Plant Physiol. (1997) 114: 539–547

Characterization of the Cell Wall Microdomain Surrounding Plasmodesmata in Apple Fruit

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In fleshy fruits ripening is generally associated with a loss in tissue firmness resulting from depolymerization of wall components and separation of adjacent cells. In the regions of the wall that contain plasmodesmata, the usual sequences of ripening events, i.e. depolymerization of the middle lamellae and splitting of the walls, are not observed. In the present study we attempted to characterize in apple (*Malus domestica* Borkh.) fruit the structural microdomain of the cell wall that surrounds the plasmodesmata by in muro visualization of the cell wall components. Anionic sites of galacturonic acids were labeled with cationic gold. Low-esterified homogalacturonans were labeled with the monoclonal antibody JIM 5. In addition, a polyclonal antibody directed toward β(1→3)-glucopyranose was used to target callose in situ. The results indicated that the plasmodesmata-wall complexes were surrounded by a pectic microdomain. This domain was composed of low-esterified homogalacturonans that were not involved in calcium cross-bridging but were probably surrounded by a cationic environment. These structural features may result in the prevention of normal cell wall separation in regions containing plasmodesmata. However, observations by low-temperature scanning electron microscopy suggested that splitting of these walls ruptured the plasmodesmata and ultimately resulted in the spatial separation of adjacent cells.

In higher plants different developmental processes are associated with a regulated cell separation, which results in loosely attached cells that are surrounded by extensive intercellular spaces (Knox, 1992). For example, fruit softening, which occurs during ripening, results from the loss of cell cohesion (Labavitch, 1981; Brady, 1987; Poovaiah et al., 1988; Van Buren, 1991). Pectolytic enzymes and changes in pectin composition are responsible for the decreased adhesion between cells and have been the focus of numerous studies of fruit ripening (for a review, see Fischer and Bennett, 1991) as well as investigations to evaluate the commercial potential of genetically modified fruits (Brady, 1992; Grierson and Schuch, 1993).

Ultrastructural studies show that fruit softening involves a disruption of the middle lamella and cell wall autolysis to different extents, depending on the degree of juiciness of the fruits (Mohr and Stein, 1969; Ben Arie et al., 1979; Platt-Aloia et al., 1980; Crookes and Grierson, 1983). The development of affinity methods, which use specific probes, has enabled investigators to use new approaches to understand the microheterogeneity of the cell wall (Carpita and Gibeaut, 1993; Roberts, 1994). Targeting the hydrolytic enzymes (Dallman et al., 1989; Morré, 1989; Pogson et al., 1992) and visualizing in muro the subtle changes that the substrates undergo (Roy et al., 1992; O'Donoghue et al., 1994) have led to the conclusion that fruit tissue is a mosaic of microdomains where minute changes can occur during the ripening process (Roy et al., 1992).

In the cell wall regions that contain plasmodesmata, the normal sequence of ripening events, i.e. depolymerization of the middle lamella and swelling and splitting of the cell wall, is usually not observed (Ben-Arie et al., 1979; Hallett et al., 1992; Martin-Cabrejas et al., 1994). Plasmodesmata form an important symplasmic pathway for cell-to-cell transport in higher plants (for a review, see Robards and Lücas, 1990; Lucas et al., 1993). Their ultrastructural features are becoming more clearly defined because of improvements in the preservation of the tissues (Ding et al., 1992; Badelt et al., 1994; Turner et al., 1994). However, because it is difficult to isolate plasmodesmata, the cell walls that surround these structures are difficult to characterize. Investigators have found that, in many tissues, regions of the cell wall that contain plasmodesmata stain differently and have denser fibrillar structures (Vian and Rougier, 1974; Ben-Arie et al., 1979; Hallett et al., 1992; Martin-Cabrejas et al., 1994). These observations suggest that the cell wall regions around the plasmodesmata may differ in composition.

In the present investigation we attempted to characterize the cell wall microdomain that surrounds plasmodesmata to understand its behavior during cell separation. To achieve this objective several probes were used to visualize the cell wall components: (a) a cationic poly-L-Lys colloidal gold complex was used to localize anionic sites in muro (Roy et al., 1994a), (b) a previously characterized JIM 5 monoclonal antibody against low-esterified homogalacturonans (Knox et al., 1990) was used to characterize the differentiation pattern of the pectic matrix components, and (c) a polyclonal antibody directed toward β(1→3)-glucopyranose (Northcote et al., 1989) was used to visualize callose in situ. These probes can be used either alone or after dissecting treatments. The results suggest that a

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Abbreviations: LTSEM, low-temperature scanning electron microscopy; PG, polygalacturonase; PME, pectin methyl esterase.

clearly defined cell wall microdomain surrounds the plasmodesmata and may be involved in the modulation of cell wall autolysis in this area.

