Maintenance of Turgor by Rapid Sealing of Puncture Wounds in Leaf Epidermal Cells

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ABSTRACT

When leaf epidermal cells are puncture wounded with a glass microcapillary tip, a small droplet of fluid is discharged and then evaporates, leaving a solid residue on the cell surface. For puncture wounds of about 3.5 micrometers in diameter, this process is complete within 2 to 3 seconds. A second puncture wound also exhibits a similar discharge, indicating the persistence of some turgor pressure within the cell, despite damage to the cell wall. Direct measurement of turgor on the large epidermal cells of Tradescantia virginiana L. demonstrated that turgor was substantially maintained (91–96%) after puncture wounding. Anatomical and histochemical evidence suggests that the damaged portion of the cell wall was sealed with an amorphous plug of material comprised of pectinaceous polysaccharides. Rapid sealing of puncture wounds and the maintenance of turgor in epidermal cells may be an important functional component of plant adaptation to physical damage such as that caused by insect feeding.

The pressure microprobe, the only method available for the direct measurement of turgor pressure in higher plant cells (8), requires the physical puncture of the cell wall by insertion of a glass microcapillary tip. For the most part, measurement of turgor in higher plants has been limited to large cells, such as the 100-μm epidermal cells of Tradescantia virginiana L. (10). The outer wall of an epidermal cell is unsupported, however, and under strain when the cell is turgid. Hence, it is interesting that, despite the structural damage and presumed weakening of the punctured area of the wall itself, these large epidermal cells have been found to support turgor of 0.6 to 0.7 MPa continuously throughout periods of many hours (11). In the absence of an independent method, evidence concerning the structural integrity of the cell between the glass microcapillary tip and the measured cell, and hence the reliability of the microprobe measurements themselves, has been indirect. In many of the early studies (e.g. ref. 14), stable turgor values were found, which could be reversibly and predictably influenced by external osmotic changes or imposed cell volume changes, suggesting at least that the presence of the puncture wound did not interfere with cell membrane integrity. Cell to cell reproducibility (2), stability immediately after penetration (12), and agreement with independent measurements of tissue-level water relations (10) have all indicated that, in the absence of physical disturbance, pressure measured with the microprobe accurately reflects cell turgor pressure. Using structural and cytochemical evidence together with an improved microscopic system (12), we document in this report the presence of a sealing mechanism at the cell level which may account for the reported reliability of turgors measured with the pressure probe.

MATERIALS AND METHODS

Plant Material

Epidermal cell turgor measurement and most of the structural studies were performed on leaves of greenhouse-grown Tradescantia virginiana L. Greenhouse-grown plant specimens representing 11 other vascular plant taxa (Juglandaceae, Liliaceae, Myrtaceae, Oleaceae, Orchidaceae, Pteridaceae, Punicaceae, Rosaceae, Rutaceae, Solanaceae, and Vitaceae) representing primitive, advanced, woody, herbaceous, monocot, and dicot types were also used.

Turgor Measurement, Optical System, and Cell Puncture Wounding

The system used to position, penetrate, and measure the turgor of individual epidermal cells was largely identical with that described earlier (13). Briefly, cells and microcapillary tips were observed at a total magnification of ×200, through a vertically illuminated microscope (BHMP system, Olympus Corp., New York) using a long working distance (11 mm) ×20 objective. Glass microcapillary tips were pulled (Koh model 750 micropipette puller) and, when used for turgor measurements, were opened with a jet stream of polishing compound (12). Puncture wounds were made by impaling cells with unopened glass microcapillary tips, with the degree of wounding determined by the depth of microcapillary penetration into the cell. In some cases, the behavior of the wounded cell was recorded on a videotape system (Sharp XCB-10 camera and Ampex/Nagra VPR-5 recorder using 1-inch “C” format), and photographic prints were made from digitized images to document the events which rapidly followed cell wounding.

Microscopy and Cytochemistry

Surface characteristics of wounded epidermal cells were observed in situ with the same microscopic system used for the turgor measurements. Wounded cells were also prepared for SEM1 and light microscopy. Tissue for SEM was fixed in 4% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8, de-

1 Abbreviation: SEM, scanning electron microscopy.
hydrated in a graded ethanol series, critical point dried, and sputter coated with gold-palladium. For thin sections, every epidermal cell in an approximately 10-mm² area of the adaxial leaf surface was punctured once to increase the likelihood of obtaining sections through puncture wounds. This area was dissected from the leaf, fixed in 4% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8, dehydrated, embedded in glycol methacrylate resin, and sectioned at 1 to 2 μm. Sections were stained with coriphosphine (Bio/Medical Specialties, Santa Monica, CA) specific for pectic polysaccharides (16) and calcofluor white M2R (Polysciences, Warrington, PA), specific for β-1-4 glucans (7).

