Plasma membrane H\(^+\)-ATPase in the root apex: Evidence for strong expression in xylem parenchyma and asymmetric localization within cortical and epidermal cells

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The cell and subcellular localization of plasma membrane P-type H\(^+\)-ATPase in root apices from Zea mays L. (maize) seedlings was investigated by immunofluorescence microscopy. H\(^+\)-ATPase was highly abundant in cells of epidermal and endodermal tissues as well as in phloem companion cells. Strong immunodetection was also observed in a subset of xylem parenchyma cells forming a connection between the endodermis and metaxytem. Evidence that these cells are equipped for active membrane transport raises the potential that they play a special role in xylem loading. Significant amounts of H\(^+\)-ATPase were also observed in outer cortical cells. Progressively less H\(^+\)-ATPase was seen in cortical cells further away from the root-soil interface. The H\(^+\)-ATPase was asymmetrically localized within both epidermal and outer cortical cells, with higher levels detected on cell surfaces closest to the root-soil interface. This asymmetric localization of H\(^+\)-ATPase is consistent with the hypothesis that transport systems for uptake of nutrients from the soil are selectively targeted to cell surfaces most exposed to nutrients.

**Abbreviations** – CC, companion cell; ED, endodermis; EP, epidermis; IC, inner cortex; IM, intercellular membranes; MP, microsomal pellet; MTSB, microtuble stabilizing buffer; MX, metaxytem; OC, outer cortex; PBS, phosphate-buffered saline; PM, plasma membrane; PS, postmitochondrial supernatant; QC, quiescent center; RC, root cap.

**Introduction**

In plants P-type H\(^+\)-ATPases function to maintain an electrical and pH gradient across the plasma membrane. This enables the plant cell to take up ions and nutrients against their concentration gradients (reviews, Michelet and Boutry 1995, Palmgren 1998). For this reason, all living plant cells are thought to express a plasma membrane H\(^+\)-ATPase. However, it has been shown that the abundance of this enzyme varies in different cell types and tissues (e.g. Parets-Soler et al. 1990, Villalba et al. 1991, Samuels et al. 1992, Bouchez-Pillon et al. 1994a,b, DeWitt and Sussman 1995, Froemland et al. 1995). For example, immunohistochemical studies have shown that H\(^+\)-ATPase is very abundant in phloem companion cells, guard cells, transfer cells, and root epidermal cells. These examples support the prevailing hypothesis that high levels of H\(^+\)-ATPase are present in cells engaged in active transport (Serrano 1989).

The root is a specialized organ that functions in uptake of nutrients from the soil and translocation of those nutrients to other parts of the plant. Using immunohistochemistry, two studies have examined the distribution of the H\(^+\)-ATPase in roots of seedlings,
showing high expression in epidermal and vascular tissues (Paredes-Soler et al. 1990, Samuels et al. 1992). The root epidermal cells are thought to be the primary site for nutrient uptake from the soil. The root vascular system functions in loading and long distance transport of water and nutrients through both xylem and phloem. Thus, the relative abundance of H\(^+\)-ATPase in these tissues is consistent with an expected high level of membrane transport activity.

Here we examine the localization of H\(^+\)-ATPases in root apex tissues. To improve the sensitivity and resolution, we employed a gentle embedding-sectioning technique using Steedman's wax (Baluska et al. 1992). Our results confirm that H\(^+\)-ATPases are most abundant in root epidermal, endodermal and phloem companion cells. In addition, we provide new evidence for relatively high levels of H\(^+\)-ATPase in a group of xylem parenchyma cells forming a 'bridge' between endodermal cells and the metaxylem. This observation raises the question of whether these cells are involved in actively loading the metaxylem, perhaps analogous to the role of companion cells in loading sieve tubes. The improved sensitivity of our immunodectection also showed relatively high levels of H\(^+\)-ATPase in outer cortical cells, consistent with a model that these cells provide secondary sites of nutrient uptake. H\(^+\)-ATPase in both outer cortical and epidermal cells showed an asymmetric localization with more pumps in membranes closest to the soil surface, suggesting that root epidermal and cortical cells have the capacity to sense and reorganize their membrane cytoskeletal system for efficient nutrient uptake.