MATERIALS AND METHODS

Plant Material and Specimen Preparation

Golden delicious apples (*Malus domestica* Borkh.) were harvested from a commercial orchard in Pennsylvania and stored at 0°C for a few days. Small pieces of the pericarp were cut and chemically fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 3 h, then washed in cacodylate buffer, and postfixed overnight in 1% osmium tetroxide. After dehydration in an alcohol series, the samples were embedded in London Resin White[1] methacrylate resin (Polysciences, Warrington, PA), as described by Roland and Vian (1991).

Labeling with Cationic Colloidal Poly-L-Lys Gold Complexes

The labeling was performed as a one-step procedure by the direct use of the gold complex. Ultrathin sections were incubated in 3% acetic acid at pH 2.6 for 20 min. The sections were treated for 1 h at room temperature with a cationic poly-L-LYS colloidal gold complex consisting of gold particles of either 10 nm (CGC10, British BioCell International, Cardiff, UK) or 5 nm (CGC5, British BioCell International), diluted 1/60 (v/v) in 3% acetic acid. The sections were then rinsed thoroughly in 3% acetic acid, followed by distilled water. A control experiment was performed by preincubating sections with a 1 mg mL^{-1} solution of poly-L-LYS (molecular weight > 350,000; Sigma) prior to the labeling.

Immunolabeling of Homogalacturonan Sequences

Ultrathin sections were incubated for 30 min in normal goat serum diluted 1/30 in 0.05 M TBS (pH 7.4) containing 0.5% BSA. The sections were treated overnight at 4°C, with the cell culture supernatant of JIM 5, a monoclonal antibody directed toward low-esterified homogalacturonans (Knox et al., 1990), diluted 1/5 (v/v) in TBS plus BSA 0.5%. Following treatment, the specimens were rinsed thoroughly in TBS. Sections were treated for 1 h with a 10-nm colloidal gold goat anti-rat immunoglobulin complex (British BioCell International) diluted 1/30 in TBS. They were then washed thoroughly in TBS and distilled water. To assess the specificity of the labeling, the cell culture supernatant JIM 5 was incubated with polygalacturonic acid from citrus fruit (Sigma).

Cytochemical Dissection

In some cases sections were subjected to chemical or enzymatic treatments prior to labeling. For in situ chemical de-esterification of pectins, sections were preincubated with 0.1 M Na_2CO_3 at 4°C for 16 h (Fry, 1989). For in situ enzymatic de-esterification of pectins, sections were preincubated with a 0.1 mg mL^{-1} solution of PME from orange peel (Sigma) in 0.1 M NaCl at pH 7.5 (Vreeland et al., 1989). The control consisted of preincubation with NaCl solution only. For in situ chelation of calcium, sections were preincubated in 0.2 M EDTA (Sigma), pH 8.0, at 60°C for 1 h (Wick and Hepler, 1980).

Immunolabeling of β(1→3)-Glucopyranose

Ultrathin sections were incubated for 30 min in normal goat serum diluted 1/30 in 0.05 M TBS, pH 7.5, containing 0.5% BSA. The sections were treated overnight at 4°C with a polyclonal antibody directed toward β(1→3)-glucopyranose (Euromedex, Souffelweyersheim, France), diluted 1/25 (v/v) in TBS plus BSA 0.5% (Northcote et al., 1989). The specimens were rinsed thoroughly in TBS. Sections were treated for 1 h with a 10-nm colloidal gold goat anti-rabbit immunoglobulin complex (British BioCell International) diluted 1/30 in TBS; they were then washed thoroughly in TBS and distilled water.

LTSEM

A field emission scanning electron microscope (S-4100, Hitachi, Tokyo, Japan) equipped with an Oxford CT-1500HF Cryotrans system (Oxford Instruments, Eynsham, UK) was used for LTSEM observations. Specimen preparation consisted of removing 1-cm^2 segments of the pericarp and mounting them onto a complementary holder with a cryoadhesive (Tissue Tek, Miles Scientific, Naperville, IL). The holder was rapidly plunge-frozen in liquid nitrogen and cryotransferred under vacuum to a cold stage in the prechamber of the cryosystem. The frozen specimen was etched in the prechamber by raising the temperature of the stage to −95°C, sputter-coated with Pt, and then transferred to the cryostage in the SEM for observations. An accelerating voltage of 10 kV was used to view the specimens.

