed patterns suggest roles for these glycoproteins in the establishment of cellular identity and, hence, anatomical structures in plant tissues.

The available anti-polysaccharide monoclonal antibodies include those against homogalacturonans (Liners et al., 1989; VandenBosch et al., 1989; Knox et al., 1990), callose [(1→3)-β-glucan] (Meikle et al., 1991), and the (1→3) and (1→4) mixed-linked β-glucans of cereals (Meikle et al., 1994). These antibodies have been used to locate polysaccharide epitopes within plant cells (Vian and Roland, 1991; Liners and Van Cutsem, 1992; Van Aelst and Van Went, 1992; Vian et al., 1992; Zhang and Staehelin, 1992; Sherrier

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* Corresponding author; e-mail hahn@mondl.ccrc.uga.edu; fax 1–706–542–4412.

Abbreviations: KPB, 50 mM potassium phosphate buffer, pH 6.9; KPBS, 10 mM potassium phosphate, pH 7.2, containing 500 mM NaCl.

and VandenBosch, 1994; Casero and Knox, 1995; Geitmann et al., 1995) and tissues (VandenBosch et al., 1989; Bonfante-Fasolo et al., 1990; Knox et al., 1990; Peretto et al., 1990; Meikle et al., 1991, 1994; Rae et al., 1992). The antibodies against homogalacturonans have been used to obtain evidence that pectins of different degrees of methyl-esterification localize to different sites in plant cells and tissues (Knox et al., 1990; Liners and Van Cutsem, 1992; Vennigerholz, 1992; Li et al., 1994; Liners et al., 1994; McCann et al., 1994; Wells et al., 1994).

We have generated a small panel of monoclonal antibodies that recognize carbohydrate epitopes in plant cell-wall polysaccharides (Puhlmann et al., 1994). The epitopes of two of these antibodies were characterized in some detail. CCRC-M1 recognizes a terminal (1→2)-linked α-fucosyl residue, which is present in most dicot cell-wall xyloglucans and is also a minor component of at least some species of rhamnogalacturonan I (Puhlmann et al., 1994). CCRC-M7 binds to an arabinosylated (1→6)-linked β-galactan epitope that is present in rhamnogalacturonan I as well as in arabinogalactan-proteins (Puhlmann et al., 1994; Steffan et al., 1995). Monoclonal antibody CCRC-M2 binds exclusively to rhamnogalacturonan I, although the epitope structure has not been characterized. We are using these monoclonal antibodies to determine the distribution patterns of cell-wall polysaccharide epitopes in developing roots of *Arabidopsis thaliana*.

A. thaliana roots offer several advantages for the study of plant cell-wall dynamics. The architecture of the root is simple and well described (Dolan et al., 1993), permitting the unambiguous identification of cell types in root sections. The outer cell layers of the root (epidermis, cortex, endodermis, and pericycle) each consist of a single layer of cells, except for the presence of a second layer of cortical cells in that portion of the root closest to the hypocotyl. The columellar root cap and one to three layers of lateral root-cap cells are present at the root tip. Developmental lineages of each cell type in the root have been determined (Dolan et al., 1994; Scheres et al., 1994), allowing correlations to be made with the appearance or disappearance of epitopes. A number of root developmental mutants have been isolated in Arabidopsis (Benfey et al., 1993; Benfey and Schiefelbein, 1994; Dolan et al., 1994; Galway et al., 1994; Holding et al., 1994; Aeschbacher et al., 1995; Hauser et al., 1995; Scheres et al., 1995) that may help to elucidate specific functions of wall components. The value of Arabidopsis for these studies is enhanced by the demonstration that the polysaccharide components of the cell walls of *A. thaliana* are similar in quantity and structure to those found in the cell walls of many other higher plants (Zablackis et al., 1995). We report here that different cell-wall polysaccharide epitopes show distinct distribution patterns within cells and tissues of wild-type *A. thaliana* roots, and we demonstrate that at least one polysaccharide epitope in the cell walls is developmentally regulated.

MATERIALS AND METHODS

Plant Culture Conditions

Seeds of *Arabidopsis thaliana* (Columbia) were surface sterilized by immersion in 70% (v/v) aqueous ethanol for 2 min followed by a 10-min immersion in 1% (w/v) sodium hypochlorite (20% Chlorox) containing 0.02% (v/v) Triton X-100. The seeds were then rinsed three times with sterile distilled H_2O and germinated, and the seedlings were grown in sterile Petri dishes on 1% (w/v) agar containing 1% (w/v) Suc, 3 mM KNO_3, 2.3 mM Na_2HPO_4, 2.0 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 50 μM EDTA (disodium salt), 50 μM $FeSO_4$, 50 μM H_3BO_3, 50 μM $MnSO_4$, 18 μM $ZnSO_4$, 2.5 μM KI, 2 μM MoO_3, 0.05 μM $CuSO_4$, and 0.05 μM $CoCl_2$ (at pH 6.0). The Petri dishes were oriented vertically, maintained at 23°C, and received 12 h of fluorescent illumination (100 $\mu E\ m^{-2}\ s^{-1}$) daily.

Antibodies

The generation of murine monoclonal antibodies CCRC-M1, CCRC-M2, and CCRC-M7 has been described (Puhlmann et al., 1994). The characteristics of these antibodies are listed in Table I. Goat anti-mouse IgG (catalog No. M-8642), goat anti-mouse IgG-gold conjugate (10 nm) (catalog No. G-7652), and goat anti-mouse IgG-fluorescein isothiocyanate conjugate (catalog No. F-0257) were purchased from Sigma. Monoclonal antibodies $6D_4$ (Hussey, 1989) (an IgM) and $6F_{11}$ (Davis et al., 1992) (an IgG), which recognize secretory granules within the esophageal glands of *Meloidogyne* spp., were obtained from R.S. Hussey (Department of Plant Pathology, University of Georgia, Athens).

Colloidal Gold Conjugation

Colloidal gold (approximately 15 nm) was prepared from tetrachloroauric acid by the trisodium citrate reduction method of Frens (1973) as modified by Roth (1983). The secondary antibody (goat anti-mouse IgG) was conju-

Table I. *Characteristics of the monoclonal antibodies CCRC-M1, -M2, and -M7*

Antibody	Isotype	Polysaccharides Recognized	Epitope Characteristics	Competitive Ligands	Ref.
CCRC-M1	IgG_1	Xyloglucan Rhamnogalacturonan I	Terminal α-fucosyl residue (1→2)-linked to a galactosyl residue	Xyloglucan (sycamore maple) Fucose	Puhlmann et al., 1994
CCRC-M2	IgM	Rhamnogalacturonan I	Unknown	Rhamnogalacturonan I	Puhlmann et al., 1994
CCRC-M7	IgG_1	Arabinogalactan-proteins Rhamnogalacturonan I	(1→6)-Linked β-galactan carrying one or more arabinosyl residues	Rhamnogalacturonan I 6-*O*-β-D-galactopyranosyl-D-galactose	Steffan et al., 1995

gated to colloidal gold using a modification of the procedure described by Roth (1983). Briefly, 1 mg of lyophilized antibody was dissolved in 50 μL of 80 mM sodium borate buffer, pH 9.0, and briefly sonicated (5 s) to ensure dissolution. Ten milliliters of the 15-nm colloidal gold suspension, adjusted to pH 9.0 with 0.2 M K_2CO_3, was rapidly added to a 15-mL siliconized round-bottom centrifuge tube containing the antibody solution. After a 5-min adsorption period, 500 μL of 1% (w/v) PEG (Carbowax PEG 20M, catalog No. P162–1; Fisher Scientific) were added to the tube and mixed by repeatedly inverting the tube. The preparation was centrifuged at 48,000*g* for 30 min at 5°C. The centrifuge tube was then placed at an angle in a small beaker to permit the mobile pellet containing the stabilized protein-bound colloidal gold particles to separate from the hard pellet containing the unstabilized gold particles. The mobile pellet was recovered with a minimum of supernatant and resuspended in 500 μL of 10 mM potassium phosphate buffer, pH 8.0, containing 500 mM NaCl and 0.02% (w/v) PEG to yield the gold-labeled secondary antibody, which was stored at 4°C until use (no longer than 3 months).

EM

Four-day-old Arabidopsis seedlings, with roots 10 to 12 mm in length, were initially fixed by flooding the Petri plates at room temperature with fixative (KPB containing 2.5% [v/v] glutaraldehyde). After 1 h, the root tissue was gently removed from the agar and transferred to 1-dram vials containing 2 mL of fresh fixative. Vials containing tissue and fixative were cooled to 4°C, and subsequent fixation, dehydration, and embedding procedures were carried out at 4°C. Following 1 h in glutaraldehyde fixative, the tissue was washed with three changes (10 min each) of KPB and postfixed for 1 h in KPB containing 1% (w/v) osmium tetroxide. The fixed tissue was again rinsed three times with KPB before dehydration with a graded aqueous ethanol series (20, 35, 50, 60, 70, 80, 90, 100, 100, 100% [v/v]; 30 min each step). The dehydrated tissue was gradually infiltrated with LR White embedding resin (Ted Pella Inc., Redding, CA) according to the following schedule: 20, 40, 60, and 80% (v/v) LR White:ethanol, each step for 24 h, and 100% LR White for 24 h with a change of resin every 8 h. The infiltrated tissue was transferred to BEEM capsules (Ted Pella) containing 100% LR White for embedding. Polymerization was accomplished by exposure of the capsules to 365-nm UV light at 4°C for 48 h in a UV Cryochamber (Ted Pella).