In addition, filaments extruded from punctured epidermal cells of strawberry (see “Results” for details) were collected on slides and examined using a range of cytochemical procedures: 1-anilinonaphthyl-sulfonic acid (CalBiochem, La Jolla, CA), a fluorescence stain for proteins (6); nile red (Molecular Probes, Eugene, OR), a fluorescence stain for lipids (5); Calcofluor White M2R (Polysciences), a fluorescence stain for cellulose and β-1-4 glucans (7); periodic acid-Schiff reaction, a fluorescence and bright-field stain for insoluble polysaccharides (9); Coriphosphine (Bio/Medical), a fluorescence stain for pectic polysaccharides (16); and decolorized aniline blue (Polysciences), a fluorescence stain for β-1-3 glucans (13).

RESULTS AND DISCUSSION

During routine measurement of turgor on epidermal cells of T. virginiana L., close observation of the glass microcapillary tip and the cell surface in the zone of penetration indicated that there was a surprisingly strong adhesion between the cell wall and the glass microcapillary tip. For instance, the capillary tip could be moved to one side far enough to cause flexing of the cell wall and bending of the capillary tip glass, without causing any apparent leaks or any detectable change in cell turgor. In some cases, the adhesion was strong enough to tolerate a small amount of outward pull on the capillary tip. This behavior indicated that the seal formed between the cell wall and the glass capillary tip was not simply analogous to a compressive seal between a circular hole and a conical plug. Hence, we hypothesized that, if a punctured cell wall could seal around a foreign object, then a mechanism may exist to seal damaged areas of the cell wall in the absence of foreign objects. Qualitative evidence that puncture-wounded epidermal cell walls were resealed and that the cells were still turgid following damage was obtained by performing multiple punctures in the same cell (Fig. 1, A–D). The first time a capillary tip was inserted into a cell (Fig. 1A), a droplet of fluid was discharged from the damaged spot when the capillary tip was withdrawn (Fig. 1B). The size of the droplet depended approximately on the degree of damage, with deeper penetration giving a larger droplet size. The fluid would subsequently evaporate, leaving a residue on the surface of the cell. Occasionally, some of the fluid adhered to the withdrawing capillary tip, and this fluid exhibited the same rate of evaporation and final residue as the fluid on the cell surface. Because the cells were turgid, the discharge itself was not unexpected; however, the suddenness of appearance and disappearance of the droplet was surprising: the entire process was complete in 2 to 3 s. Furthermore, as soon as the capillary

Figure 1. Puncture wounding of individual leaf epidermal cells in T. virginiana (A–D) and F. × annassa (E and F). The cell wall was punctured with a glass microcapillary tip to a depth of about 10 μm (A) and a discharge of cell fluid occurred during capillary tip withdrawal (B). About 15 s later, the same cell was punctured in a new position (C) and exhibited a similar discharge of fluid (D), indicating the continued presence of turgor in the cell. A solid residue is also visible at the site of the first puncture (C, arrowhead). On some occasions, capillary tip withdrawal was not associated with fluid discharge but, rather, with the pulling of filamentous material from the puncture site (E: arrowhead, end of the microcapillary tip and beginning of the filament). In this case, after pulling to a length of about 100 μm, a pause in the withdrawal motion allowed the filament to stiffen, and resumption of withdrawal reopened the wound and caused the typical fluid discharge (F). Bar, 100 μm.
tip could be repositioned and a second site damaged on the same cell (within 10–15 s), the same droplet behavior was observed (Fig. 1C and D). This behavior was consistent with the hypothesis that at least some turgor remained after the initial damage. Further discharges could be observed from the same cell following additional damage but with progressively smaller and less sudden droplet appearance.