### Materials and methods

#### Plant material

Maize (Zea mays L.) seeds were soaked for 6 h in tap water and seedlings were grown on moistened filter paper at 25°C in darkness to a root length of 4–5 cm.

#### Biochemical characterization and localization

Maize roots (10 g) were homogenized in 50 ml of 250 mM Tris-HCl, pH 8.0, 300 mM sucrose, 25 mM EDTA, 5 mM DTT, 5 mM ascorbate, 0.6% (w/v) insoluble polyvinylpolypyrrolidone and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 10,000 g for 15 min and the postmitochondrial supernatant (PS) was subsequently centrifuged for 55 min at 50,000 g. The resulting microsomal pellet (MP) was subjected to partitioning in an aqueous polymer two-phase system (8-g systems) composed of 6.2% (w/w) Dextran T500 (Pharmacia, Uppsala, Sweden), 6.2% (w/w) polyethylene glycol 1500 (The British Petroleum Company, London, UK), 330 mM sucrose, 5 mM potassium phosphate, pH 7.8, 3 mM KCl, 0.1 mM EDTA, 1 mM DTT (4°C) according to Larsson et al. (1994). The plasma membranes (PM) were yielded from the final top phases while intracellular membranes (IM) remained in the first bottom phase. Twenty µg of protein of each fraction was subjected to SDS-PAGE (7.5–15% [w/v] acrylamide) according to Lasemli (1970). Proteins were either stained with Coomassie Brilliant Blue R 250 or transferred electrophoretically to an Immobilon polyvinylidifluoride transfer membrane (Millipore) for immunostaining. The monoclonal antibody 46E5B11F6 raised against purified H\(^+\)-ATPase from maize coleoptiles (Villalba et al. 1991, Palmgren and Christensen 1994, Baur et al. 1996) was used to decorate the membrane. Protein was estimated according to Bearden (1978) with bovine serum albumin (BSA) as a standard.

#### Fixation and embedding

Fixation and embedding were done as described in Baluska et al. (1992). Apical portions (1.5 cm long) of the roots were cut and fixed in 3.7% formaldehyde in 20 mM PIPES-KOH, pH 6.9, 5 mM MgSO\(_4\) and 2 mM EGTA (MTSB) for 1 h at room temperature. The roots were then washed 3 x 10 min in MTSB and 30 min in 6.5 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 140 mM NaCl and 2.7 mM KCl, pH 7.3 (PBS). Samples were subsequently dehydrated in an ascending ethanol series from 15 to 100% ethanol and embedding was performed at 37°C in steps of 2:1, 1:1, 1:2 ethanol/wax followed by 3 steps in pure wax.

#### Immunofluorescence microscopy

Seven µm thick sections of root apices were mounted on glycerol albumin covered slides and allowed to spread on distilled water. Sections were dewaxed in 3 x 100, 90, 70 and 50% ethanol in PBS and finally transferred to PBS (10 min per step). Dewaxed sections were incubated in MTSB for 30 min. After a short dip in methanol at –20°C to improve antigenicity, they were again incubated in MTSB for 30 min. Slides were then exposed to monoclonal antibody 46E5B11F6 diluted 200-fold in PBS plus 0.1% (w/v) BSA for 1.5 h at room temperature followed by washing in MTSB for 10 min. The secondary rabbit anti-mouse IgG-antibody + FITC conjugate (Sigma, Munich, Germany) was diluted 100-fold in PBS plus 0.1% (w/v) BSA. Specimens were observed using a Zeiss Axiovert 405 and photographed on Kodak T-Max 400.

Presented data are representatives out of 6 independent occasions.
Results

Antibody specificity

The monoclonal antibody 46E5B11F6 has previously been used as a specific probe to localize H\(^+\)-ATPases (Villalba et al. 1991, Baur et al. 1996). The antibody binds to multiple H\(^+\)-ATPase isoforms, as shown by its cross reaction with three Arabidopsis isoforms (AHA1-3) individually expressed in yeast (Palmgren and Christensen 1994). To further test the specificity of this antibody for use on maize roots we used it to probe protein gel blots of root protein extracts from seedlings (Fig. 1). Only one band at the expected size of 100 kDa (Harper et al. 1989, Pardo and Serrano 1989) was detected in a postmitochondrial supernatant (PS) containing microsomes and soluble proteins. When microsomal membranes were further subjected to fractionation by an aqueous polymer two-phase system (Larsson et al. 1994), the 100-kDa polypeptide was enriched in the upper phase, consistent with a plasma membrane (PM) localization. No other polypeptides were labeled.