Osmium was omitted from the fixation in some cases, and, in other cases, the tissue was infiltrated and embedded in Epon-Araldite or Spurr's embedding medium (both purchased from EM Sciences, Fort Washington, PA). However, no labeling was observed, under the conditions described above, with tissue embedded in Spurr's medium. Frequently, little or no labeling occurred in sections embedded in Epon-Araldite, but when labeling was successful, the labeling patterns were identical with those seen in LR White resin.

Thin sections (<100 nm) were cut with an MT 6000-XL (RMC Inc., Tucson, AZ) ultramicrotome and collected on Formvar-coated, gilded copper slot grids (Ted Pella) and placed on Formvar bridges to dry (Rowley and Moran, 1975). Immunolabeling of the sections is described in the next section. After immunolabeling, the sections were poststained for 3 min with 4% (w/v) aqueous uranyl acetate and for 30 s with lead citrate (Reynolds, 1963). The sections were examined at 80 kV with a Zeiss EM 902A electron microscope. The location of each section in the root is expressed relative to the basal wall of central cells at the root tip in accordance with the practice used by Dolan et al. (1993).

Immunolabeling Procedures

All incubations were carried out at room temperature unless otherwise indicated. Thin sections mounted on gilded slot grids were first hydrated by floating the grids for 10 min, section side down, on 10-μL droplets of KPBS containing 0.02% (w/v) PEG. The sections were then treated with 0.1 N HCl for 10 min to remove glutaraldehyde and to increase exposure of epitopes. This was followed by a 10-min incubation with KPBS. Nonspecific antibody-binding sites on the sections were blocked by incubating the sections for 45 min on droplets of 3% (w/v) nonfat dried milk in KPBS. The sections were then incubated for 60 min on droplets of hybridoma supernatant undiluted or diluted 1:1 or 1:2 (v/v) in KPBS as indicated, followed by a 30-s rinse with KPBS. The sections were labeled by incubating them for 30 min on droplets of goat anti-mouse IgG conjugated to 15-nm colloidal gold (prepared as described above) or, in some cases, commercially available goat anti-mouse IgG conjugated to 10-nm colloidal gold, diluted 1:1 or 1:3 (v/v) as indicated in 10 mM potassium phosphate, pH 8.0, containing 500 mM NaCl and 0.02% (w/v) PEG. The sections were washed with one 30-s rinse each of KPBS and distilled water.

Light Microscopy

Light microscopy was carried out on an Axioscop microscope (Carl Zeiss) equipped with differential interference contrast and epifluorescence optics. Epifluorescence microscopy was carried out on the same processed tissue used for EM. Thicker sections (250 nm) were cut using a microtome and mounted on glass microscope slides previously coated with a solution of 0.4% (w/v) gelatin, 0.04% (w/v) Chrom-Alum (Fisher), and 0.02% (w/v) sodium azide. Immunolabeling was carried out as described for EM except that solution droplets were placed on the glass slide-mounted sections and the secondary antibody was goat anti-mouse IgG-fluorescein isothiocyanate conjugate. Epifluourescence of the immunostained tissue sections was viewed through a Zeiss UV filter set (excitation filter 450–490 nm, beam splitter 510 nm, barrier filter 520 nm) and photographed with T-max 100 35-mm film (Kodak).

Immunological Controls

Several control experiments were performed for each monoclonal antibody used in this study. The controls included omission of primary antibody from the immunolabeling experiments or substitution of the primary antibody with another murine antibody of the same immunoglobu-

lin class that does not bind to any plant antigen. The specificity of the labeling observed with the monoclonal antibodies was tested by preincubation of the antibodies with antigen prior to application to tissue sections as follows. CCRC-M1 was preincubated with either sycamore maple xyloglucan (100 μg/mL) or Fuc (2 M), both of which have been shown to compete for this antibody's combining site (Puhlmann et al., 1994). CCRC-M2 was preincubated with sycamore maple rhamnogalacturonan I (1 mg/mL), which is recognized by this antibody (Puhlmann et al., 1994). CCRC-M7 was preincubated with either sycamore maple rhamnogalacturonan I (1 mg/mL) or with 6-*O*-β-D-galactopyranosyl-D-Gal (25 mg/mL), both of which have been shown to compete for this antibody's combining site (Steffan et al., 1995). The preincubations were carried out at room temperature for 1 to 2 h.

RESULTS

The locations of the carbohydrate epitopes recognized by monoclonal antibodies CCRC-M1, CCRC-M2, and CCRC-M7 (Table I) were determined in the roots of young Arabidopsis seedlings. Immunohistochemical studies were carried out at several points along the length of the root from the root tip to the junction between root and hypocotyl. All classically defined zones of cellular activity (Schiefelbein and Benfey, 1991) (e.g. meristematic zone, elongation zone, specialization zone) were included. The results of these immunohistochemical studies, which are described in more detail in the following sections, are summarized in Table II.

Distribution of Polysaccharide Epitopes at the Arabidopsis Root Tip

Immunohistochemical studies carried out on longitudinal sections and cross-sections of Arabidopsis root tips demonstrated differential distribution of the carbohydrate epitopes recognized by the monoclonal antibodies CCRC-M1, CCRC-M2, and CCRC-M7. The rhamnogalacturonan I epitope recognized by CCRC-M2 was not found in any cells within approximately 1.0 mm of the root tip. On the other hand, the arabinosylated (1→6)-linked β-galactan epitope recognized by CCRC-M7 (Table I) was present in the walls of all root tip cells except those of the lateral root cap (Fig. 1, C and D), and the terminal fucosyl-containing epitope recognized by CCRC-M1 (Table I) was present in the walls of all cells (Fig. 1, A and B). Furthermore, differential staining was seen in the various walls of single cells. For example, the outer walls of lateral root-cap cells and of epidermal cells showed more intense immunofluorescent labeling with CCRC-M1 than did other walls in sections of the same cells. Immunogold labeling (Fig. 2) determined that the more intense immunofluorescence observed in the lateral root cap and outer epidermal walls probably resulted from immunolabeling of a larger area, because these walls are thicker than other cell walls in the root. However, this was not the reason for differential labeling of walls seen elsewhere in the root (see below). Immunogold labeling also revealed that in lateral root-cap cells CCRC-M1 labeling of the anticlinal walls and the periclinal wall adjacent to epidermal cells was significantly reduced when compared with labeling of the periclinal wall facing the root exterior (Fig. 2). The unequal distribution of CCRC-M1 labeling was also observed by immunofluorescence microscopy of walls of cells sloughed off the root cap (Fig. 1A). Thus, carbohydrate epitopes are located in a cell-type-specific manner and, at least in some cases, are unequally distributed over the several walls of a single cell, a pattern also documented in other parts of the root (see below).

Longitudinal sections of the root tip occasionally yielded sections through cells undergoing cytokinesis, permitting visualization of epitopes within the developing cell plate.

Table II. *Summary of epitope distribution results in Arabidopsis roots using monoclonal antibodies CCRC-M1, -M2, and -M7*

Cell Type	Distance[a]	Antibody[b]			Figure(s)	Comments
		CCRC-M1	CCRC-M2	CCRC-M7		
Columellar root cap		+	−	+	1	
Lateral root cap	0–0.5 mm	+	−	−	1, 2	Labeling of anticlinal and interior periclinal walls is less than outer periclinal wall (Fig. 2).
Epidermis	<1 mm	+	−	+	1, 2	CCRC-M1 labeling of the outer periclinal wall is stronger than labeling of other walls (Fig. 4E).
Atrichoblast[c]	>1 mm	+	+	+	4, 8	
Trichoblast[c]	1–2 mm	+	−	+	8	
	>2 mm	+	+	+	4, 8	
Cortex	<2 mm	+	−	+	8	
	>2 mm	+	+	+	4, 5, 6, 8	
Endodermis		+/−	−	+	4	CCRC-M1 does not label the radial cross-walls (Fig. 4F). The Casparian strip is not labeled (Fig. 4, F and H).
Cell plate	0.2 mm	+	−	+	3	
Pericycle		+	−	+	4, 6, 8	
Phloem		+	−	+	4, 5	

[a] Distance from the root tip is measured as distance from the basal walls of the central cells in the root apical meristem (Dolan et al., 1993). Where no distance is given, labeling is observed throughout the root. [b] Labeling: +, present; −, absent. [c] Trichoblast and atrichoblast refer to epidermal cells that do and do not form root hairs, respectively (Dolan et al., 1994; Galway et al., 1994).