RESULTS

The general ultrastructural features of the parenchyma cells in apple fruits are similar to those previously described (Ben-Arie et al., 1979; Roy et al., 1994a, 1995). The fruit pericarp is composed of (a) superficial layers of tightly cemented isodiametric cells having thick cell walls and numerous plasmodesmata that are concentrated in discrete areas, and (b) in deeper areas of the pericarp, large parenchyma cells that are separated from one another along a portion of their thin cell walls, thereby forming intercellular spaces (data not shown). Plasmodesmata were rarely observed within the latter area. Further observations were focused on the small, isodiametric cells region, and secondary plasmodesmata, as described by Ding and Lucas (1996), were mainly observed. Figure 1 illustrates an "H-shaped" secondary plasmodesmata with the development of a central cavity within the middle lamella (arrows, Fig. 1). In sections that were stained with lead citrate, the cell wall domains that surround plasmodesmata appeared

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

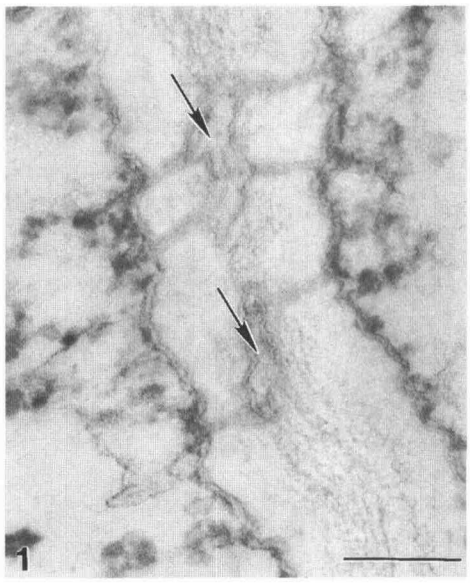

Figure 1. Structure of secondary plasmodesmata in cell wall from apple pericarp. The secondary plasmodesmata is characterized by protoplasmic branches and a central cavity within the middle lamella region (arrows). Bar = 0.2 μm.

more electron-opaque than other areas of the wall (data not shown).

Distribution of the Pectic Matrix Component

To characterize the molecular environment of the cell wall surrounding the plasmodesmata, cationic colloidal gold was used to label the anionic sites of the wall (Fig. 2a). Cross-sections through the cell wall showed that the cationic gold labeling was dense and evenly distributed throughout most of the wall; however, the areas that consisted of plasmodesmata-wall complexes were not labeled with cationic gold (arrowheads, Fig. 2a), except along a small zone of the cell wall that bordered the plasmalemma. Control sections that were preincubated with a solution of unlabeled poly-L-Lys only rarely exhibited cationic gold labeling (data not shown).

Following immunolabeling, the low-esterified homogalacturonans epitope recognized by JIM 5 was intensively localized in the plasmodesmata-wall complexes but was only weakly seen in the primary cell wall (Fig. 2b). Preabsorption of the monoclonal antibody JIM 5 with a solution of polygalacturonic acid greatly reduced the labeling (data not shown).

Influence of Preincubation Treatment prior to the Labeling

Specific enzymatic or chemical treatments were used to discriminate the various forms in which the homogalacturonans may exist, i.e. low-esterified, highly esterified, or bound with calcium ions. Neither the calcium-chelator treatment with EDTA (Fig. 3a) nor hydrolysis of the methyl groups (Fig. 3b) resulted in labeling with cationic gold in the plasmodesmata-wall area. Conversely, chemical de-esterification (Fig. 3c), enzymatic de-esterification (data not shown), or calcium-chelator treatment (Fig. 4) prior to labeling dramatically modified the distribution pattern of low-esterified homogalacturonans epitopes recognized by JIM 5. JIM 5 labeling was abundant throughout the primary cell wall adjacent to the plasmodesmata-wall complexes after de-esterification (compare Figs. 2b and 3c) and after calcium-chelator treatment (compare Figs. 2b and 4). The results are summarized in Table I.

LTSEM Observation and Callose Distribution

To further characterize the wall regions that were rich in plasmodesmata, immunogold labeling with $\beta(1\rightarrow3)$-glucopyranose was carried out on sectioned material. The results clearly show the association of callose with protoplasmic branches or strands (Fig. 5).

LTSEM allowed us to observe the frozen, fully hydrated cells of the pericarp. The frozen tissues did not exhibit the structural artifacts that are usually associated with chemical fixation, dehydration, and critical point drying (Wergin and Erbe, 1991). This technique enabled us to visualize small, round protuberances on the surfaces of walls from cells that had separated, i.e. cells found in the deep areas of the pericarp (Fig. 6a). The protuberances are believed to consist of cytoplasmic material that is surrounded by a layer of cell wall (Fig. 6b). They probably represent the plasmodesmata that were mechanically ruptured during cell separation.

DISCUSSION

Although apple fruits never reach the degree of juiciness found in tomatoes or peaches, Ben-Arie et al. (1979) have shown a progressive splitting and dissolution of the pectin-rich apple middle lamella. We assume that the pattern of pectin dissolution in apple fruits follows the sequence visualized in tomato fruits (Roy et al., 1992, 1994b). By means of in situ visualization of cell wall components, we have clearly illustrated that there are differences in the cell wall that surrounds the plasmodesmata. Because during softening of juicy fruits, plasmodesmata-wall complexes do not show swelling and splitting of the cell wall (Ben-Arie et al., 1979; Hallett et al., 1992; Martin-Cabrejas et al., 1994), the occurrence of a cell wall microdomain suggests modulation of cell separation in these areas.

