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Ultrastructural Localization of Polysaccharides in Apple Leaf Cuticles[1, 2]

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Abstract. **The ultrastructural localization of polysaccarides in *Malus domestica* Borkh. leaf cuticles was investigated by electron microscopy staining. The cuticle/cell wall interface was not stained by ruthenium red or hydroxylamine-ferric chloride, indicating the lack of a distinct pectin layer. The inner region of the cuticle was intensely and uniformly stained by phosphotungstic acid (PTA) and was lightly stained by silver proteinate, indicating the presence of polysaccharides, which in part may be pectin as indicated by staining with ruthenium red. Fibrils in the inner region of the cuticle were not stained by ruthenium red, hydroxylamine-ferric chloride or silver proteinate, but appeared lightly stained by PTA; this suggests by deduction that the fibrils may consist of cutinized cellulose. The outer region of the cuticle was not stained by any of the staining procedures, indicating the lack of polysaccharides.**

The cuticle covers all exposed primary plant parts and is a cutin matrix impregnated with waxes and polysaccharides (11). Knowledge of cuticle structure and composition has been derived mainly by light microscopy and chemical analysis (11, 21), with little work on the precise ultrastructural localization and identification of cuticular components and structures. The chemical composition and physical arrangement of cuticular components should influence strongly the movement of substances across cuticles. Waxes decrease permeability to water and ionic compounds due to their hydrophobic nature (11), and polysaccharides should impart increased permeability due to their hydrophilic nature. These studies attempted to ultrastructurally localize polysaccharides in apple leaf cuticles as a basis for determining structure-function relationships involved in penetration of compounds into leaves.

Materials and Methods

The 8th and 9th fully expanded leaf from the apex of greenhouse-grown 'Northern Spy' apple seedlings was used for standard tissue preparation. Leaf pieces, 1–2 mm, were excised, avoiding major veins, and fixed for 3 hr with 1.5% paraformaldehyde/3% glutaraldehyde, and 5 mg/ml $CaCl_2$ in 0.1 M Na cacodylate buffer, pH 7.0. The tissue was buffer-washed for 1 hour in 0.1 M Na cacodylate buffer, pH 7, then post-fixed for 3 hr with 2% OsO_4 in 0.1 M Na cacodylate, pH 7.0, then buffer-washed for 1 hr. The fixed tissue was dehydrated in a water:ethanol gradient and was infiltrated and embedded in propylene oxide: Epon/Araldite (15, 17).

For uranyl acetate/lead citrate staining, thin sections on formvar-coated copper grids were floated for 30 min on 2% aqueous uranyl acetate, rinsed 3 times in water, floated for 10 min on lead citrate (19), and then water-rinsed.

For ruthenium red staining, 3 procedures were followed: 1) 0.1% ruthenium red was added to the paraformaldehyde/glutaraldehyde and OsO_4 fixatives of the standard procedure (10); 2) a 1 hr soak in 1% ruthenium red-0.3 M $MgCl_2$ followed by 3 water-rinses was inserted between the first buffer wash and OsO_4 fixation of the standard procedure (23); and 3) thin sections on uncoated nickel grids were immersed for 1 hour in 0.5% ruthenium red containing either 0.1 M acetic acid (9), 0.3 M $MgCl_2$, or 0.1 M Na cacodylate, pH 7.0, followed by 3 water-rinses.

For hydroxylamine-ferric chloride staining (modified from 1, 2, 18), leaf pieces were immersed for 1 hr in 7% NaOH-7% hydroxylamine hydrochloride-60% ethanol after the 60% ethanol dehydration step of the standard procedure, then acidified by two 5-min soaks in 0.1 N HCl-60% ethanol, followed by immersion for 1 hr in 2% $FeCl_3$-0.1 N HCl-60% ethanol, then the dehydration procedure was continued at 70% ethanol. All of the above steps were carried out at 0–4°C.

For silver proteinate staining (modified from 20, 28), thin sections on uncoated nickel grids were immersed for 30 min in 1% periodic acid; thoroughly water-rinsed; immersed 30 min in 1% thiosemicarbizide-10% acetic acid; rinsed for three 5-min soaks in 10% acetic acid then 5 min each in 5% acetic acid, 1% acetic acid and water; immersed for 30 min in 1% silver proteinate in a dark room under red safe lights; then water-rinsed.