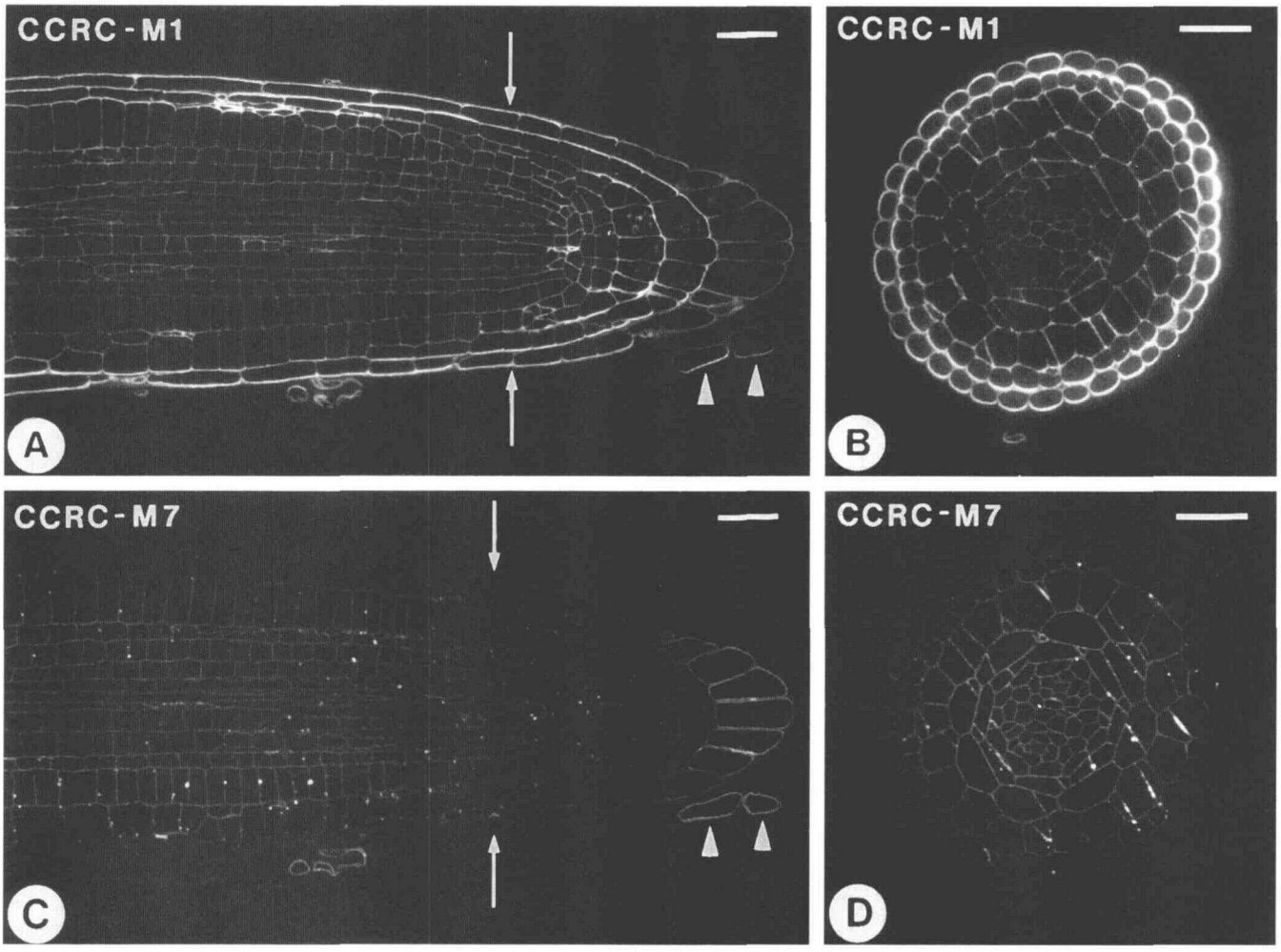

Figure 1. Immunofluorescent labeling of serial longitudinal (A and C) and serial transverse (B and D) sections of Arabidopsis root apices with CCRC-M1 (A and B) and CCRC-M7 (C and D). Arrows in A and C indicate approximate position of transverse sections shown in B and D. Arrowheads in A and C indicate cells sloughed off the lateral root cap. Measurement bars are 20 μm.

The carbohydrate epitopes recognized by CCRC-M1 (Fig. 3) and CCRC-M7 (not shown) were present in the vesicles of the cell plate at all observed stages of plate formation.

Distribution of Polysaccharide Epitopes in Mature Parts of Arabidopsis Roots

The distribution of the carbohydrate epitopes recognized by CCRC-M1, CCRC-M2, and CCRC-M7 in cells 5 mm from the Arabidopsis root tip (Fig. 4), i.e. beyond the zone of cell differentiation, was notably different from that observed at the root tip. At this stage of root development, the arabinogalactan epitope recognized by CCRC-M7 was present in the walls of all cells, as visualized by indirect immunofluorescence of root cross-sections (Fig. 4G). However, the Casparian strips, which constitute a portion of the radial walls of endodermal cells, were not labeled with CCRC-M7 (Fig. 4H). The CCRC-M7 epitope was also present in cell corners, particularly in older parts of the root (Fig. 5D).

The terminal α-fucosyl-containing epitope recognized by CCRC-M1 was also present in the walls of all root cells (Fig. 4E), with the exception that CCRC-M1 labeling was absent from the entire radial endodermal walls that contain the Casparian strips (Fig. 4F). Labeling with CCRC-M1 was particularly heavy in the thicker outer walls of the epidermis (Fig. 4E). No labeling with CCRC-M1 was observed in cell corners (data not shown). These results again demonstrate that carbohydrate epitopes are not uniformly distributed among the walls that enclose plant cells.

The extensive distribution of the CCRC-M1 and CCRC-M7 epitopes across all cell types in the mature root was not mimicked by the CCRC-M2 epitope. The rhamnogalacturonan I epitope recognized by CCRC-M2 was present only in the epidermal and cortical cell layers (Fig. 4, C and D); no label was observed in any endodermal, pericycle, or vascular tissues, even in those parts of the root closest to the hypocotyl. In the oldest parts of the root, where a second layer of cortical cells is formed (Dolan et

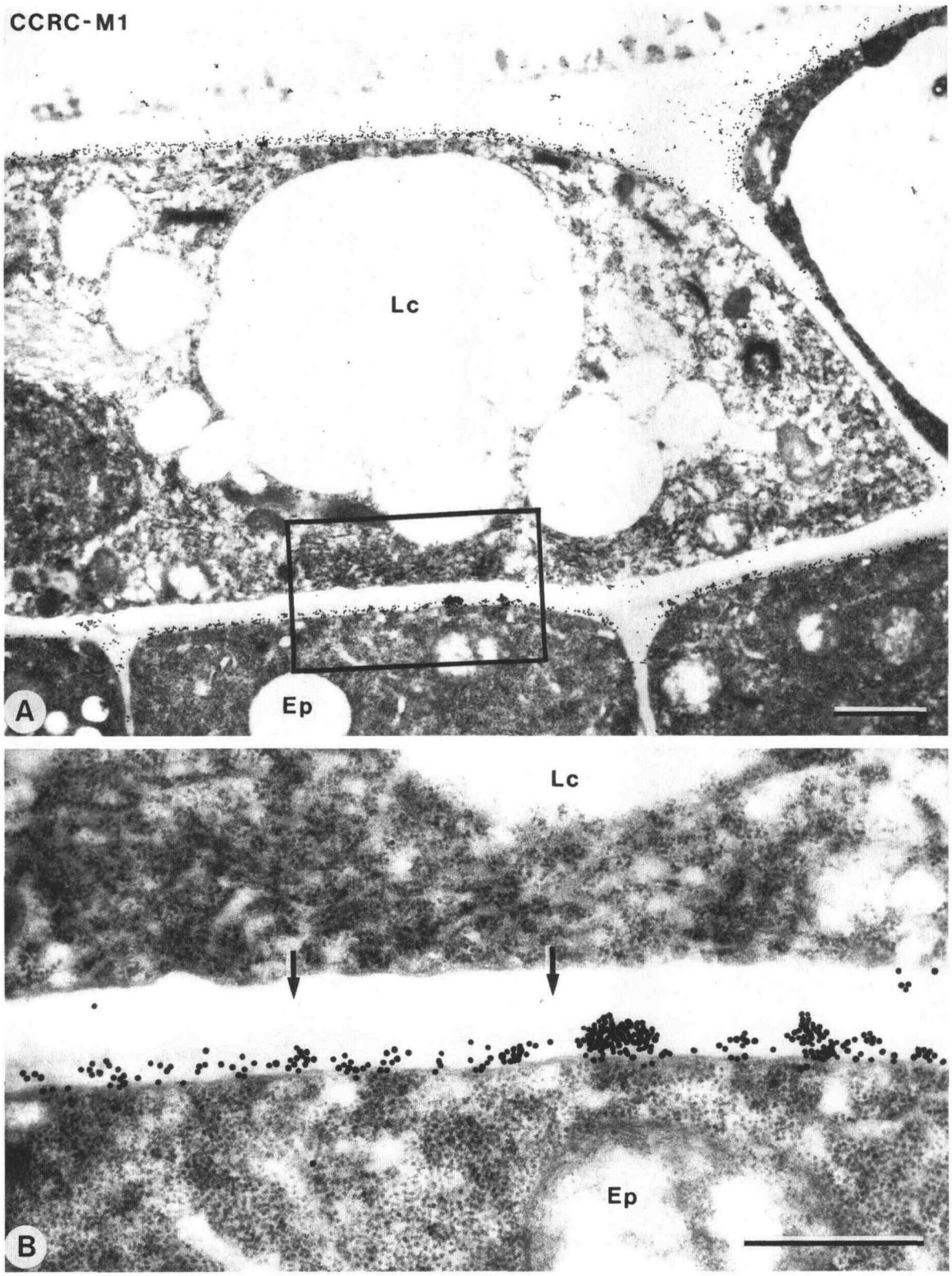

Figure 2. Immunogold labeling with CCRC-M1 of lateral root-cap (Lc) and epidermal (Ep) cells of an Arabidopsis root about 100 μm from the root apex. The box in A indicates the area enlarged in B. Arrows point to the sparser labeling of the interior wall of the lateral root-cap cells. Measurement bars are 1 μm in A and 0.5 μm in B.

