

Changes in Pectin Structure during Epidermal Cell Elongation in Pea (*Pisum sativum*) and Its Implications for Cell Wall Architecture

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Changes in cell wall architecture during elongation of epidermal cells in pea epicotyls were visualized by rapid-freezing and deep-etching (RFDE) techniques. The abundant network structure composed of the association of granular substances disappeared from the cell wall during elongation. The granular substances were demonstrated to be pectic polysaccharides by their disappearance upon EDTA treatment and by chemical analysis of the EDTA-extractable substances. Labeling with the monoclonal antibody JIM5, which recognizes unesterified pectins, was much more extensive in the cell walls of the non-elongating region than in those of the elongating region. The pore size of the cell wall was larger in the non-elongating region than in the elongating region. These observations suggest that the formation of the pectic gel itself is not involved in the control of the wall porosity. We proposed that the association of the granular substances is involved in the swelling of the cell walls in the elongating region.

Key words: Cell wall — Deep-etching — Epidermal cell — Immunogold labeling — Pectin — *Pisum sativum*.

Pectins are one of the major components of the primary cell wall in higher plants and are proposed to function in the control of cell wall porosity, in cell adhesion and in plant defense responses (Baron-Epel et al. 1988, Jarvis 1984, Hahn et al. 1989). Pectins are grouped into three classes of acidic polysaccharides: polygalacturonic acids, rhamnogalacturonans-I, and rhamnogalacturonans-II. Upon association with calcium ions, these polyuronic galacturonan backbones can aggregate and form gels (Jarvis 1984). It is generally accepted that pectins are synthesized in the Golgi apparatus and secreted into the cell wall in a highly methylesterified form (Zhang and Staehelin 1992). The methylesterification of carboxyl groups prevents the newly secreted pectins from forming calcium bridges. A cell wall localized pectin methylesterase deesterifies the secreted pectins, enabling them to form calcium bridges.

In the cell wall, the pectin matrix is independent of the cellulose-xyloglucan framework, but it plays important roles in elongation growth. Pectins may act as physical bar-

riers between xyloglucan and xyloglucanase (Carpita and Gibeaut 1993). Furthermore, the movement and activity of enzymes for wall metabolism during cell elongation could be controlled by the extent of pectic gel. The extent of pectin methylesterification differs between cell walls of elongating cells, in which the pectins are relatively highly esterified, and cell walls of non-elongating cells, where the pectins are relatively unesterified (Asamizu et al. 1984, Goldberg et al. 1986). It is reasonable to expect that the formation of a gel upon the demethylesterification of pectins in non-elongating cells should affect the molecular architecture of the cell wall. The variations in degree of demethylesterification in cell walls from different tissues could lead to different pore sizes in those walls. In fact, there is evidence that the pectin matrix controls pore size within the wall (Baron-Epel et al. 1988). However much remains to be learned about the role of pectins in the molecular architecture of the cell wall.

The utilization of antibodies against pectins in indirect immunofluorescent and immunogold electron microscopic techniques has demonstrated different degrees of methylesterification among different cell types within a plant tissue during the course of plant development (Casero and Knox 1995, Knox et al. 1990, Liners et al. 1994, Steele et al. 1997). There has been reported to be a distinct zonation of pectic polysaccharides, as defined by their degree of esterification, in both dicots and monocots; unesterified pectins are confined to the outer third of the outer epidermal cell wall, and highly esterified pectins are present throughout the cell wall (Schindler et al. 1995, Sherrier and VandenBosch 1994). This zonation may affect the cell wall architecture.

We focused on epidermal cells because they may control the rate of elongation of a stem or a coleoptile (Bret-Harte and Talbott 1993). It is of great interest to clarify whether the formation of the pectic gel affects the molecular architecture of the cell wall. The purpose of this investigation is to visualize the pectin structure during elongation of epidermal cells in pea by rapid-freezing and deep-etching (RFDE) techniques and its implications for cell wall architecture.

Materials and Methods

Plant materials and growth measurement—In all experiments, we used the third internode of pea (*Pisum sativum* L. cv.

Abbreviations: Ara, arabinose; Fuc, fucose; Gal, galactose; Glu, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

Alaska) epicotyls grown in the dark at room temperature. On the 7th day after germination, ink marks were made on the third internode at 5-mm intervals starting at the hook base and extending towards the node. To determine the elongating and non-elongating regions the distance between the marks was measured, daily for 3 d.

Chemical fixation, dehydration and embedding—The two regions were cut from the epicotyl and intact or EDTA-treated (0.1 M EDTA pH 7.0 at 85°C for 4 h and washed thoroughly) segments were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 50 mM cacodylate buffer pH 7.0 for 1 h at room temperature under gentle vacuum followed by another hour not under vacuum at 4°C. All samples were washed in the buffer, then postfixed with 1% osmium tetroxide in 50 mM cacodylate buffer pH 7.0. Dehydration was performed with an acetone series for Spurr's resin and an ethanol series for LR White resin. Sections were cut with an ultramicrotome (Reichert Ultracut E, Germany), then collected on Formvar coated copper or nickel grids for conventional electron microscopy and for immunogold labeling, respectively.