In this study, the monoclonal antibody 46E5B11F6 has been used to study the cell- and subcellular localization of the H\(^+\)-ATPase in the maize root apex. The schematic drawing in Fig. 2 demonstrates the parts of the root from which the single sections shown in Figs 3–5 were derived.

H\(^+\)-ATPase is most abundant in epidermal and phloem companion cells

To survey the cell specific expression of H\(^+\)-ATPase in maize roots, cross sections were made through the elongation zone at the root apex and immunodecorated with the anti-H\(^+\)-ATPase monoclonal 46E5B11F6, as shown in Figs 3 and 4. The strongest immunostaining was observed in epidermal (Fig. 3A) and phloem companion cells (Fig. 4). In the epidermis (Fig. 3A), some signal in the interior of the cells was also visible resulting from the view on transversal cell surfaces which were included in the sections. Moderate staining was seen in the outer cortical cells (Fig. 3A,C), endodermis, metaxylem and a subset of xylem parenchyma cells (Fig. 4). Other cells showed very weak staining. In controls lacking the primary antibody, there was no significant fluorescence (Fig. 3B). In addition, controls using anti-actin and anti-z-tubulin antibodies (Baluska et al. 1996, 1997, Vitha et al. 1997) confirmed that patterns seen here were specific for monoclonal 46E5B11F6.

H\(^+\)-ATPase is highly expressed in specific xylem parenchyma cells

A subset of xylem parenchyma cells showed relatively high levels of H\(^+\)-ATPase, equivalent to levels seen in endodermal cells (Fig. 4). These parenchyma cells formed a ‘bridge’ between the endodermis and metaxylem. The staining of these cells showed an even distribution of pumps around the cell perimeter.

H\(^+\)-ATPase is asymmetrically localized in epidermal and outer cortical cells

Epidermal cells appeared to be asymmetrically decorated showing several times more signal in plasma membranes facing the root-soil interface (Fig. 3A). Along this surface, the most intense labeling was consistently observed between epidermal cells, with the exterior cell tip showing significantly less staining (Figs 3A and 5A).
Considerable labeling was also observed in the outer two or three layers of cortical cells below the epidermis (Fig. 3A). In these cell layers, the strongest staining was observed in the outermost cortical cells with progressively less staining towards the root interior. Similar to epidermal cells, outer cortical cells showed an asymmetric localization of H⁺-ATPase with higher amounts on the cell surface facing the root exterior. The resolution was sufficient to see staining of individual plasma membranes (e.g., Fig. 3C), making it possible to confirm that a strong signal corresponded to a specific cell surface.

**Localization in epidermal cells changes from uniform to asymmetric**

To determine the spatial and temporal origin of H⁺-ATPase asymmetry, we examined the developmental series of epidermal cells at the root apex, as shown in

![Image](image1.png)

**Fig. 3.** Immunofluorescent detection of H⁺-ATPase in epidermal and cortical cells of the maize root apex. A, Immunofluorescence image showing asymmetric localization of H⁺-ATPase of epidermal cells and outer cortical cells. B, Control without primary antibody: Part of a cross section including epidermal cells and cortical cells as in A. C, Immunofluorescence image of outer cortical cells directly underlying the epidermis; arrows indicate plasma membrane domains facing the root exterior; arrowheads indicate plasma membrane domains facing the central stele. EP, epidermis; OC, outer cortex; IC, inner cortex. Scale bars 5 μm.

![Image](image2.png)

**Fig. 4.** Immunofluorescent detection of H⁺-ATPase in the central stele of the maize root apex. ED, endodermis; star, protoxytem; MX, metaxylem; snowflake, protophloem; CC, companion cell. Scale bar 10 μm.