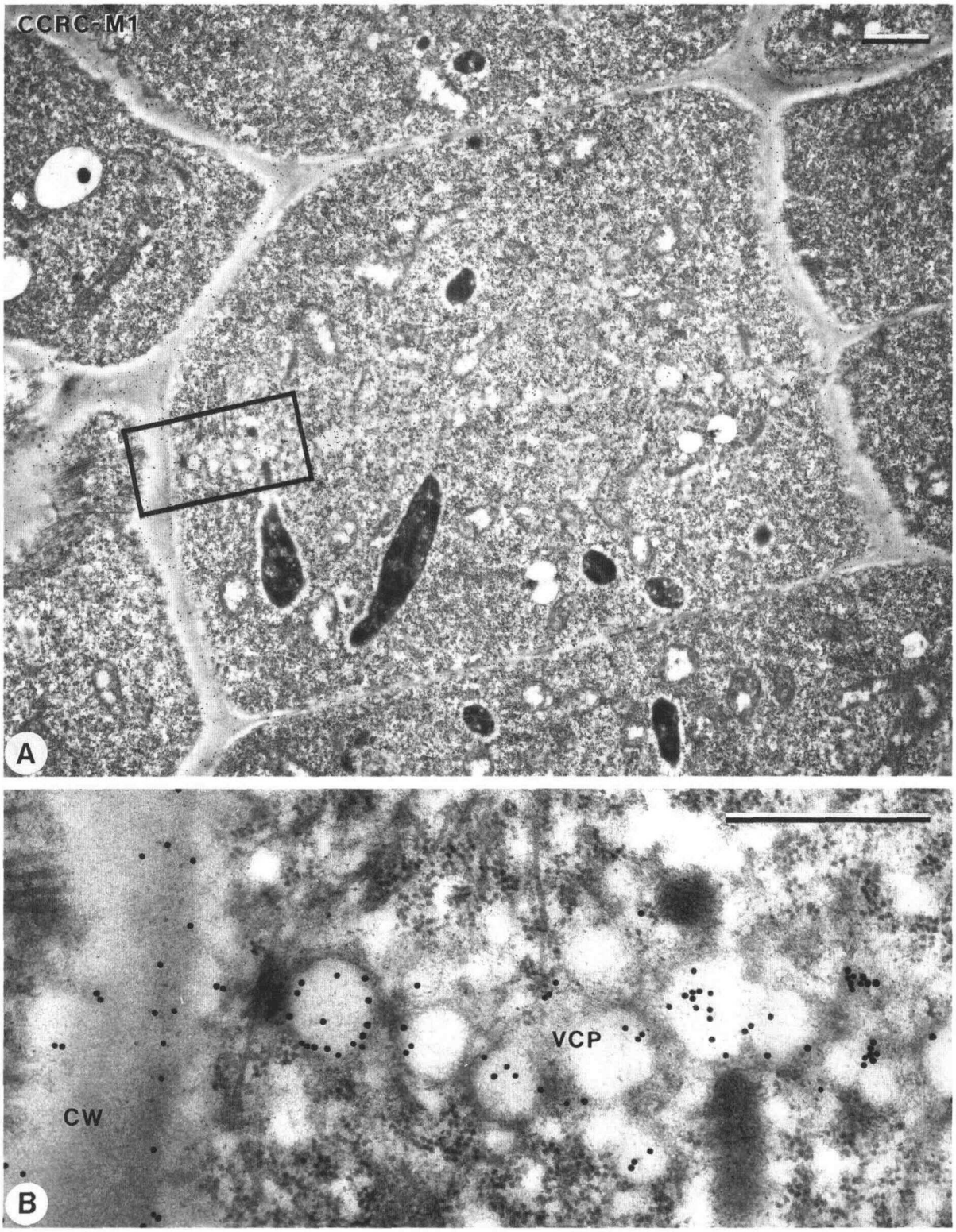

Figure 3. Immunogold labeling with CCRC-M1 of the cell wall and developing cell plate in a dividing Arabidopsis root endodermal cell about 0.2 mm from the root tip. The box in A indicates the area enlarged in B. The cell wall (CW) of the mother cell and the vesicles of the developing cell plate (VCP) are indicated. Measurement bars are 1 μm in A and 0.5 μm in B.

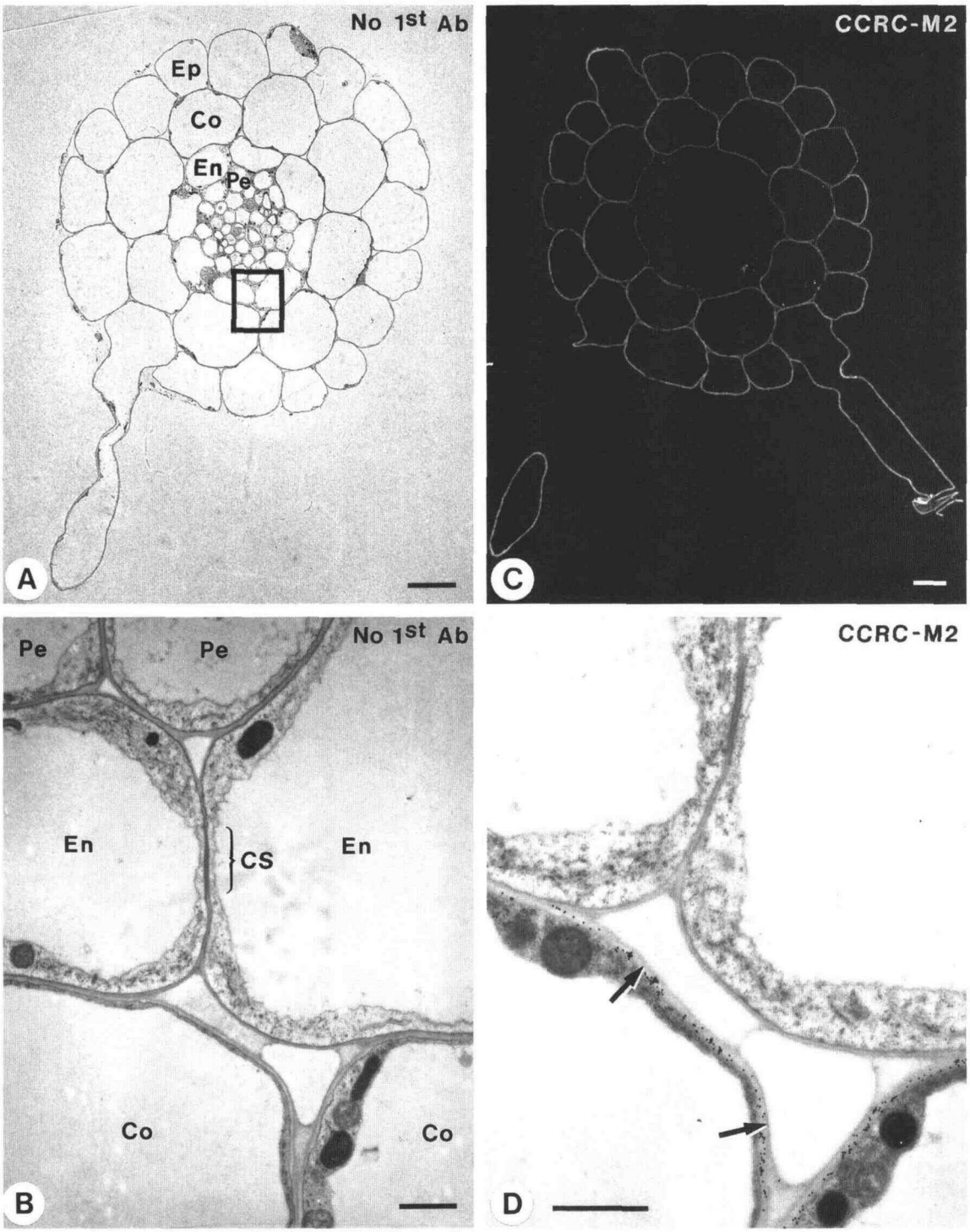

Figure 4. (Continued on facing page.)

al., 1993), the epidermis and both cortical layers were labeled (Fig. 6). In this part of the root, near the junction with the hypocotyl, decreased labeling with CCRC-M2 was observed in certain epidermal cells, particularly in the trichoblasts (Fig. 6). These results confirm that cells modify the structures of their wall polysaccharides depending on their developmental stage.