A Cell Wall Microenvironment

The specificity of the cationic colloidal gold probe has been previously characterized (Roy et al., 1994a). Cationic gold recognizes the COO^- groups of galacturonic acid of unesterified pectins and glucuronic acid in xylans. Because large biochemical quantities of the former and small amounts of the latter are present in the primary cell walls of all dicotyledons analyzed so far (Carpita and Gibeaut, 1993), we assume that cationic gold probes the carboxylic groups of galacturonic acids (Roy et al., 1994a). Three possible reasons account for the low amount of gold labeling detected in the plasmodesmata-wall complexes after probing the sections with cationic gold (Fig. 2a): (a)

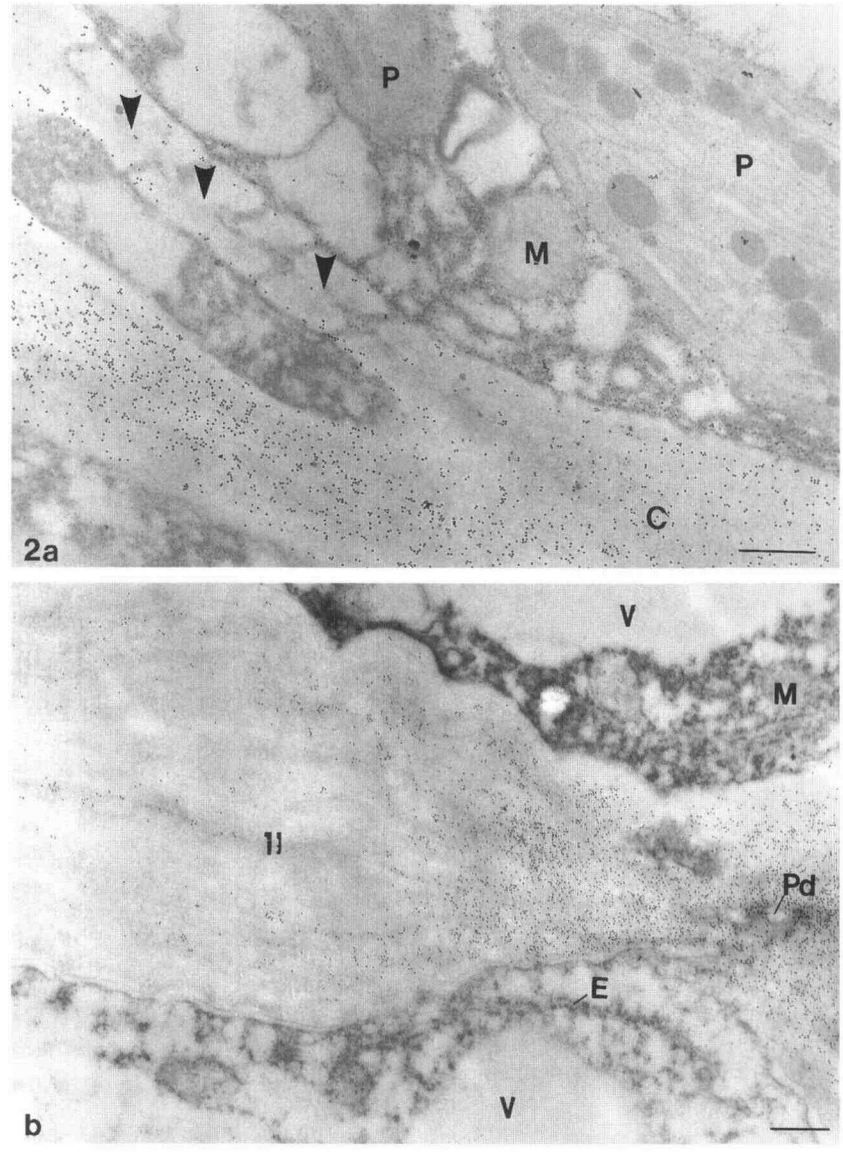

Figure 2. Labeling of the pectic network around the plasmodesmata. a, In muro visualization of anionic sites labeled with cationic colloidal gold. Labeling is intense and evenly distributed in the cell wall with the exception of the plasmodesmata area (arrowheads). b, Distribution of low-esterified homogalacturonic sequences by immunolabeling with JIM 5. Intense labeling is restricted to the area where the cell wall narrows and surrounds the plasmodesmata. In the primary cell wall, the labeling is less intense. C, Primary cell wall; E, ER; M, mitochondrion; Ml, middle lamella; P, plastid; Pd, plasmodesmata; and V, vacuole. Bars = 0.5 μm.

the cell wall components that were targeted were not abundant, (b) methyl groups or calcium ions may have masked the anionic sites, or (c) a cationic environment prevented the area from being labeled. To investigate these possibilities, immunogold labeling and cytochemical dissection were used.