![Image](image3.png)

**Fig. 5.** Longitudinal sections through the apex showed very little H⁺-ATPase staining for epidermal initials (root tip). However, strong staining was observed in young cells starting abruptly, less than 10 cells from the tip (Fig. 5C). These young epidermal cells showed uniform staining around their cell perimeter (i.e., no asymmetry). A gradual change towards asymmetry occurred in cells further from the root tip. A change towards asymmetry was observed in relatively young epidermal cells still covered by the root cap (Fig. 5B). A complete change towards an asymmetric localization was observed already less than 40 cells from the tip (Fig. 5A).
Discussion

Evidence presented here supports the hypothesis that high levels of plasma membrane H⁺-ATPase are present in a specific subset of root cells involved in active solute transport. Our results indicate that H⁺-ATPases are most abundant in epidermal and phloem companion cells, and to a lesser extent in the endoderms, as shown using the monoclonal antibody 46E5B11F6. This general pattern confirms two previous studies on roots, using different antibodies (Parets-Soler et al. 1990, Samuels et al. 1992). However, this study also detected significant H⁺-ATPase expression in a subset of outer cortical and xylem parenchyma cells. This new finding may be the result of using a different antibody which recognizes a broader spectrum of H⁺-ATPase isoforms, or more likely reflects technical improvements in sensitivity and resolution.

Radial transport and xylem loading in the root apex

Radial transport of ions and water to the stele has been studied in relation to the development of the endodermis, a barrier for passive leakage of ions (Harrison-Murray and Clarkson 1973, Robards et al. 1973, Sanderson 1983). According to Sanderson (1983), highest rates of uptake into barley roots of two-week-old plants take place at around 5 mm from the tip, but also very young regions of the root contribute to absorption of ions and water (Sanderson 1983). Here we show that in the root apex (at 4 mm from the tip) a subset of xylem parenchyma cells have relatively high levels of H⁺-ATPase and form a previously unrecognized unique connection (‘bridge’) between the endodermis and living metaxylem. Interestingly, the work of Peterson and Perumalla (1984) gives proof that at the apex of the maize root (up to 5 mm from the very tip) the diffusion of Calcofluor white is limited to the outer tangential wall of epidermal cells. This shows that the radial cell walls constitute a barrier for passive movement of dye molecules and indicates that these cell walls are not freely permeable for ions. Thus, ions must enter a symplastic pathway already at the epidermis allowing a selective uptake of ions across the root. Our data suggest such a pathway due to the presence of high amounts of plasma membrane H⁺-ATPase in both epidermal and xylem parenchyma cells.

The sensitivity of our immunodetection allowed us to observe a clear asymmetry in outer cortical cells, with more H⁺-ATPase on the side of the cell facing the root exterior. The degree of polarization diminished with cells closer to the interior of the root. We offer the speculation that a nutrient gradient may provide an important environmental cue to trigger the development of these polarized cells. One consequence of such polarized architecture is to localize active membrane transport systems to the cell surface best situated for high efficiency in nutrient uptake.

Asymmetric localization of H⁺-ATPase has also been reported in transfer cells of broad bean embryos (Bouché-Pillon et al. 1994b) and in phloem transfer cells of broad bean (Bouché-Pillon et al. 1994a). In these other examples, the asymmetric localization of the pump correlates with the expected high transport activity of a specific cell surface.

An important question is how the H⁺-ATPase becomes asymmetrically localized. In animal cells, the analogous primary ion pump, a Na⁺/K⁺-ATPase, is asymmetrically localized in gut epithelial cells; in this case generating a sodium gradient to drive sodium-coupled cotransport systems for nutrient uptake from the gut. The differentiation of gut epithelial cells has been extensively studied (Caplan 1997). Asymmetry occurs only after cells have attached to a matrix and formed tight junctions between neighboring cells. The process of generating asymmetry is accompanied by the selective targeting of ATPases to one surface. Membrane targeting in these cells appears to be complex and regulated since some proteins go to the opposite surface, and still other proteins go to both surfaces. Alternatively, asymmetric localization of the H⁺-ATPase could be the result of either differential retention or selective degradation.

In plants, the H⁺-ATPase in the root epidermis provides an excellent marker for investigating the development of a polarized cell. The root epidermis also provides an experimentally accessible cell layer. Since young epidermal cells initially express the H⁺-ATPase in a non-polarized pattern, it would be interesting to determine: (1) if a nutrient gradient triggers polarization, and (2) what is the mechanism for selectively localizing the H⁺-ATPase (and maybe other transporters) to the lateral and apical membrane surfaces.

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