Immunohistochemical studies of the thick outer cell walls of epidermal cells and their attached root hairs demonstrated that cell-wall polysaccharide epitopes are located in well-defined domains within the walls, which, in the case of the three epitopes examined here, are more or less mutually exclusive. In the walls of root-hair cells, CCRC-M1 labeling was located primarily in the outer

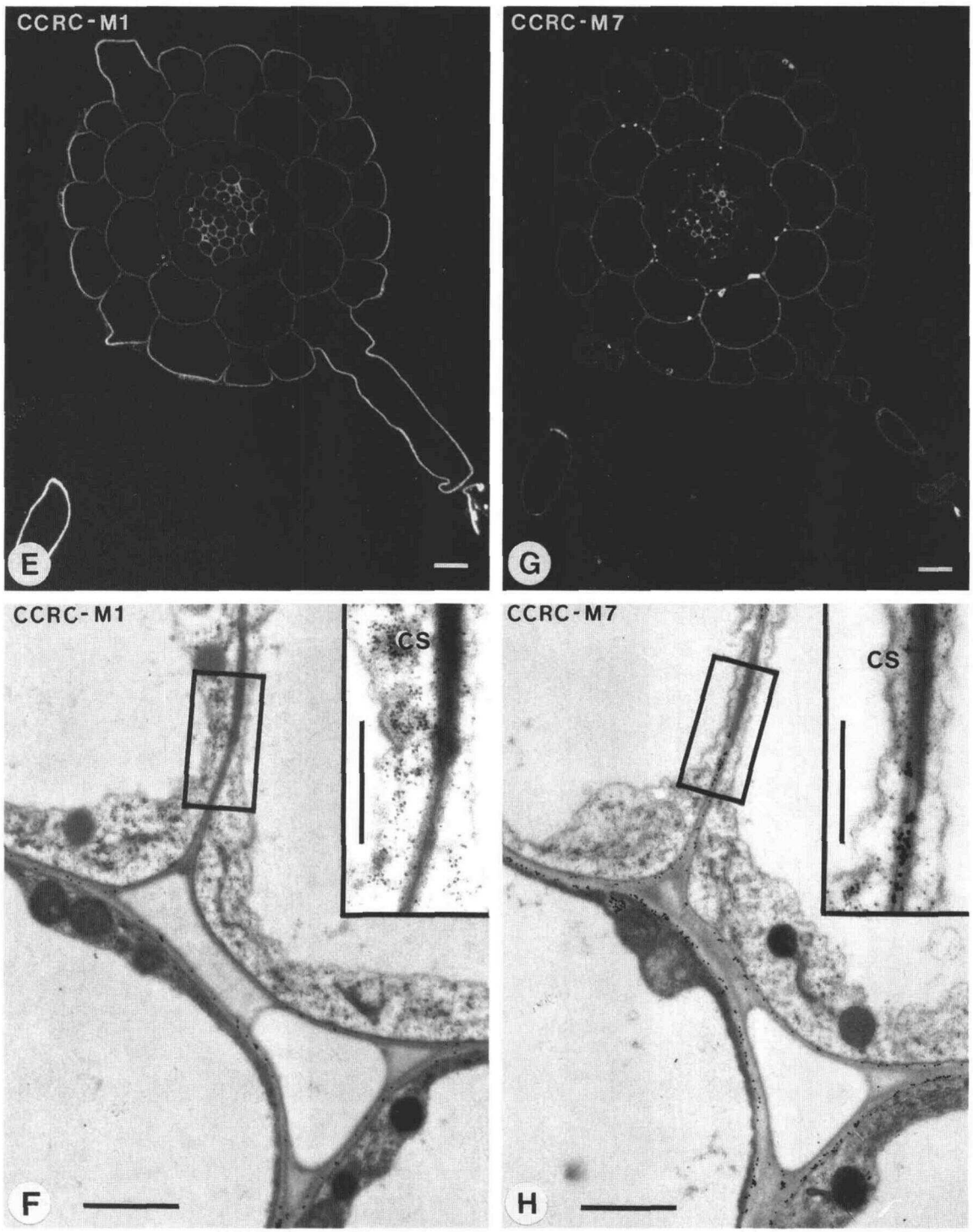

Figure 4. (Continued from facing page.) Distribution of complex carbohydrate epitopes in transverse sections of Arabidopsis roots about 5 mm from the root tip. A and B, Sections stained in the absence of primary antibody. The box in A highlights the area enlarged in B. The epidermal (Ep), cortical (Co), endodermal (En), and pericycle (Pe) cell layers are indicated in A; the Casparian strip (CS) located in the endodermal cross-wall is identified in B. C, E, and G, Immunofluorescent labeling; A, B, D, F, and H, immunogold labeling. Arrows in D indicate the wall of a cortical cell labeled by CCRC-M2. The inset in F highlights the absence of CCRC-M1 labeling in the endodermal cross-wall. The inset in H highlights labeling by CCRC-M7 of the endodermal cross-wall, except in the Casparian strip (CS). Measurement bars are 10 μm in A, C, E, and G, 1 μm in B, D, F, and H, and 0.5 μm in the insets in F and H.

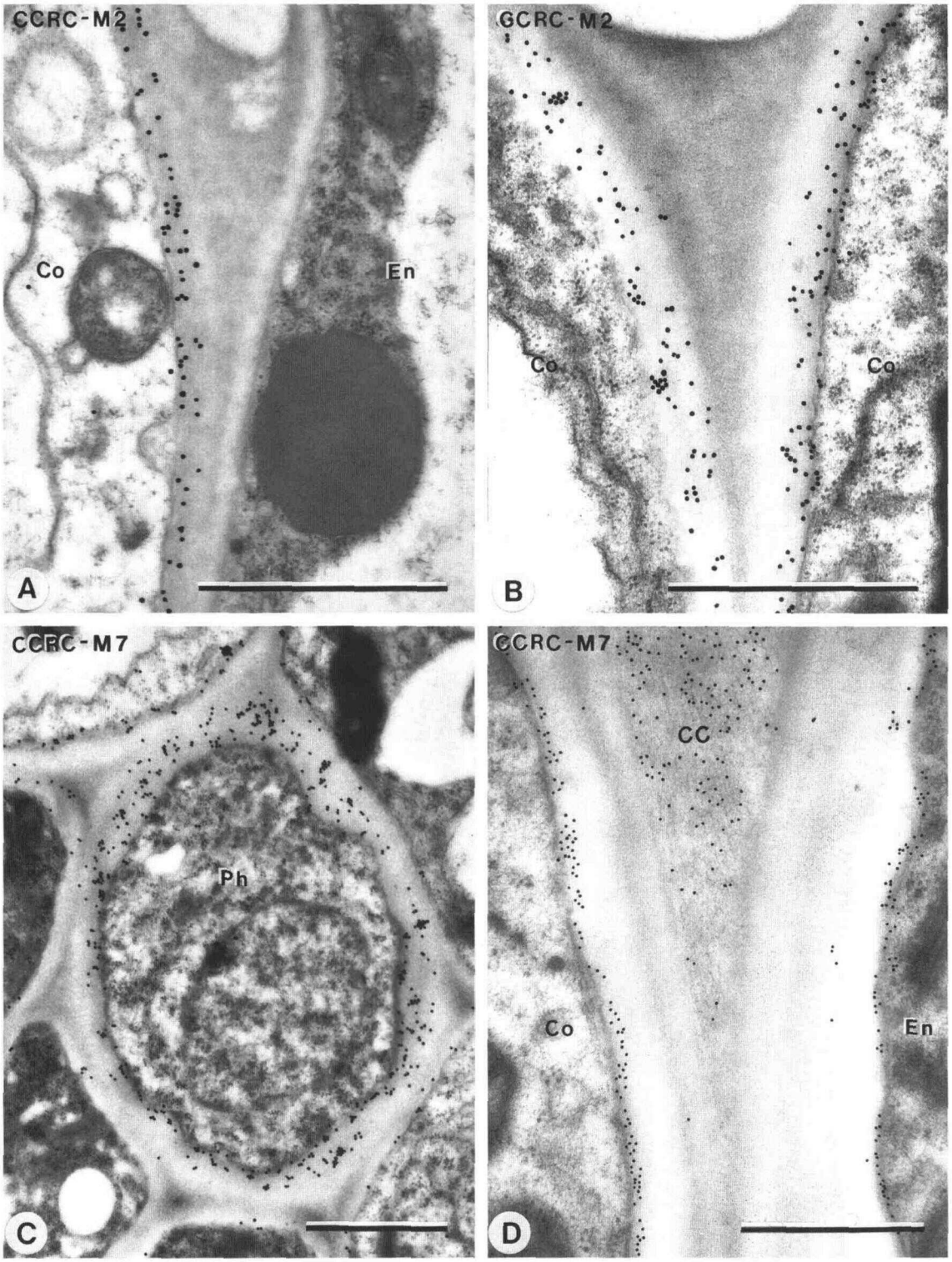

Figure 5. *Immunogold labeling of cell walls in mature* Arabidopsis roots. A and B, Labeling with CCRC-M2 about 9 mm from the root tip. A, Labeling of the cell walls separating a cortical (Co) and an endodermal (En) cell. B, Labeling of the cell walls between two cortical cells. C, Labeling of the cell wall of a phloem cell (Ph) with CCRC-M7 about 5 mm from the root tip. D, Labeling of the cell walls of a cortical (Co) and an endodermal (En) cell with CCRC-M7 about 9 mm from the root tip (note that 10-nm gold was used for immunostaining of this section). The cell corner (CC) is also labeled with this antibody. Measurement bars are 1 μm in A, B, and C and 0.5 μm in D.