Immunogold labeling—The sections on the nickel grids were incubated for 30 min at room temperature with blocking solution of 1% BSA in PBST (10 mM phosphate buffer pH 7.2 containing 50 mM NaCl, 0.1% NaN₃ and 0.5% Tween 20). The grids were drained and then placed on a drop of primary antibody diluted (1 : 1) in blocking solution and incubated at 4°C overnight. The rat monoclonal antibodies, JIM5 and JIM7 (Knox et al. 1990) were a kind gift from Dr. K. Roberts (John Innes Centre Norwich, U.K.). After washing in a continuous stream of PBST, the secondary antibody (goat anti-rat IgG, Biocell, U.K.; diluted 1 : 100 in blocking solution) conjugated to 10 nm gold particles was applied for 2 h at room temperature. The grids were rinsed for 30 s with PBST followed by distilled water and were stained with saturated aqueous uranyl acetate.

Rapid-freezing and deep-etching techniques—Specimens for RFDE were either cut from intact tissues or were from the EDTA extracted tissues. In neither case were the specimens treated with fixatives. They were mounted on an aluminum disk, rapid-frozen with a liquid helium-cooled machine (Meiwa QF5000, Japan), then deep-etched and replicated with platinum and carbon as described previously (Fujino and Itoh 1994, Heuser and Salpeter 1979). Replicas were examined at 100 kV with a JEOL 2000EXII electron microscope. All deep-etched micrographs in this paper are presented as negative images.

Isolation of cell wall materials and EDTA extraction—Elongating and non-elongating regions were frozen in liquid nitrogen and homogenized in a mortar with a pestle in 80% ethanol. The homogenates were extracted once with chloroform : methanol (1 : 1, v/v) and twice with acetone, oven dried at 60°C overnight, and weighed as cell wall materials.

The extracted and dried cell wall material was suspended in 0.1 M EDTA (pH 7.0) and incubated at 85°C for 6 h (the extraction solution was changed every 2 h). At the end of the extraction, the residue was washed twice with distilled water. The various extract and wash solutions were combined and dialyzed thoroughly against distilled water. The dialyzed extract solutions were concentrated by a rotary evaporator and finally freeze dried.

Amounts of total sugar and uronic acid in the extract solutions were determined by the phenol-sulfuric acid method (Dubois et al. 1956) with glucose as a standard, and the *m*-hydroxydiphenyl method (Filisetti-Cozzi and Carpita 1991) with galacturonic acid as a standard, respectively.

Neutral sugar analysis—Extract pellets were hydrolyzed with ice-cold 85% trifluoroacetic acid for 1 h, which was then diluted

to 2 M by addition of distilled water and heated at 100°C for 5 h. The hydrolyzed material was converted to alditol acetate by reduction and acetylation (Blakeney et al. 1983). The materials were analyzed on a gas chromatograph (Shimadzu GC-14B, Japan) with an HR-SS-10 capillary column, 0.25 mm × 25 m (Shinwa Chemical Industries, LTD, Japan) at 210°C and a flame ionization detector.

Results

Determination of elongating and non-elongating regions—The elongation rate was greater for those intervals closer to the hook base. On the 8th day after germination, the region, 0–5 mm below the hook base had elongated from 5 mm on the previous day to 21.4 mm and further

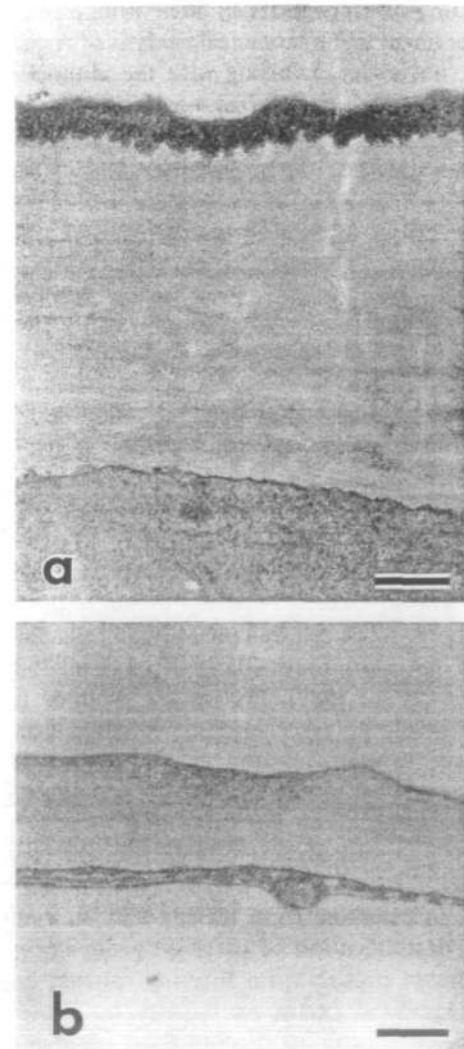


Fig. 1 Cross section of the outer epidermal cell of pea epicotyl. (a) The outer epidermal wall in elongating region, (b) the outer epidermal wall in non-elongating region. Note that the thickness of the cell wall in the non-elongating region decreased to one third of that in the elongating region after cessation of elongation growth. Bars: 500 nm.