JIM 5 was used to recognize low-esterified homogalacturonans. The intense reaction that we observed indicated that plasmodesmata-wall complexes were probably composed of low-esterified homogalacturonans (Fig. 2b). The segregation of the distribution for cationic gold and JIM 5 labeling was surprising because these two probes should target the same component, i.e. low-esterified galacturonic acid chains. This may suggest that the sensitivity of the cationic gold, which recognizes a single unit of galacturonic acid in pectic chains, is higher than that of JIM 5. However, these data do not explain why the plasmodesmata-wall complexes were void of cationic gold labeling.

In the plasmodesmata-wall area, anionic sites may have been masked by either methyl groups or calcium ions. We have shown that the homogalacturonans are not esterified, because de-esterification prior to probing does not restore the cationic gold labeling in the plasmodesmata-wall complexes (Fig. 3b), as it does in the primary cell wall with JIM 5 labeling (Fig. 3c). Similarly, pretreatment with a calcium chelator does not enhance the cationic gold labeling in the plasmodesmata-wall regions, indicating that calcium cross-bridges do not exist (Fig. 3a), as they do in the primary cell wall (Fig. 4).

In the plasmodesmata-wall complexes anionic sites seem to be masked in some way that is different from methyl groups or calcium ions. Cationic gold labeling, which is based on electrostatic interactions, may have been prevented by the presence of a cationic environment. This may reflect pectin interaction with other cell wall components. Although we do not yet have direct evi-