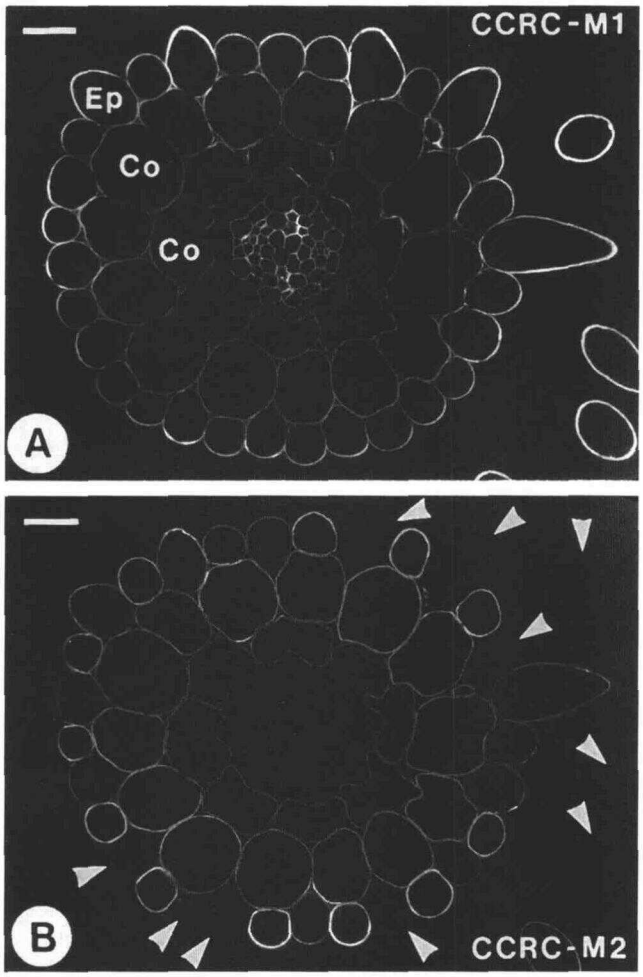

Figure 6. Immunofluorescent labeling of Arabidopsis roots about 9 mm from the root tip near the root-hypocotyl junction. A, Labeling with CCRC-M1. B, Labeling with CCRC-M2. The epidermal (Ep) and cortical (Co) cell layers are indicated in A. Arrowheads in B indicate epidermal cells and root hairs in which labeling with CCRC-M2 is decreased. Measurement bars are 20 μm.

three-fourths of the wall (Fig. 7A). CCRC-M2 labeled primarily the quarter of the cell wall closest to the plasma membrane (Fig. 7B), which was the part of the wall that was not labeled with CCRC-M1. A similar distribution of the CCRC-M2 epitope was often observed in cell walls of epidermal and cortical cells in older parts of the root where the walls were sufficiently thick to permit regional epitope distribution to be visualized (Fig. 5, A and B). In general, CCRC-M7 labeled the plasma membrane and perhaps that portion of the cell wall immediately adjacent to the plasma membrane (Figs. 5D and 7C); the resolution of the technique did not allow a distinction between the two. The cell walls of sieve tube elements of the phloem were an exception, because their entire thickness was intensely labeled with CCRC-M7 (Fig. 5C). These results also document that the distribution of carbohydrate epitopes varies across individual walls and among walls of different cell types.

Developmental Regulation of Polysaccharide Epitope Distribution

The absence of CCRC-M2 labeling at the root tip and the presence of labeling with this antibody in the cell walls of epidermal and cortical cells in more mature parts of the root led us to investigate when the epitope recognized by CCRC-M2 was first inserted into (or un-

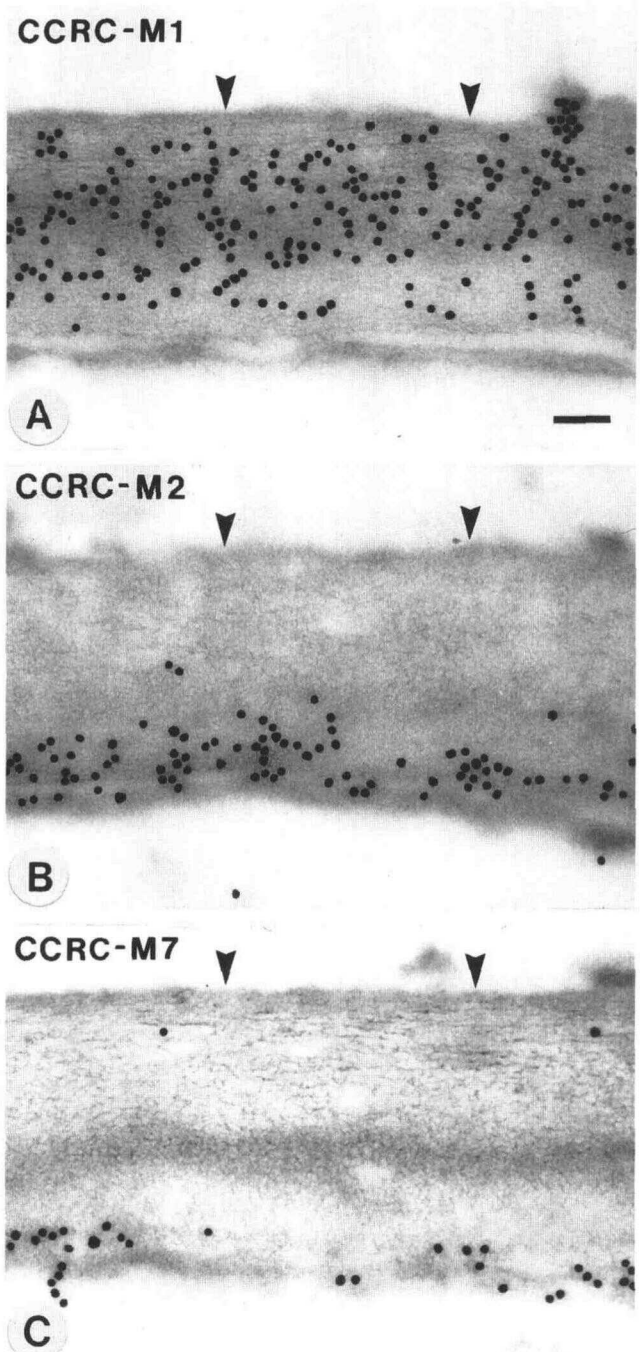

Figure 7. Immunogold labeling of root-hair cell walls of Arabidopsis epidermal cells. A, Labeling with CCRC-M1. B, Labeling with CCRC-M2. C, Labeling with CCRC-M7. Arrowheads indicate the outer wall surface. Measurement bar is 0.1 μm.

masked in) the walls of these two cell types. Labeling of cell walls with CCRC-M2 was first observed in atrichoblasts (epidermal cells that do not form root hairs) between 1 and 1.5 mm from the root tip (Fig. 8B). The walls of trichoblasts (root hair-forming epidermal cells) and cortical cells did not bind CCRC-M2 at this distance from the root tip, i.e. at this stage of development (Fig. 8C). Weak labeling of trichoblast and cortical cell walls was observed beginning 2 to 2.5 mm from the root apex, but the label was significantly less intense than labeling in atrichoblast cell walls (Fig. 8, E and F). Equally intense labeling in the walls of atrichoblasts, trichoblasts, and cortical cells was first observed at about 3 mm from the root apex and continued throughout the remainder of the root (Fig. 4C). We also observed CCRC-M2 labeling of Golgi in those cortical and epidermal cells whose walls were labeled with this antibody (data not shown). These results further document that cells can regulate the structure of their walls in a cell-type-specific manner and as a function of their developmental stage.

Immunological Controls

A variety of control experiments was performed to demonstrate the specificity of the labeling of Arabidopsis root sections with CCRC-M1, CCRC-M2, and CCRC-M7. Omission of the primary monoclonal antibody resulted, in each case, in the complete absence of labeling (Fig. 4, A and B). Substitution of any of the CCRC-M-series antibodies with monoclonal antibodies generated against nematode esophageal gland granules that are of either IgG or IgM isotype yielded no labeling of cell walls in any Arabidopsis root sections (data not shown). Finally, preincubation of CCRC-M1, CCRC-M2, and CCRC-M7 with antigens known to bind to the antibodies (Table I) abolished labeling of Arabidopsis root-hair cell walls (Fig. 9), as well as labeling in all other root sections examined (data not shown).

