Plant Physiol. (1992) 99, 1509–1514
0032-0889/92/99/1509/06/$01.00/0

Received for publication November 1, 1991
Accepted February 24, 1992

Immunofluorescent Localization of Plasma Membrane H^+-ATPase in Barley Roots and Effects of K Nutrition[1]

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ABSTRACT

The plasma membrane H^+-ATPase (PM-H^+-ATPase) of barley (*Hordeum vulgare* L. cv Klondike) roots was assayed by cross-reaction on western blots and cryosections with an antibody against the PM-H^+-ATPase from corn roots. Under conditions of reduced K availability, which have previously been shown to increase K influx by greater than 25-fold, there were only minor changes detected in PM-H^+-ATPase levels. Antibody labeling of cryosections showed the relative distribution of PM-H^+-ATPase among cell types in root tips and mature roots. Epidermal cells, both protoderm and mature root epidermis, including root hairs, had high levels of antibody binding. In mature roots, the stelar tissue showing the highest antibody binding was the companion cells of the phloem, followed by pericycle, xylem parenchyma, and endodermis.

The PM-H^+-ATPase[2] (EC 3.6.1.35) has been called a "master enzyme" responsible for a broad range of physiological processes. These include cell wall acidification, cytoplasmic pH regulation, and the provision of proton motive force for secondary active transport (25). In higher plants, there is now substantial evidence that solutes such as sucrose, various amino acids, nitrate, and K are absorbed by means of proton-mediated transport systems (1, 23).

Most of the information regarding the characteristics of the PM-H^+-ATPase has come from biochemical studies in which whole plant roots or other tissues have been homogenized to obtain PM-H^+-ATPase preparations. As a consequence, the isolated PM-H^+-ATPase complement represents the average of the component cell types. However, the expression of PM-H^+-ATPase may be quite different among the cell types of which the tissue is composed, *e.g.* root hair cells may express more PM-H^+-ATPase than cortical cells. This information is important in assessing theories of ion uptake and xylem loading in the root.

Studies of the distribution of the PM-H^+-ATPase in roots have encountered technical difficulties (14, 20) that have not allowed descriptions of the relative amounts of PM-H^+-ATPase at the cellular level. In the present study, the distribution of PM-H^+-ATPase among cell types of the barley root was observed by applying anti-PM-H^+-ATPase antibodies to thawed 3-μm cryosections. Use of secondary antibody-FITC allowed visualization of the primary antibody by fluorescent light microscopy. In contrast with previous protocols, this technique allows good cellular resolution while preserving protein antigenicity (28).

The present work was initiated to investigate the distribution of the PM-H^+-ATPase among cell types in barley roots and effects of K nutrition on the expression of the PM-H^+-ATPase.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. cv Klondike) was grown in hydroponic solution cultures as described previously (5). Control plants were grown in one-tenth Johnson's media, $[K^+]$ = 600 μM; low-K plants were obtained by transferring control plants to media containing no added K for periods varying from 1 to 4 d.

Western Blots

Plasma membrane-enriched fractions were isolated according to the methods of Dupont *et al.* (3) with modifications (4). Protein concentrations of membrane fractions were determined according to Markwell *et al.* (18). Equal amounts of protein (20–25 μg) from plasma membrane-enriched fractions were heat denatured at 70°C for 20 min in the sample buffer (consisting of 0.125 M Tris-HCl, 25% [v/v] glycerol, 2.5% [v/v] SDS, either 2.5% [v/v] 2-mercaptoethanol or 50 mM DTT, and 0.05% [w/v] bromphenol blue). Solubilized protein was then subjected to electrophoresis in 10% (w/v) acrylamide gels (15). Western blotting was carried out according to White and Green (29) with slight modifications.

Immunofluorescence

The apical 5 cm of the roots were cut into 2- to 3-mm segments, immediately fixed with 4% formaldehyde (w/v, freshly prepared from paraformaldehyde) in 50 mM Pipes buffer, pH 7.0, for 3 h. For subsequent rinsing and infiltration steps, the buffer contained 50 mM Pipes plus 5 mM EGTA and 0.5% (w/v) formaldehyde. Samples were infiltrated in mixtures of increasing concentration of sucrose over a 24-h period. The roots were frozen in liquid nitrogen with 2.3 M sucrose as cryoprotectant and stored in liquid nitrogen.

Roots were cut into 3-μm sections using tungsten-coated glass knives at −70°C on a Reichert-Jung Ultracut-E equipped with a FC-4E cryobox. Sections were picked up on a droplet of 2.3 M sucrose and transferred to poly-L-lysine-coated glass slides. After rinsing overnight in TBS (20 mM Tris, 150 mM

[1] This work was supported by a Canadian National Sciences and Engineering Research Council operating grant to A.D.M.G.