For phosphotungstic acid (PTA) staining (modified from 13, 22), thin sections on uncoated nickel grids were immersed for 30 min in 1% periodic acid; water-rinsed; immersed for 20 min in 1% PTA-10% chromic acid at pH 1.2, then water-rinsed. Chromium trioxide was used to make the 10% chromic acid solution.

All sections were photographed under identical contrast settings with a Phillips EM 201 electron microscope. All negatives were developed identically, and micrographs were exposed and then developed on high contrast paper until the epoxy resin above the cuticle was faintly visible. These precautions assured that differences in electron density between micrographs were due only to staining differences. All stains were commercial preparations, used without further purification.

Results and Discussion

The outer epidermal cell wall was stained heavily with uranyl acetate/lead citrate staining (Fig. 1). The inner region of the cuticle contained electron-dense fibrils extending from the cuticle-cell wall interface (Fig. 1, arrows), which were visualized

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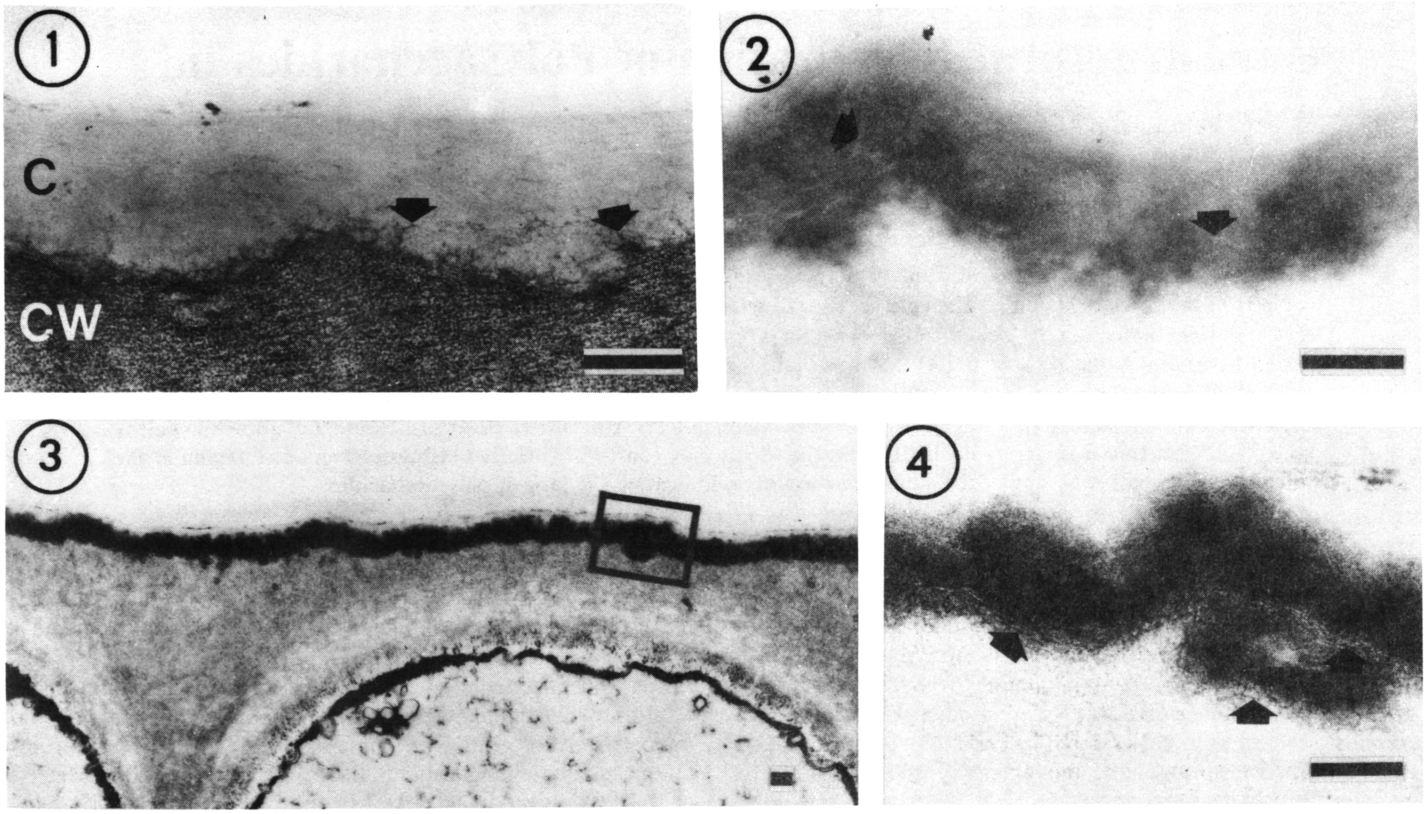