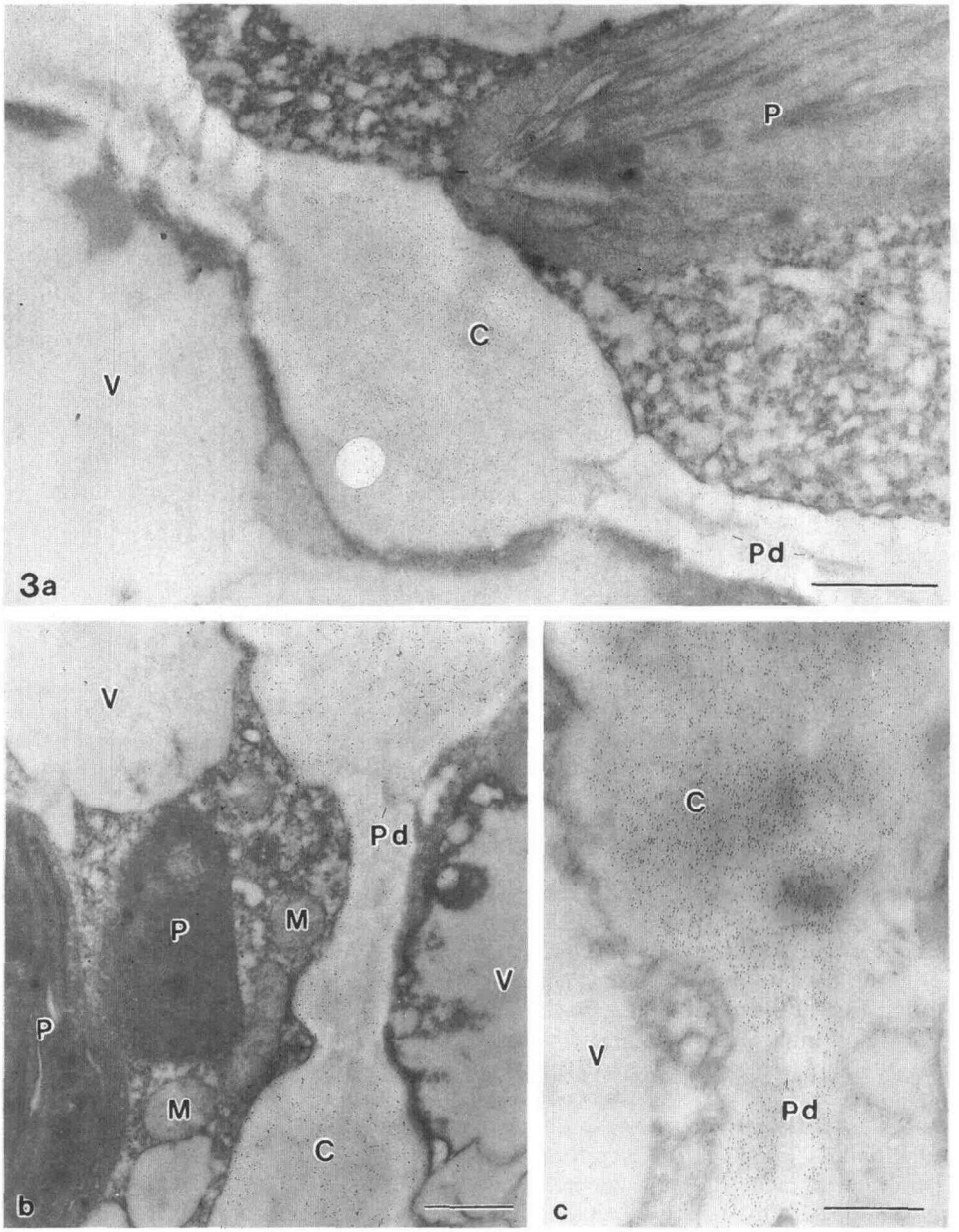

Figure 3. Effect of preincubation prior to labeling with a cationic colloidal gold probe and immunolabeling with JIM 5. a, Preincubation with EDTA and labeling with cationic 5-nm colloidal gold particles. The primary cell wall is intensively labeled. Around the plasmodesmata the labeling is restricted to the plasmalemma. b, Enzymatic de-esterification with PME and labeling with cationic 10-nm colloidal gold particles. The labeling is on the primary cell wall and very scarce in the area surrounding the plasmodesmata. c, Chemical de-esterification with Na_2CO_3 and JIM 5 labeling (10 nm). Chemical de-esterification spreads even labeling throughout the primary cell wall. C, Primary cell wall; M, mitochondrion; P, plastid; Pd, plasmodesmata; and V, vacuole. Bars = 0.9 μm.

dence, we propose that one good candidate for this cationic environment is polyamines. Polyamines are cationic molecules (Smith, 1985) that are known to form electrostatic bounds with pectic compounds (Goldberg and Perdrizet, 1984; Charnay et al., 1992). We suggest that they may have prevented labeling of the plasmodesmata-wall complexes with cationic gold by interacting with the anionic sites of the pectic polymers.

Importance for the Control of Cell Separation

Based on ultrastructural studies of different species, it has been proposed that plasmodesmata-wall complexes resist cell wall autolysis during fruit ripening (Hallett et al., 1992; Martin-Cabrejas et al., 1994). The localized distribution of low-esterified homogalacturonans in the plasmodesmata-wall region of fruit pericarp visualized

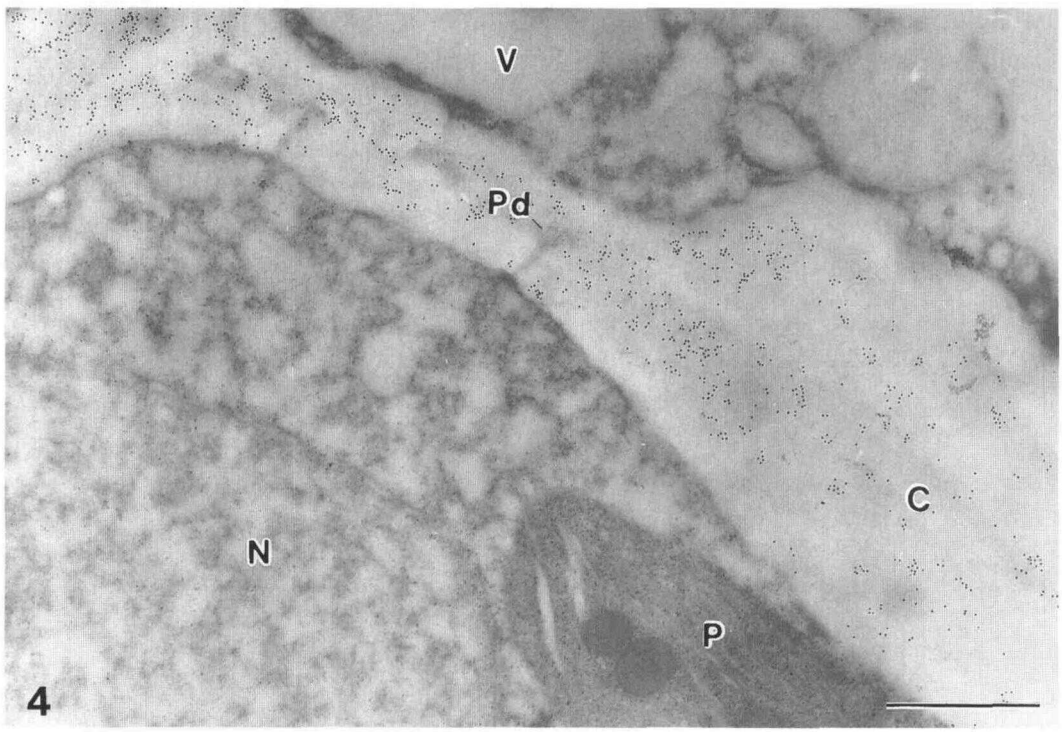

Figure 4. Effects of preincubation with EDTA prior to immunolabeling with JIM 5. Calcium-chelator treatment increases slightly the labeling in the primary cell wall. C, Primary cell wall; N, nucleus; P, plastid; Pd, plasmodesmata; and V, vacuole. Bar = 0.8 μm.