DISCUSSION

The antibody-labeling patterns described in this report demonstrate that carbohydrate epitopes are distributed in distinct patterns in the walls of Arabidopsis root cells, patterns that range from well-delineated locations within a single cell wall and among the walls of individual cells to restricted distribution in the walls of specific cell and tissue types. Thus, the dynamics observed with glycoprotein components of the plant extracellular matrix apply also to at least some of the polysaccharide components of the cell wall.

Two characteristics of cell-wall carbohydrates are relevant to the interpretation of immunohistochemical studies such as those described here. First, structural features (epitopes) of complex carbohydrates are sometimes present in more than one macromolecule. Thus, an antibody that recognizes such an epitope will cross-react with more than one polysaccharide in the wall (e.g. CCRC-M1 binds to both xyloglucan and rhamnogalacturonan I, albeit with different affinities [Puhlmann et al., 1994]). This makes it difficult to locate specific polysaccharides or proteoglycans within the wall, and hence we refer in this report to the location of carbohydrate epitopes rather than polysaccharides. The second relevant characteristic of cell-wall polysaccharides is that cell-wall polysaccharides are often found as families of structurally related macromolecules. For example, rhamnogalacturonan I exists as a family of related polysaccharides that differ in the degree of branching and in the monosaccharide compositions of their side chains (Thomas et al., 1987; Ishii et al., 1989). As a consequence, a monoclonal antibody may identify the distribution pattern of some but not all members of a polysaccharide family, depending on whether or not the epitope recognized by that antibody is present in all polysaccharides of a family. This could explain the difference in the epitope distribution patterns observed with CCRC-M2 and CCRC-M7 (Table II), both of which bind to epitopes on rhamnogalacturonan I.

A number of studies using monoclonal antibodies have examined the distribution of carbohydrate epitopes present on arabinogalactan-proteins in carrot (*Daucus carota*) roots and embryos (Knox et al., 1989, 1991; Stacey et al., 1990; Smallwood et al., 1994) and oilseed rape (*Brassica napus*) flowers (Pennell et al., 1991). The studies in carrot root, in particular, noted specific distribution of arabinogalactan-protein epitopes in cells associated with developing vascular tissues, suggesting that these glycoproteins might be involved in positional distinctions between cells and therefore might be useful molecular markers for the formation of particular tissue patterns. The arabinogalactan epitope on rhamnogalacturonan I and arabinogalactan-proteins that is recognized by CCRC-M7 is different from the epitope(s) recognized by the previously described monoclonal antibodies against arabinogalactan-proteins (Steffan et al., 1995); CCRC-M7 does not specifically label (proto)vascular tissues (Figs. 1, C and D, and 4G), although phloem cell walls are more heavily labeled than are other cell types (Fig. 5C).

The arabinogalactan epitope recognized by CCRC-M7 is reduced in or absent from the lateral root-cap cells but present in the columellar root-cap cells (Fig. 1C). The ability of CCRC-M7 to distinguish between these two cell types correlates with data from cell-fate-map studies of Arabidopsis seedlings, which show that the columellar and lateral root-cap cells originate from different progenitor cells (Dolan et al., 1994; Scheres et al., 1994). Thus, our data provide evidence that the different origins of these two cell types results in their synthesizing cell walls with different structural elements.

The nonuniform distribution of complex carbohydrate epitopes within plant cell walls, such as that seen most prominently in the thicker outer walls of epidermal cells (Fig. 7) and the walls of cells located in the upper part of the root closer to the hypocotyl (Fig. 5), has been observed by other researchers. In particular, the close association of arabinogalactan epitope recognized by CCRC-M7 with the plasma membrane (Figs. 5D and 7C) and the presence of these epitopes in cell corners (Fig. 5D) coincides with the findings of related studies that used antibodies against

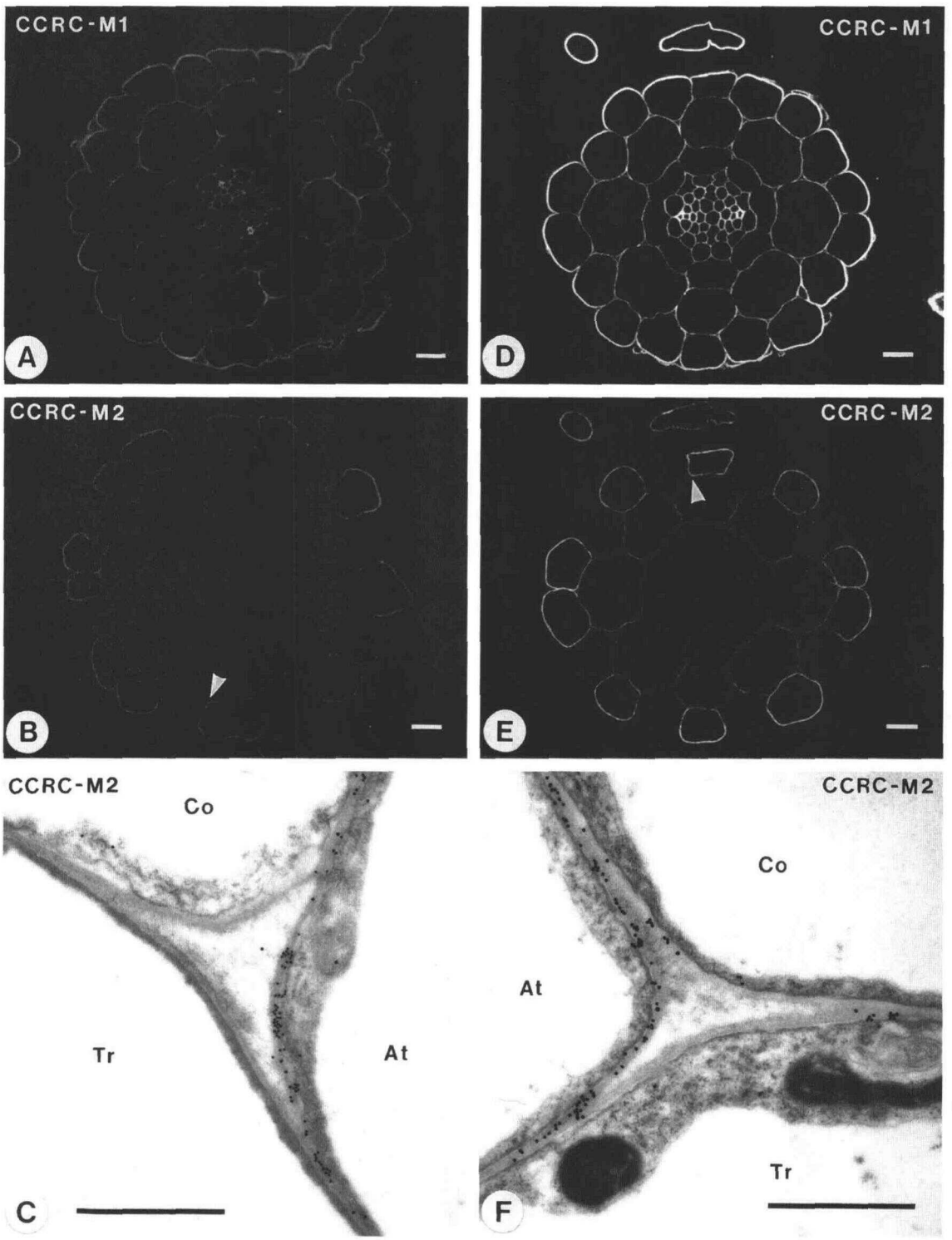

Figure 8. Developmental stage-specific labeling of cell walls in Arabidopsis roots with CCRC-M2 (B, C, E, and F). Labeling with CCRC-M1 (A and D) is shown for reference. Sections were taken 1.5 mm (A–C) and 2.5 mm (D–F) from the root tip. Arrowheads in B and E indicate the cell junctions enlarged in C and F, respectively. Cell types are indicated in C and F as follows: Tr, trichoblast; At, atrichoblast; Co, cortical cell. Measurement bars are 10 μm in A, B, D, and E and 1 μm in C and F.