from 21.4 mm to 28 mm on the following day. On the other hand, 45–50 mm region showed little elongation. The elongating region was defined as a 3-mm long region below the hook base; the non-elongating region was defined as a

3-mm long region in the upper side of the node. The third internode, approximately 45 mm long, which included both regions, was cut from the epicotyls on the 8th day after germination.

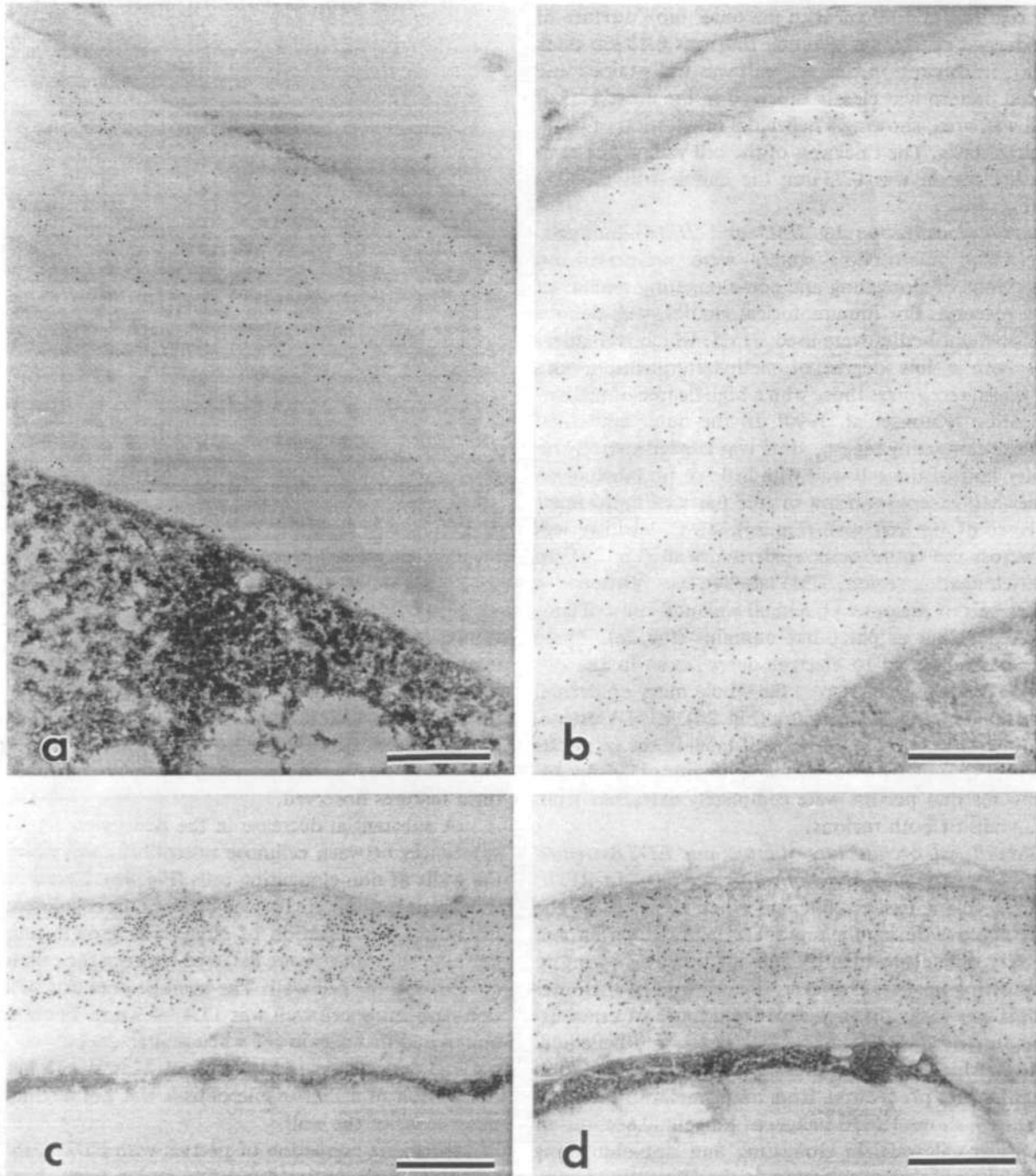


Fig. 2 Immunogold labeled sections of both elongating (a and b) and non-elongating (c and d) regions in intact tissue. The outer wall of the epidermal cell was labeled by JIM5 (a), JIM7 (b). (a) The label was confined to outer half of the wall. (b) The label was abundant throughout the wall. The outer wall of the epidermal cell was labeled by JIM5 (c), JIM7 (d). (c) The label was confined densely to outer half of the wall, (d) the label was abundant throughout the wall. Bars: 500 nm.