with JIM 5 (Fig. 2b) has been previously reported (Casero and Knox, 1995). These authors concluded that the specific distribution of low-esterified pectin epitope reflects regionalization of plant cell wall architecture. In our study the localized pattern also illustrates in muro the changes in the degree of esterification of the pectic chains. Indeed, enzymatic de-esterification (Table I), as well as chemical de-esterification (Fig. 3c), allows recognition by JIM 5 in the primary cell wall adjacent to the plasmodesmata-wall complexes and suppresses the localized pattern of JIM 5 labeling distribution illustrated in Figure 2b. Therefore, at an earlier stage of fruit development, PME may demethylate the pectic polysaccharides in the wall that surrounds the plasmodesmata. During fruit ripening progressive and programmed de-esterification prepares the cell wall for potential pectolytic attack, which results in cell separation (Hobson, 1963; Koch and Nevins, 1989; Roy et al., 1992).

PME also affects the ionic properties of pectins in the cell walls (Tieman et al., 1992) by enhancing calcium cross-linking as the degree of esterification decreases (Burns and

Table I. *Summary of the labeling experiments in the primary cell wall and the plasmodesmata-wall complexes*

Minus (−) and plus (+) refer to the negative or positive labeling.

Labeling Experiment	Plasmodesmata-Wall Complex	Primary Cell Wall
Cationic gold	−	+
Cationic gold after EDTA	−	+
Cationic gold after PME	−	+
Cationic gold after Na_2CO_3	−	+
JIM 5	+	+/−
JIM 5 after Na_2CO_3	+	+
JIM 5 after PME	+	+
JIM 5 after EDTA	+	+

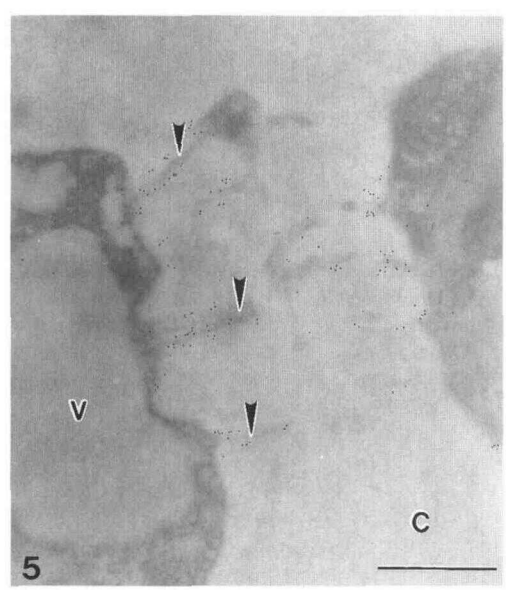

Figure 5. Distribution of callose by immunolabeling with anti-$\beta(1\rightarrow3)$-glucopyranose antibody. Callose appears to be closely associated with the strands of the plasmodesmata (arrowheads). C, Primary cell wall; V, vacuole. Bar = 1.7 μm.