Puncturing epidermal cells and observing their behavior was a simple qualitative test for the persistence of turgor after damage, and so this test was performed on plant specimens from a number of diverse taxonomic groups. Epidermal cells in the leaves of the 11 species tested (Adiantum diaphanum Blume, Citrus sinensis [L.] Osbeck, Epidendrum cochleatum L., Fejoo sellowiana Berg., Juglans regia L., Lilium tigrinum L., Lycopersicon esculentum L., Potentilla glandulosa Lindl., Punica granatum L., Vitis vinifera L.) showed the same evidence of turgor persistence as did those of T. virginiana. Hence, this property may be common to the epidermal cells of many higher plants. Substantial maintenance of turgor despite damage was confirmed quantitatively by direct turgor measurements in damaged cells of T. virginiana (Table I). The loss of turgor following a single puncture damage (0.03–0.07 MPa, depending on the degree of damage) was a surprisingly small fraction (4–9%) of the initial turgor in these cells. Thus, these cells are able to rapidly seal damaged portions of their walls and maintain >90% of their initial turgor pressure. These results underscore conclusions from an earlier study (12) that the criterion for reliability of turgor pressure measurements cannot be based solely on stability, because stable but erroneously low values can be obtained from damaged and resealed cells that have lost a substantial quantity of fluid during penetration.

The sealing phenomenon is difficult to understand on physical principles alone. In theory, the surface tension of water (0.073 J m⁻²) could support the observed pressure of about 0.7 MPa (Table I) but only for a maximum 0.4-μm diameter hole through a hydrophobic wall. If the hole were through a wall of hydrophilic material (such as cellulose), then no pressure could be supported. Involvement of the plasma membrane would only reduce the value based on water alone, because the maximum surface tension of biological membranes, beyond which irreversible plastic deformation occurs, is about 0.08 × 10⁻³ J m⁻² (4), three orders of magnitude less than that of water. Hence, the hole that remained in the outer wall of these epidermal cells after damaging was either (a) much smaller than the value based on the diameter of the capillary tip at each depth of penetration (as assumed in Table I) or (b) sealed with solid material. The involvement of some solid adhesive material was suggested by occasional observations of a filament adhering to the capillary tip as it was withdrawn from a cell (Fig. 1E). The appearance and behavior of these filaments suggested that they resulted from an extrusion-like process, in which a viscous fluid was being pulled out through the puncture hole and was hardening on contact with the air. Connection of the filament to the puncture site was clearly demonstrated for the case depicted in Figure 1 when, after a pause in the withdrawal motion allowed the filament to harden (Fig. 1E), a pull on the filament reopened the wound and caused the typical droplet discharge (Fig. 1F).

The occurrence of filaments was an infrequent event for the epidermal cells of most species but was frequent enough in strawberry (Fragaria × annassa) that filament specimens could be collected, mounted on glass microscope slides, and characterized cytochemically with a range of staining reactions. Negative reactions were obtained following staining for proteins (1-anilinonaphthyl-sulfonic acid [6]), lipids (nile red [5]), and cellulose/β-1–4 glucans (Calcofluor White M2R [7]). Strongly positive reactions were obtained for polysaccharide (periodic acid-Schiff [9]) and pectic materials (Coriphosphine [16]). No results were obtained for callose/β-1–3 glucans (decolorized alkaline aniline blue [13]) because the filaments were completely soluble in the high pH solution required for this staining reaction. These results indicate that the filaments are composed primarily of pectinaceous carbohydrate material, with negligible amounts of proteins, lipids, and β-1–4-linked carbohydrates.

Even though the pectinaceous composition of these filaments was apparent, the importance of pectinaceous material to the wall-sealing process itself remained unclear. The puncture wounds made by the capillary tip may have caused structural damage to the wall only on a very limited scale (e.g. 0.4 μm) with elastic stretching of the wall occurring as the capillary tip was further inserted. According to this hypothesis the rescaling of the wall against pressure might be due to an elastic relaxation back to an effective hole size small enough for surface tension or membrane strength to hold, and the pectinaceous material may be simply a residue of the discharged fluid. However, this hypothesis was inconsistent with the appearance of the surfaces of damaged cells (Fig. 2). A residue that remained on the surfaces of these cells could be clearly seen in situ (Fig. 2A). Most of this residue was easily removed with water, but the damaged spot on each cell was still visible at the light microscope level (Fig. 2B). SEM of similar specimens (Fig. 2, C–E) revealed that these spots corresponded to persistent holes in the cell wall of the same