Fig. 1. Apple leaf cuticle thin section stained with uranyl acetate and lead citrate; C-cuticle; CW-cell wall; arrows denote electron-dense fibrils, 62,000x; Bar = 0.2μm.

Fig. 2. Unstained control apple leaf cuticle; arrows denote electron-translucent areas; 62,000x; Bar = 0.2μm.

Fig. 3. Apple leaf cuticle thin section treated with 1% periodic acid, then stained with phosphotungstic acid (PTA)-chromic acid; boxed area enlarged in Fig. 4; 12,000x; Bar = 0.2 μm.

Fig. 4. Enlargement and photographic underexposure of the boxed area in Fig. 3; arrows denote an electron-dense network at the cell wall-cuticle interface; 75,000x; Bar = 0.2μm.

as translucent areas on unstained controls (Fig. 2, arrows). The outer region of the cuticle was amorphorus, often with very faint electron-translucent lamellae parallel to the cuticle surface. These observations on apple are consistent with the ultrastructure of most cuticles (11).

The inner region of cuticles is reported to be composed of an amorphous matrix of cutin, variously impregnated with cellulose, pectin, hemicellulose, and wax (11, 21). Staining of thin sections with acidic phosphotungstic acid (PTA) resulted in very intense staining of the inner region of cuticle and the cytoplasmic material lining the cell lumen, with lesser staining of the cell wall, and no staining of the outer region of the cuticle (Fig. 3, 4). PTA is specific for polysaccharides if applied at an acidic pH (13, 14), but looses its specificity and also stains proteins if applied at neutral and alkaline pH (5). Thus acidic PTA staining indicates the presence of polysaccharides in the inner region of the cuticle, which probably is not cellulose due to the much lighter staining of the subjacent cell wall. The presence of polysaccharides is also indicated by light granular staining of the inner region of the cuticle with silver proteinate (Fig. 5), compared to unstained controls (Fig. 2). Silver proteinate is specific for 1, 2-glycol containing polysaccharides (8, 20). If periodic acid oxidation was omitted, no staining was obtained (not shown), which indicates that endogenous 1, 2-glycols did not cause the staining observed (Fig. 5).

Staining of leaf segments with 1% ruthenium red-0.3 M $MgCl_2$ between formaldehyde/glutaraldehyde and OsO_4 fixation resulted in greater electron density in the inner region of the cuticle compared to the outer region (Fig. 6) and compared to unstained controls (Fig. 2). The cell wall was not stained. Results with 0.1% ruthenium red added to the formaldehyde/glutaraldehyde and OsO_4 fixatives was similar but not as intense. Staining of thin sections was not successful. Ruthenium red is specific for pectin by forming a salt bridge between adjacent nonesterified galacturonic acid monomers (27), thus the ruthenium red staining indicates the presence of pectin in the inner region of the cuticle. Polyuronides (pectin) also were reported in the inner region of oat coleoptile cuticles (12), and ruthenium red also lightly stained the inner region of *Agave americana* leaf cuticles (29), but did not stain barley leaf cuticles (26). It is not known if ruthenium red (Fig. 6) silver proteinate (Fig. 5) and PTA (Fig. 3) were all staining the same or different polysaccharides. Hydroxylamine-ferric chloride did not stain the cuticle, although it lightly stained the cell wall (Fig. 7), compared to controls where the electron-dense $FeCl_3$ step was omitted (Fig. 8). Hydroxylamine-ferric chloride is specific for esterified pectin (18), and the lack of staining tentatively suggests that the pectin stained by ruthenium red might be nonesterified. To verify this, methylation of leaf segments was attempted (17), but disruption of ultrastructural detail by the harsh procedure obscured results.