[2] Abbreviations: PM-H^+-ATPase, plasma membrane H^+-ATPase; TBS, Tris-buffered saline; FITC, fluoroscein isothiocyanate.

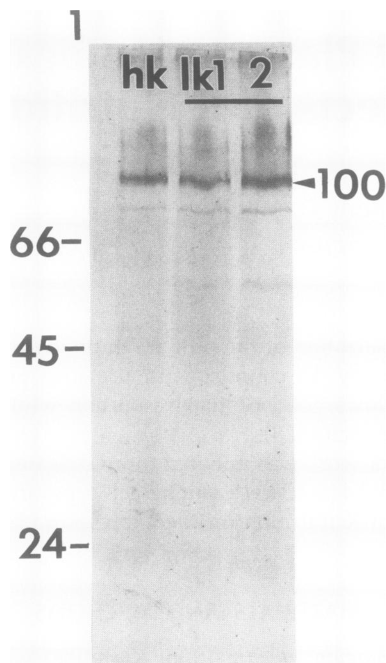

Figure 1. Western blot of plasma membrane-enriched fractions from barley roots, cross-reacted with antibody against corn PM-H$^+$-ATPase. Barley plants were grown in hydroponic solution containing control K levels (hk = high K, lane 1) or solutions lacking K (lk1, low K for 1 d, lane 2; 2, low K for 2 d, lane 3).

NaCl, pH 8.0), slides were treated with 50 mM NH_4Cl in TBS for 10 min to block free aldehydes. The antibody incubation protocol is a modification of the method used by Parets-Soler *et al.* (20). For all subsequent incubation steps, TBS was supplemented with 0.05% Tween-20 (v/v) (TBST) and 2% nonfat dried milk (w/v) to decrease nonspecific protein binding. After 30 min in TBST with milk, slides were inverted on staining chambers that contained about 200 μL of specific antibody solution, diluted 1:500, or a control solution (buffer or nonimmune serum) and incubated for 3 h in a moist environment at room temperature. Polyclonal antibodies for the corn root PM-H$^+$-ATPase were the kind gift of Drs. R.T. Leonard and S. Gallagher; the antigen was isolated from a Triton X-114-treated plasma membrane fraction by one-dimensional phenol-acetic acid-urea-PAGE (8, 9). After antibody incubation, slides were washed with TBS rinse buffer (0.5% Tween-20 [v/v] and 2% nonfat dried milk) three times for 10 min per wash. Slides were again inverted on staining chambers this time containing anti-rabbit immunoglobulin G-FITC conjugate (Sigma F-1262) diluted 1:100 and incubated for 1 h. Slides were rinsed three times for 10 min per wash in TBS rinse buffer, followed by 10 min in clear TBS. Slides were prepared with glycerol:TBS mounting media (4:1, v/v, pH 8.0) and viewed with a Zeiss Universal Photomicroscope equipped for epifluorescence (Zeiss filter set 11 for FITC, photographed on Kodak TMAX-400 film).

RESULTS

Antibodies raised against corn root PM-H$^+$-ATPase cross-reacted on western blots with barley plasma membrane polypeptides in the 100-kD region (Fig. 1). Although the influx of K^+ has been shown to increase up to 25-fold by the second day in low K conditions (4, 5), there was no evidence from these western blots to indicate a corresponding increase in the expression of PM-H$^+$-ATPase. In addition to the strongly staining 100-kD region, there was weak cross-reaction with some minor bands.

Cryosections of barley roots treated with primary antibody against the PM-H$^+$-ATPase and secondary antibody-FITC showed fluorescent labeling of the plasma membrane (Fig. 2). Where cells were slightly plasmolyzed, the labeling pattern followed the contours of the protoplast surface. The epidermal cell layer had higher levels of fluorescence than adjacent cell types. This was especially apparent in the root tip, where the protodermal layer was labeled more strongly than the ground meristem; the light labeling pattern of procambium and ground meristem was homogeneous across the root tip (Fig. 3, A and B). The root cap cells were also strongly labeled (not shown). In mature roots, there was strong labeling of epidermal cells, including root hairs (Fig. 3, C and D).