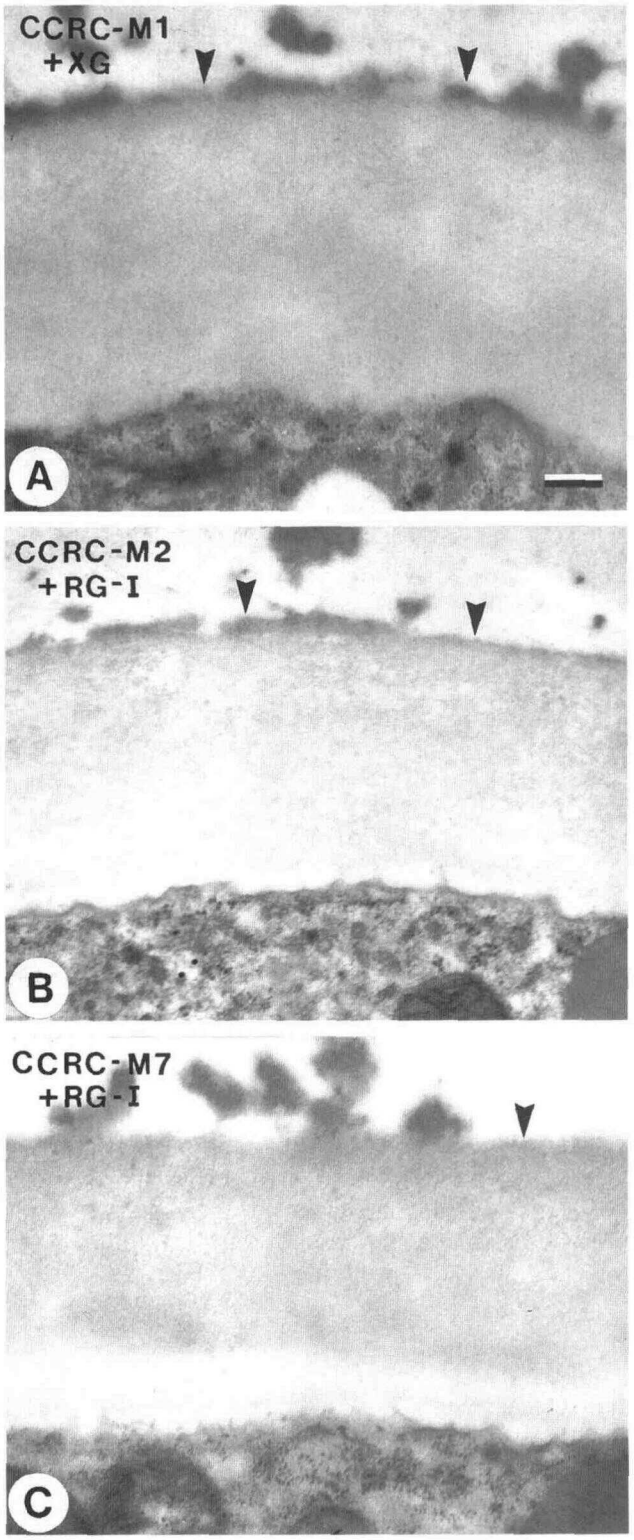

Figure 9. Preadsorption controls for immunogold labeling of root-hair cell walls of Arabidopsis epidermal cells. A, Labeling with CCRC-M1 that had been preadsorbed with sycamore maple xyloglucan. B, Labeling with CCRC-M2 that had been preadsorbed with sycamore maple rhamnogalacturonan I. C, Labeling with CCRC-M7 that had been preadsorbed with sycamore maple rhamnogalacturonan I. Arrowheads indicate the outer wall surface. Measurement bar is 0.1 μm.

other arabinogalactan epitopes (Knox et al., 1989, 1991; Horsley et al., 1993; Schindler et al., 1995).

Studies, using polyclonal antisera, of the location of xyloglucan within the walls of suspension-cultured sycamore maple cells and clover and oat roots indicated a fairly uniform distribution of this polysaccharide throughout the walls (Moore et al., 1986; Lynch and Staehelin, 1992, 1995). Using CCRC-M1 to label cell walls of tightly appressed interior cells of Arabidopsis roots, we also observed a fairly uniform distribution in the walls of terminal α-fucosyl residues (Fig. 4F), an epitope most prevalently located on xyloglucan but also present in rhamnogalacturonan I (Puhlmann et al., 1994). However, in the thicker walls separating the epidermal cells from lateral root-cap cells, this epitope is located in that part of the epidermal cell wall closest to the plasma membrane (Fig. 2), whereas in the root-hair cell wall of more mature epidermal cells, the same fucosyl-containing epitope is located in the outer three-quarters of the wall (Fig. 7A). Thus, the distribution of a carbohydrate epitope across a cell wall depends on the cell type and its stage of development.

The asymmetric distribution in two cell types (endodermal and lateral root-cap cells) of the terminal α-fucosyl epitope recognized by CCRC-M1 (Figs. 2B and 4F) is likely to reflect structural differentiation of the walls in those cells. The different structural characteristic of the inner wall of lateral root-cap cells adjacent to epidermal cells may reflect modification of this wall to permit sloughing off of the lateral root-cap cells. Likewise, the different structure of the radial walls separating endodermal cells compared with the walls facing cortical and pericycle cells correlates with the presence of the Casparian strip in a portion of the radial walls.

Our data do not permit conclusions about the mechanism(s) used by lateral root-cap and endodermal cells to achieve the observed asymmetric distribution of the fucosyl-containing epitope among different walls of a single cell (Figs. 2 and 4F). Possible mechanisms for asymmetric distribution of molecules in the different walls of a single cell include selective targeting of secretory vesicles to specific walls, selective in muro addition and/or removal of polysaccharide epitopes from specific walls, and selective masking of epitopes. Irrespective of the mechanism(s) responsible, our data clearly indicate that individual plant cells do selectively modify polysaccharide structures in their various walls.

The data presented in this and previous reports provide insight into the biosynthesis and ontogeny of plant cell walls. The plant extracellular matrix begins to be laid down during the fusion of Golgi-derived vesicles that have collected at the developing cell plate (Samuels et al., 1995). In Arabidopsis, these vesicles are labeled by both CCRC-M1 (Fig. 3) and CCRC-M7 (data not shown), confirming that xyloglucan and rhamnogalacturonan I and/or arabinogalactan-proteins are among the first components laid down in the newly synthesized wall. The xyloglucan and arabinogalactan epitopes recognized by these two monoclonal antibodies are synthesized in the Golgi, as evidenced by labeling of the *trans*-cisternae of Golgi and the *trans*-Golgi

network (Zhang and Staehelin, 1992; and unpublished observations of the authors). Earlier research using polyclonal antisera also demonstrated the presence of xyloglucan in cell plates of dividing red clover (*Trifolium pratense*) cortical cells (Moore and Staehelin, 1988) and of arabinogalactan-proteins in the cell plates of bean (*Phaseolus vulgaris*) root cells (Northcote et al., 1989). The fact that cytokinesis results in the synthesis of one new wall in each of the daughter cells suggests one mechanism by which a cell could selectively control and alter the structural characteristics of one of the walls that encloses it.

The much greater growth of periclinal walls and longitudinally oriented anticlinal walls compared to transverse anticlinal walls makes it apparent that plant cells selectively target cell-wall precursors to the different walls that surround them (Roberts, 1994). The different compositions of the cutin-containing outer walls of epidermal cells and of the Casparian strip in the radial walls of endodermal cells also establish the principle of asymmetric synthesis of a cell's walls. The differential distribution of the CCRC-M1 epitope in lateral root-cap and endodermal cells provides experimental evidence of the ability of plant cells to control the compositions of the walls that enclose them. Significant differences have also been observed in the intensity of labeling of longitudinal versus cross-walls in cortical cells of clover roots using a polyclonal antiserum raised against xyloglucan (Lynch and Staehelin, 1992). Those researchers hypothesized that the various Golgi in cortical cells produce different populations of secretory vesicles carrying different combinations of complex carbohydrates that are targeted to the different walls of the cells. Double-labeling experiments with antibodies against different cell-wall components in clover root tips were interpreted as providing evidence for the existence of different populations of secretory vesicles (Moore et al., 1991), although similar experiments in vetch (Sherrier and VandenBosch, 1994) yielded no evidence for different types of secretory vesicles.

The extracellular matrix laid down at cytokinesis rapidly differentiates into two cell walls as additional components are added to the matrix by each of the daughter cells. These newly synthesized wall components include polysaccharides, such as callose and cellulose, that are synthesized at the plasma membrane after vesicle fusion begins to occur in the developing cell plate (Samuels et al., 1995). Additional Golgi-synthesized components (e.g. xyloglucan, pectins, arabinogalactans) also continue to be added after cytokinesis, as evidenced by labeling of Golgi in Arabidopsis root cells no longer undergoing mitosis (unpublished observations of the authors). The labeling patterns observed with CCRC-M2 provide additional evidence for such post-cytokinetic wall differentiation. The rhamnogalacturonan I epitope recognized by CCRC-M2 is not present at the initial stages of wall synthesis as indicated by the absence of labeling in the meristematic zone of Arabidopsis roots and in cell plates of cells undergoing cytokinesis. Rather, this epitope is first detected in walls later in root development after differentiation of the epidermal cells has occurred (i.e. after root-hair formation by trichoblasts has been initiated). This epitope appears first in the walls of atrichoblasts (Fig. 8, B and C) and then in those of trichoblasts and cortical cells (Fig. 8, E and F). CCRC-M2 labeling of Golgi in these cells (data not shown) suggests that this rhamnogalacturonan I epitope appears in the walls of the epidermal and cortical cells via de novo synthesis rather than by alteration of existing polysaccharides in muro. In the upper parts of the root near the root-hypocotyl junction, decreased labeling with CCRC-M2 is observed in some epidermal cells (Fig. 5). This decreased labeling could result from either in muro masking or removal of the epitope from the polysaccharide. Thus, our data clearly indicate that plant cell-wall differentiation continues to occur in a carefully regulated manner after cytokinesis and that this differentiation is reflected in the altered structural characteristics of the walls of different cell types at different stages of development.

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