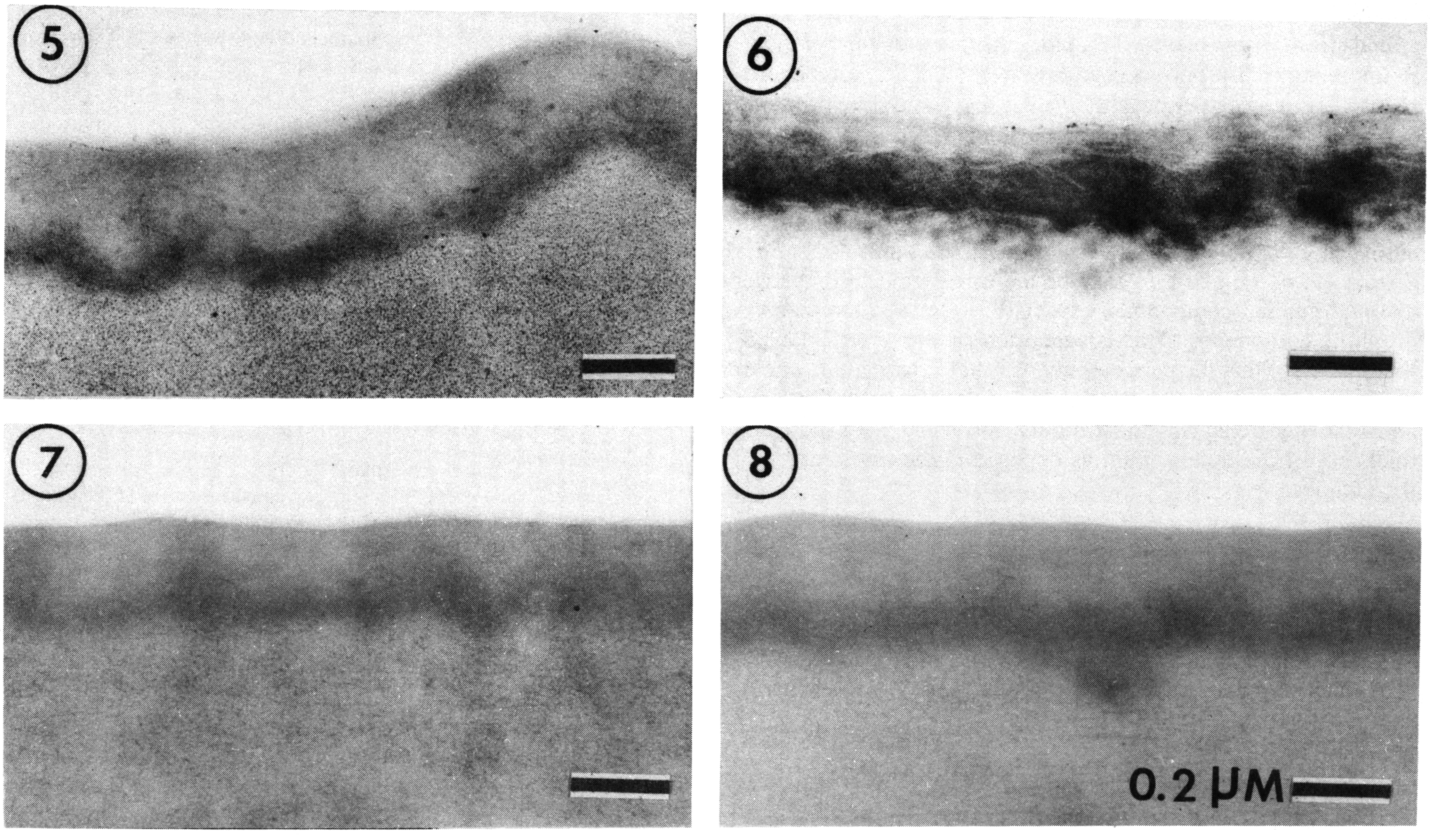

Fig. 5. Apple leaf cuticle thin section treated with 1% periodic acid, then stained with silver proteinate; 57,000x; Bar = 0.2μm.

Fig. 6. Apple leaf cuticle block stained with 1% ruthenium red-0.3 M $MgCl_2$ between formaldehyde/glutaraldehyde and OsO_4 fixatives; 62,000x; Bar = 0.2μm.