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<th>Time after Damage</th>
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<td>5–30 min</td>
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approximate size as was estimated from the capillary tip dimensions. Most of the holes appeared to be filled with a plug of amorphous material. Thin sections through the damaged portion of the wall showed a plug of material in the damaged area (Fig. 3, A, D, and E) in some cases extending into the cell (Fig. 3D). The material plugging the wound gave a negative staining reaction with Calcofluor White M2R (Fig. 3B), indicating that β-1,4 glucan polymers, including cellulose, were not a significant component of the plug (?). The plug material was autofluorescent at long wavelengths (>650 nm) when near-UV blue (365–395 nm) excitation was used (Fig. 3C). Although the nature of this autofluorescent material is uncertain, numerous plant phenolic products show this type of fluorescence and may be involved here. The plugs gave a positive staining reaction with coriphosphine (Fig. 3F), a response that is consistent with the occurrence of pectinaceous polysaccharides as a component of the plugging material (16).

Hence, evidence from both SEM and light microscopy indicated that the cell wall sustained permanent structural damage as a result of puncturing and that this damage exceeded the 0.4-μm limit needed to explain maintenance of turgor through wall elasticity and surface tension effects alone. A solid adhesive material, possibly pectinaceous, may be responsible for the surprisingly rapid and effective sealing of these puncture wounds in epidermal cells, but the mechanism of this process remains unclear. Regardless of its mechanism, however, the fact that leaf epidermal cells do maintain turgor and can rapidly seal puncture wounds may itself be of substantial adaptive significance in plants. One salient feature of plants is that living cells, rather than layers of dead tissue, compose a large portion of the epidermal surface. In addition to structural support, leaf epidermal cells, which have little cytoplasm and contain no chloroplasts, clearly must allow a high percentage of light energy to reach photosynthetic tissues. Maintenance of turgor in these epidermal cells may be central to the maintenance of their structural and optical properties and, hence, one of their primary functions.

Figure 2. Surface views of puncture-wounded leaf epidermal cells under light microscopy (A and B) and SEM (C–E). A residue of discharged fluid which was clearly visible following puncture damage (arrowheads in A) was easily removed with water, but puncture sites were still apparent after washing (arrowheads in B). SEM of similar specimens (C–E) indicated that these sites corresponded to persistent holes in the external cell wall (arrowheads) with a plug of amorphous material filling most (D) but not all (E) puncture sites. Bars: A to D, 100 μm; E, 10 μm.
Another function of the leaf epidermis, for which rescaling may play an important role, is as a protective layer against predators. Many types of insects feed on plant leaves by piercing through the epidermal layer, in most cases intracellularly but, depending on insect species, also intercellularly. Piercing insects generally do not feed on nutrient-poor epidermal cells, but a rapid sealing mechanism may also make direct penetration of epidermal cells particularly difficult and, hence, may explain why epidermal cells are often avoided by piercing insects. In this context, it is interesting to note that an amorphous material described as “surface debris” was localized around intercellular spider mite (Tetranychus mcdanielli) penetration sites on the epidermal surface of apple leaves (15). Epidermal cells were also found to be essentially undamaged by mite feeding in this and other studies, and we suggest that these observations are consistent with the sealing process described in this paper. The possible involvement of carbohydrates in cell sealing suggests that, at least in the epidermal layer, these materials are readily available and have important functions other than as structural components in the cell wall. Indeed, high levels of carbohydrates for the purposes of wound repair in these outermost plant tissues may suggest an adaptive reason for the growing body of evidence (1, 3) that matrix polysaccharides play a central and two-way role as the chemical basis of recognition between plants and their predators and pathogens.

**LITERATURE CITED**


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**Figure 3.** Thin sections of punctured leaf epidermal cells of T. virginiana. A to C, same section, stained with Calcofluor White M2R, viewed with phase-contrast (A) and fluorescence illumination (B and C). Material plugging the puncture is evident (arrowhead in A). B, An UG-1 (365-395 nm) excitation filter and an FT-405 dichroic mirror were used with an LP-420 (>420 nm) and SP-650 (<650 nm) barrier filter; C, the SP-650 barrier was removed. Material comprising the plug is not stained with Calcofluor White M2R (B), although long-wavelength autofluorescence (C), seen also in unstained sections, is apparent. D, Phase-contrast image of a plugged puncture wound; arrowhead indicating the plug. E and F, The same section, stained with Coriphosphine, viewed with (E) phase-contrast and (F) fluorescence illumination (546 nm bandpass excitation filter, 580 nm dichroic mirror, and 590 nm longpass barrier filter). The plugging material gives a strong positive staining with Coriphosphine (F). Bar, 20 μm.