The change of wall thickness of epidermal cells in elongating and non-elongating regions—In order to compare the thickness of the outer epidermal cell walls in elongating and non-elongating regions, thin sections were examined. The epicotyl was sectioned perpendicularly to the longitudinal axis. The thickness of the epidermal cell wall in the elongating region was $1.99\ \mu\text{m}$ with the outer most surface of the epidermal cell having a cuticle that was $0.13\ \mu\text{m}$ thick (Fig. 1a). In this region, the cell wall was well-stained and the arced pattern was clearly observed in the inner-half of the cell wall area, showing a helicoidal orientation of cellulose microfibrils. The thickness of the cell wall in the non-elongating region was $0.73\ \mu\text{m}$; the cuticle was $0.03\ \mu\text{m}$ thick (Fig. 1b).

Immunolocalization by JIM5 and JIM7—Immunogold electron microscopic studies were performed on cross sections of elongating and non-elongating regions of the pea epicotyl. For immunological studies, well-defined monoclonal antibodies were used: JIM5, which recognizes pectins with a low degree of methylesterification, and JIM7, which recognizes those with a high degree of methylesterification (Knox et al. 1990). In the outer epidermal wall of the elongating region, JIM5 was labeled sparsely on the outer half of the cell wall, with little or no labeling on the inner half except for a row of gold particles in the inner most layer of the cell wall (Fig. 2a). JIM7 labeling was dense across the entire outer epidermal wall (Fig. 2b). In the non-elongating region, JIM5 labeling was restricted to the outer half of the outer epidermal wall and showed concentrated labeling as patch-like domains (Fig. 2c). These patches corresponded to electron-dense areas in the cell wall. JIM7 labeling was across the whole outer epidermal wall in the non-elongating region (Fig. 2d). EDTA extraction resulted in a marked decrease of label in the cell walls of both elongating and non-elongating regions (Fig. 3a, b). This ensures that pectins were completely extracted from the cell walls of both regions.

The cell wall architecture of intact and EDTA-treated cells in elongating and non-elongating regions—In RFDE techniques, plant tissues collapsed when slammed to the cold metal block during the rapid-freezing of materials and it was very difficult to identify individual tissues owing to the shadowing images. However, the cell wall of the outer epidermis was easily distinguished from those of other tissues because of its cuticle, very thick walls, and helicoidal pattern in which the change of microfibrillar orientation was regular and progressive from one lamella to the next.

Figure 4 shows RFDE images of tangential sections of outer epidermal walls in elongating and non-elongating regions. The cell wall architecture in the elongating region was composed of cellulose microfibrils and granular substances that completely infiltrated the spaces between the cellulose microfibrils (Fig. 4a). The pore size within the cell wall was $5.5 \pm 1.4\ \text{nm}$. It seemed that several granular sub-

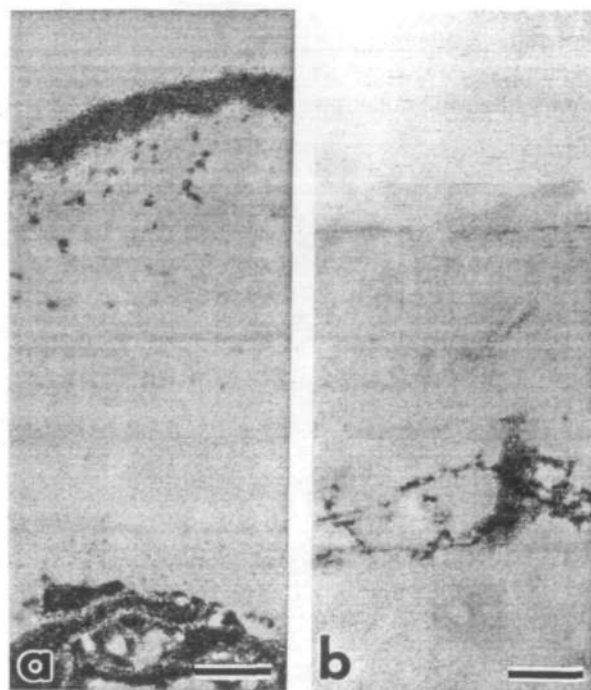


Fig. 3 Immunogold labeled sections of both elongating (a) and non-elongating (b) regions after EDTA treatment. The outer wall of the epidermal cell was labeled by JIM7. No gold particles were found in the cell wall of either region. Bars: 500 nm.

stances were connected to each other, rather than existing independently (Fig. 4 inset). This cell wall architecture of microfibrils and granular substances was continuous from the innermost wall layer just above the plasma membrane to the outermost wall layer below the cuticle. The cellulose microfibrils and granular substances were the only structural features observed.