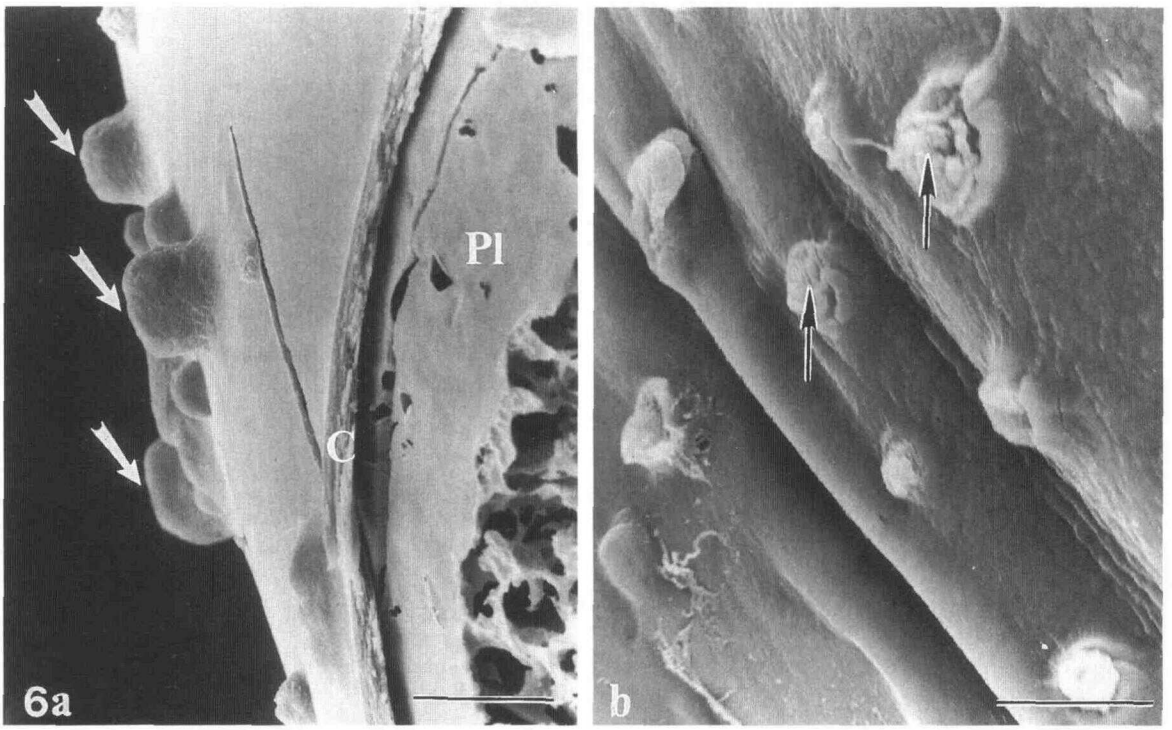

Figure 6. LTSEM observation. Cross-fractures of cells from the frozen, hydrated pericarp. a, Profile view of a pericarp cell. The fracture does not follow a single flat, horizontal plane. The plasmalemma and the cell wall could be distinguished. The outer surface of the cell wall contains circular extensions (arrows). b, Face view of the cell wall. The circular extensions appear to be composed of cell wall material surrounding cytoplasm (arrows). C, Primary cell wall; Pl, plasmalemma. Bars = 4.5 μm.

Pressey, 1987). As a result, pectolytic enzyme activities are reduced (Brady, 1987). Roy et al. (1994b, 1995) have suggested that the formation of calcium-pectin interaction complexes in target areas limits cell separation. However, the plasmodesmata-wall regions do not possess these complexes because treatment with EDTA does not restore the labeling with cationic gold (Fig. 3a), as it has been shown to do in previous studies (Roy et al., 1994a). Therefore, control of cell separation in the plasmodesmata-wall area may be partly explained by the presence of the cationic charges, as evidenced by our results of cationic gold labeling. We think that the binding of these cationic molecules will lower the electrostatic potential of the cell wall, since the negative charges of the unesterified pectins are responsible for the cell wall electrostatic potential (Nari et al., 1986). We suggest that pectolytic enzymes may be inhibited in the plasmodesmata-wall complexes by this ionic microenvironment. Our speculation is based on the findings that exogenously applied PG in unripe fruits has no effect on the autolysis of the plasmodesmata-wall complexes (Ben-Arie et al., 1979) and that polyamines modulate the activity of both PME (Charnay et al., 1992) and PG (Kramer et al., 1989). Resistance to cell wall autolysis in the plasmadesmata-wall complexes is a dramatic necessity to maintain functional exchanges throughout the process of ripening.

The Fate of the Plasmodesmata during Cell Separation

The unique collar of the cell wall that is found surrounding the plasmodesmata is an example of structural specialization of the cell wall. Our LTSEM results support the observations of Willison (1976) and Thomson and Platt-Aloia (1985), who used freeze-fracture techniques to image the plasmodesmata. LTSEM imaging allowed us to visualize the distribution of plasmodesmata complexes on the surface of separated cells. Separation of the plasmodesmata, which results from the detachment of adjacent cells occurring in deeper areas of the pericarp, ruptures the cell membranes that must subsequently seal to maintain metabolically viable cells. Localized callose deposition between the plasma membrane and the cell wall is believed to perform this sealing function (Lucas et al., 1993). Callose is present in the ring surrounding plasmodesmata in bean (*Phaseolus vulgaris*) roots (Northcote et al., 1989) and in maize (*Zea mays* L.) root tips (Balestrini et al., 1994; Turner et al., 1994). Our data, which demonstrate the presence of callose in fruit tissues, support these observations. Apparently, the callose is restricted to a peripheral collar, where it is interspersed within the cell wall and can constrict or completely seal the cytoplasmic openings associated with the ruptured plasmodesmata. Separation of the cells, which allows spatial displacement of two adjacent cells, is an important event for the softening of the fruit. This process, which usually spreads from the deep areas to the superficial layers of the pericarp, apparently involves rupture of the plasmodesmata. The reasons why plasmodesmata can enter the separation program of the cell wall within one area of the fruit is unknown.

In conclusion, we have illustrated another cell wall microdomain within the fruit tissue. We continue to support the idea that the existence of microdomains within the cell wall allows the modulation of autolysis during cell separation. Any substance, i.e. methyl groups, calcium, or cationic molecules, that is able to modify the cell wall charge density plays an important role in the localized control of pectolytic enzyme activity.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. J. Paul Knox (University of Leeds, UK) for the generous gift of JIM 5. We are grateful to Gudrun Siegert for her photographic assistance in preparing the final plates.

Received December 9, 1996; accepted March 11, 1997.

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