Fig. 7. Apple leaf cuticle block stained with complete hydroxylamine/ferric chloride procedure; 62,000x; Bar = 0.2μm.

Fig. 8. Control apple leaf cuticle block treated with hydroxylamine but not stained with ferric chloride; 62,000x; Bar = 0.2μm.

The inner region of cuticles stained with uranyl acetate/lead citrate characteristically is impregnated by electron-dense fibrils (11, Fig. 1). The fibrils stain readily with other ionic stains such as lead hydroxide or barium permanganate (6),and are sites of accumulation of silver nitrate (6) and lanthanum nitrate (24). This indicates the fibrils are hydrophilic in nature, and does not support proposals that they are sites of accumulation or pathways for the diffusion of wax precursors to the leaf surface (3, 24). The fibrils were visualized as an electron-translucent network in unstained, yet osmicated sections (Fig. 2). This indicates that the fibrils are not osmiophilic as termed by others (24, 25, 26). The fibrils were not visualized by ruthenium red (Fig. 6) or hydroxylamine-ferric chloride staining (Fig. 7) of apple leaf cuticles, or of other plants species (3, 26). This indicates that the fibrils are not composed of pectin as suggested by others (4, 12). Silver proteinate did not clearly stain the fibrils in apple leaf cuticles (Fig. 5), but reportedly stained those in *Agave americana* leaf cuticles (29). Photographically underexposing the micrographs of PTA-stained apple cuticles reveals a very fine electron-dense network at the cuticle-cell wall interface (Fig. 4, arrows), which appears analagous to the fibrils visualized with uranyl acetate/lead citrate staining (Fig. 1). These results (29, Fig. 3–4) indicate that the fibrils contain polysaccharides, which may be cellulose due to their fibrillar structure (Fig. 1) and lack of positive pectin staining (FIg. 6, 7). Cellulose has been shown to be inert to many polysaccharide staining procedures due to either its crystalline structure (8) or cutinization (21). Cellulose microfibrils might be drawn into the inner region of the cuticle during the rapid secondary stage of cuticle development as proposed by Sargent (25).

A pectin layer generally is considered to occur between the epidermal cell wall and cuticle based on light microscopy ruthenium red staining and the presence of an isotropic layer under plane-polarized light (11, 21). Increased staining of apple leaf cuticles was not observed at the cell wall-cuticle interface with ruthenium red (Fig. 6) or hydroxylamine-ferric chloride (Fig. 7), and similarly, no staining was shown at the cuticle-cell wall interface in other plants (3, 25, 26, 29). This questions the presence of a distinct pectin layer between the cuticle and cell wall. Light microscopy ruthenium red staining may be due to pectin in the inner region of the cuticle (Fig. 6), and isotropism may result from reorientation of cellulose fibrils from parallel to perpendicular at the cuticle-cell wall interface (Fig. 1).

The outer region of cuticles is proposed to be composed of a cutin matrix impregnated by wax, but not by polysaccharides (11, 21). This is supported by these studies, since no polysaccharide stain imparted electron-density into the outer region of apple leaf cuticles (Fig. 3, 5, 6, 7) or in plants studied by others (3, 25, 26, 29). However, the fibrils characteristic of the inner region of cuticles may extend very close to the outer cuticle surface (6), which would impart polysaccharides in the outer region.

The cuticle is the rate-limiting barrier to the penetration of foliar-applied compounds into leaves (7) and the loss of com-

pounds from within leaves (11). Since the inner region of cuticles is impregnated by polysaccharides, it should be more hydrophilic, hence more permeable to water and ionic or hydrophilic compounds, yet may be less permeable to hydrophobic compounds. This is supported by observations that ionic compounds preferentially penetrate cuticles via the fibrils of the inner region of cuticles (6). The outer region of the cuticle is composed of cutin and waxes (11, 21), and was shown to lack polysaccharides (Fig. 3, 5, 6, 7), and thus may be the true permeability barrier to foliar-applied compounds, especially water and ionic or hydrophilic compounds. This is supported by the observations of Reed (15, 16) that the permeability of isolated carnation cuticles to rubidium phosphate decreased as cuticle thickness increased, and that this trend was most highly correlated with increased thickness of the outer region as opposed to the inner region of the cuticles.

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