A substantial decrease in the density of the granular substances between cellulose microfibrils was observed in the walls of non-elongating cells (Fig. 4b). Larger and less numerous spaces were formed between the cellulose microfibrils after the cessation of elongation growth and fewer granular substances were detected between the cellulose microfibrils in the cell wall. The average pore size within the non-elongating cell wall was $13.4 \pm 4.3\ \text{nm}$. There were no apparent differences in cell wall architecture between the inner and outer layers in the cell wall. However, a helicoidal orientation of cellulose microfibrils was not distinct in the outer layer of the wall.

After the extraction of pectins with EDTA, the outer epidermal walls in the elongating region were observed by the RFDE technique. The granular substances completely disappeared and only the network of cellulose microfibrils was left in the wall (Fig. 5a). The surface of the cellulose microfibrils showed a smooth contour. After the EDTA treat-

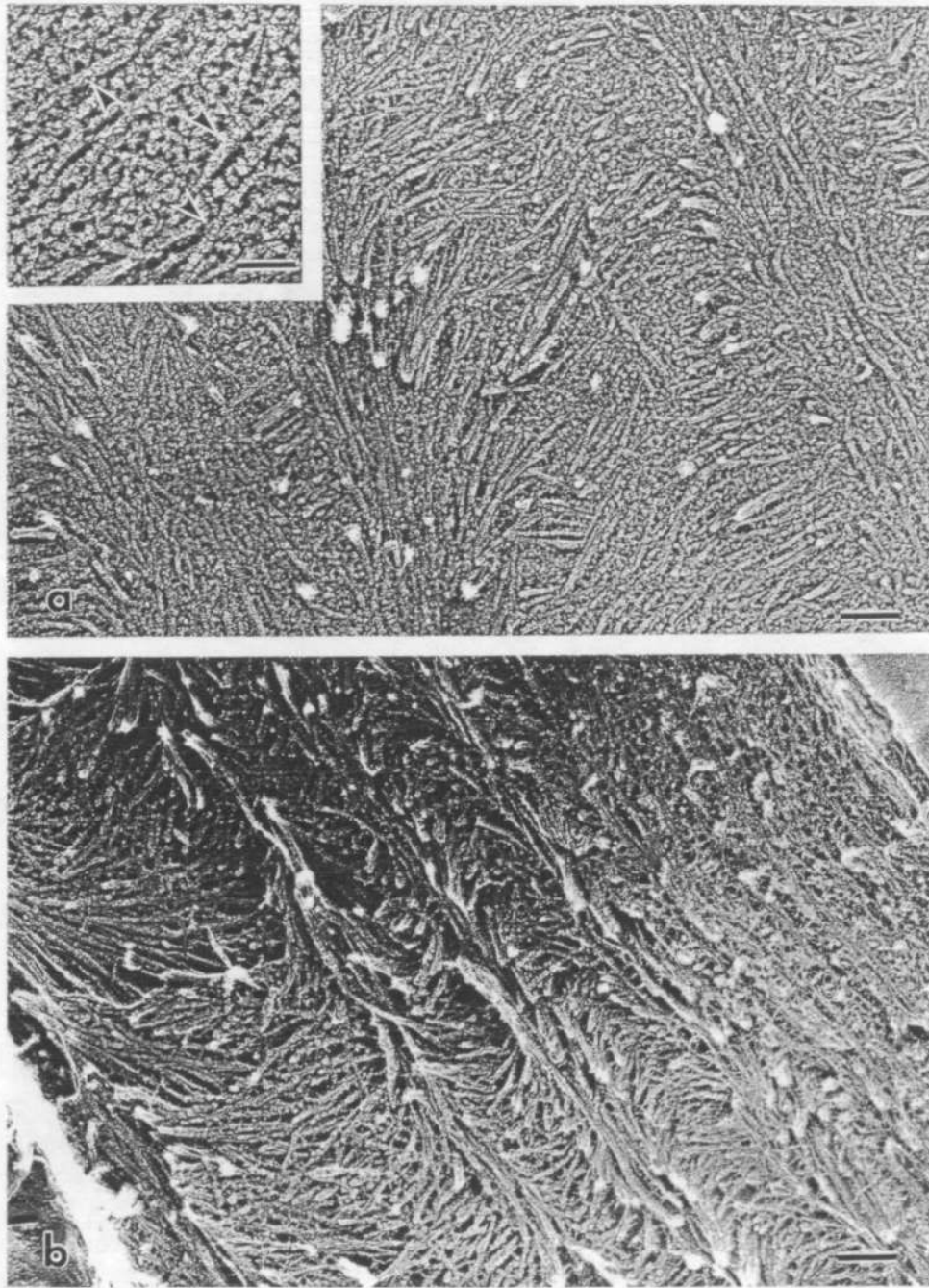


Fig. 4 RFDE images of the intact cell walls of epidermal cells in the elongating (a) and non-elongating (b) regions. (a) The cell wall architecture showed a fine porous and network structure. The spaces between the cellulose microfibrils (arrowheads) were infiltrated by granular substances. (b) The cell wall architecture of the non-elongating walls was characterized by having larger pores. Note that most of the granular substances seen in the elongating cell walls disappeared in the non-elongating region. Bars: 100 nm, bar (inset of a): 50 nm.

ment, the space between cellulose microfibrils became distinct and had a width of 30.1 ± 8.5 nm. The pore size increased significantly after EDTA treatment.

The non-elongating region of the epidermal wall was

also observed by the RFDE technique after EDTA treatment. Cellulose microfibrils were revealed more clearly than those in the non-extracted materials and the average pore size within the cell wall increased up to 20.3 ± 6.5 nm

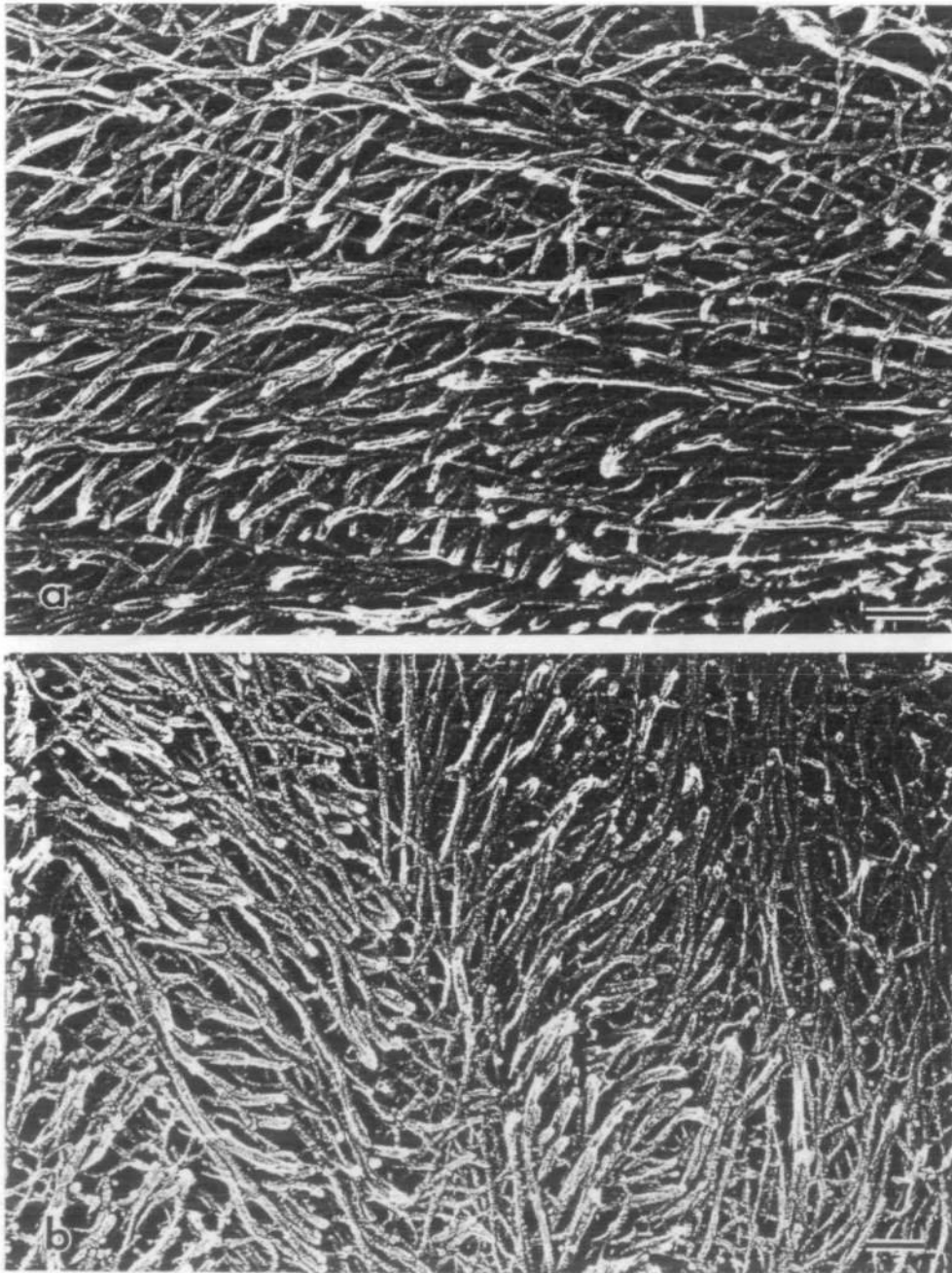


Fig. 5 RFDE images of EDTA-treated cell walls of epidermal cells in the elongating (a) and non-elongating (b) regions. (a) In appearance only the cellulose microfibrils remained. (b) The polylamellated structure of the cell wall was clearly observed. Note that the granular substances in the elongating region were completely removed by EDTA treatment. Bars: 100 nm.

(Fig. 5b). However, no substantial differences in cell wall architecture could be seen in this region between extracted and non-extracted wall.

Neutral sugar composition of EDTA-soluble pectic polysaccharides—The total sugar content of the EDTA-soluble pectic polysaccharides in the elongating region was higher than that in the non-elongating region. The uronic

acid content of the EDTA-soluble pectic polysaccharides in the elongating region was lower than that in the non-elongating region (Table 1).

The neutral sugar composition of EDTA-soluble pectic polysaccharides prepared from the elongating region was composed predominantly of Ara, Gal and Glu, with lower quantities of Xyl, Rha and Man (Fig. 6). The neutral

Table 1 Sugar contents of EDTA-soluble pectic polysaccharides extracted from elongating and non-elongating regions

Region	Uronic acid $\mu\text{g (mg cell wall)}^{-1}$	Total sugar
Elongating	59.5 ± 4.7	100.3 ± 4.2
Non-Elongating	84.5 ± 4.4	88.4 ± 0.8

The results shown as averages from four replications with standard error.

sugar composition of EDTA-soluble pectic polysaccharides in the non-elongating region was enriched in Ara and Gal, followed by Xyl, Rha, Man and Glu (Fig. 6). The amounts of Ara and Gal in pectic polysaccharides were markedly different between elongating and non-elongating regions. The ratio of the amount of Ara to that of Gal in pectic polysaccharides was 2.5 : 1 and 1.1 : 1 for the elongating and non-elongating regions, respectively. The ratio of Ara to Gal became approximately 1 : 1 after elongation growth. The high level of Glu in the elongating region and low level of Glu in the non-elongating region could be derived from starch. No attempt was made to remove starch from the cell wall materials, since both enzymatic and solvent extraction of starch could solubilize significant amounts of water-soluble pectic polysaccharides.

Discussion

We have succeeded in visualizing the structure of pectic molecules in the cell wall of the epidermal cell during

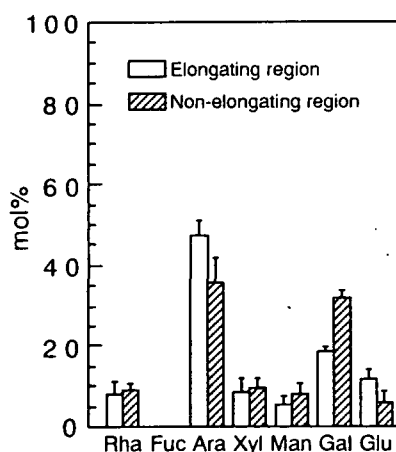


Fig. 6 Neutral sugar analysis of EDTA-soluble pectic polysaccharides extracted from elongating and non-elongating regions. The results are shown as averages of three replications with standard error.

elongation growth. The intact cell wall visualized by RFDE techniques showed dense impregnation of granular substances in the elongating region of the epidermal cell wall. These granular substances seemed to be connected to each other, thus making networks between the cellulose microfibrils. The pore size of the cell wall in the elongating region is greatly reduced by the association of the granular substances. These substances almost completely disappeared in non-elongating walls of pea epidermal cells. Furthermore, these substances completely disappeared upon pectin extraction by EDTA. As shown in Fig. 5a, the empty spaces between cellulose microfibrils were all that remained after extraction of the pectins. The distribution of the granular substances appears to determine the pore size of the cell wall by linking together the space between the cellulose microfibrils. The removal of pectins from the cell wall was confirmed by the labeling of JIM7 on the cross section of EDTA-treated segments of stem. The EDTA-soluble pectic polysaccharides extracted from both regions contained substantial amounts of uronic acid and were enriched in the neutral sugar composition of Ara, Gal, and Rha, whereas the analysis of the EDTA-soluble pectic polysaccharides was carried out in whole segments of stem. This composition would be expected from rhamnogalacturonan polymers, which bear side chains composed of Ara and Gal units attached to Rha residues in the polygalacturonic backbone (Talbot and Ray 1992). Therefore, the granular substances can be concluded to be composed of pectic polysaccharides. This is the first report on the visualization of the pectic polysaccharides in the elongating region of epidermal cells.

The granular substances disappeared from the cell wall architecture upon the cessation of cell elongation in the epidermis. However, the amount of EDTA-soluble pectic polysaccharides in the non-elongating region was higher than that in the elongating region, based upon the determination of uronic acid content. As shown by neutral sugar analysis of the EDTA-soluble pectin fraction of both regions, the molar ratio of Ara and Gal changed from 2.5 : 1 to 1.1 : 1 during elongation growth of the epidermal cell. This increase of Gal may imply changes in the galactan side chains. Cell wall properties are affected by the change of neutral sugar composition in pectic polysaccharides. For example, the change in the friability of calli is involved in a decrease of Gal content in the cell wall (Blaschek and Franz 1983, Wallner and Nevins 1974); Ara and Gal participate in the formation of cell clustering and intercellular attachment in carrot calli (Kikuchi et al. 1996); the deglycosylation of pectic polysaccharides is related to liquification of fruits (Cheng and Huber 1996). Moreover, the uronic acid backbone itself does not contribute to the processes of cell adhesion and cell separation (Liners et al. 1994). These findings suggest the involvement of neutral sugar side chains in the function of pectins. Thus, the disappearance

of the granular substances during elongation growth of epidermal cells may be caused by a compositional change of the side chains.

Differences in the labeling patterns of the monoclonal antibodies JIM5 and JIM7 were observed. JIM5 labeling was localized to the outer half of the epidermal cell wall, while JIM7 labeling occurred throughout the cell wall. This zonation of the unesterified pectin epitopes recognized by JIM5 has been found in dicots and monocots and was determined not to be involved in auxin-induced elongation growth (Schindler et al. 1995). We also found no significant differences in labeling pattern by either JIM5 or JIM7 between elongating and non-elongating regions in the epidermal wall. These data suggest that the zonation of esterification of pectic polysaccharides revealed by the antibody labeling is not involved in wall expansion. However, the unesterified pectin epitope recognized by JIM5 increased after cessation of elongation growth. Our observation that the demethylesterification of pectic polysaccharides accompanies elongation growth is consistent with previous reports that were based solely upon chemical analysis (Asamizu et al. 1984, Yamaoka and Chiba 1983). As a consequence of demethylesterification of carboxyl groups in the polyuronide backbone, pectins are able to form a calcium-bridged gel. Labeling by JIM5, which recognizes the unesterified pectins, was confined mostly to the outer part of the epidermal wall, where pectins would therefore be expected to form a calcium-bridged gel. In general, the formation of the gel might be expected to change the cell wall structure by decreasing the pore size in the cell wall. However, although JIM5 labeling was restricted to the outer part of the wall, the cell wall architecture observed by RFDE showed a homogeneous structure from the innermost to the outermost layer of the epidermal wall in both the elongating and non-elongating regions. The level of unesterified pectins increased in the non-elongating region (compare Fig. 2c with Fig. 2a), but the pore size of the wall increased. It is generally accepted that the organization of pectic substances is a major control element in defining the sieving properties of the wall (Baron-Epel et al. 1988). However, our results suggest that the formation of the pectic gel itself is not involved in determining the wall porosity. The increase of pore size in the epidermal cell wall from the elongating to the non-elongating region may be dependent upon a structural change in the pectic polysaccharides resulting from the compositional change in their neutral sugar side chains.

The thickness of the outer epidermal wall in pea epicotyls decreased during elongation growth. Sargent et al. (1974) also reported a continuous decrease in the thickness of the outer epidermal wall of etiolated pea internodes over a 24-hour growth period. Kutschera (1990) reported that wall thinning occurs during growth of plant organs such as hypocotyl, internodes and coleoptiles. Although the apposition of newly synthesized cell wall materials and its turn-

over occur, which are beyond the scope of the present analysis, the disappearance of pectic granular substances corresponded with thinning of the cell wall. Jarvis (1992) reported that pectins play a role in regulating cell wall thickness in collenchyma walls. Observation of a number of sectioned images as well as the deep-etched electron micrograph images revealed that the thickness of lamellae is decreased during elongation of epidermal cells. Originally, the wall of epidermal cells showed helicoidal orientation of cellulose microfibrils both in elongating and non-elongating regions. In the non-elongating region, the arrangement of cellulose microfibrils changed abruptly, while in the elongating region changed slowly. The presence of granular pectic polysaccharides may act as a twisting agent for the dynamic change of the helicoidal walls during elongation of epidermal cells such as sliding or loosening of the cellulose microfibrils or lamellae. In this sense, the mechanism for controlling wall thickness is dependent upon the morphological change of the pectin molecule resulting from modification of the side chains rather than from gel formation by the polyuronide backbone.

We revealed by RFDE a clear difference in the cell wall architecture between elongating and non-elongating regions. The association of pectic granular substances that was observed in the cell wall of the epidermis during its elongation growth almost disappeared after the cessation of cell elongation, in spite of the increase of pectic substances in the non-elongating region, as shown by chemical analysis. This suggests that a modification in the molecular form of pectic polysaccharides occurs during the elongation of the epidermal cell. The change in the porosity of the cell wall could be brought about by structural modification of pectic molecules, whereas aggregation of pectic polysaccharides by calcium-bridging may not play an important role in this phenomenon. We suggest that the neutral sugar side chains of pectins and not the polygalacturonan backbone play a key role in this process. The neutral sugar side chains could be involved in the interaction with other cell wall components. Further studies will be necessary to understand how the side chains of pectic polysaccharides are metabolized in the cell wall.

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