In cross-sections of mature roots, the epidermis and the stele showed strong labeling; the cortical cells, in contrast, had lower levels of antibody binding (Fig. 4). In the stele, there were several cell types that showed plasma membrane labeling: the companion cells of the phloem, pericycle, xylem

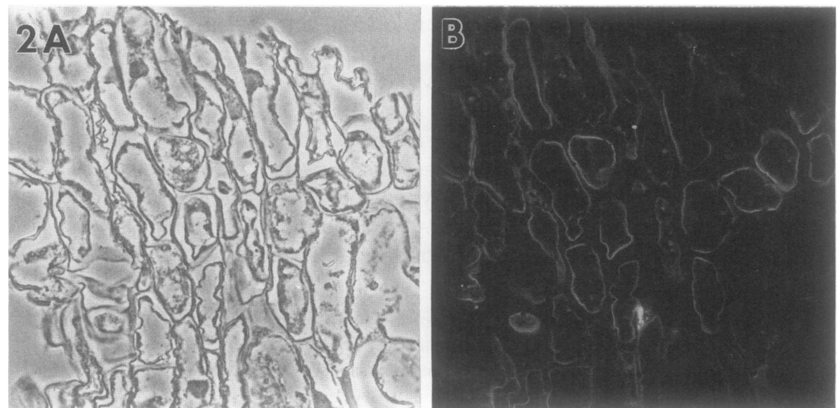

Figure 2. Cryosection of barley root, cut through late elongation zone, epidermal layer. A, Phase contrast image of cells. B, Cells labeled with antibody against PM-H$^+$-ATPase and secondary antibody-FITC. Light areas of photograph represent fluorescent label; label follows plasma membrane (600×).

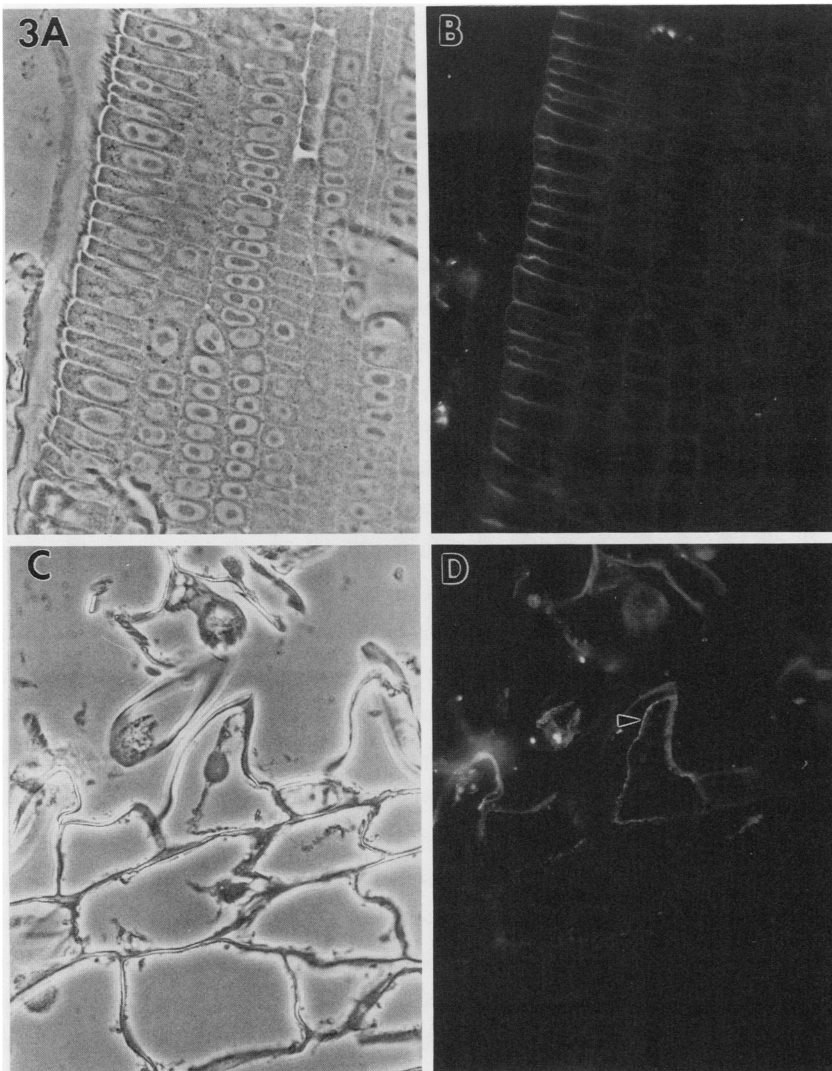

Figure 3. Cryosection of barley root tip showing protodermal layer. A, Phase contrast image of cells. B, Cells labeled with anti-PM-H^+-ATPase and secondary antibody-FITC. High antibody binding is seen in protodermal layer. (780×). C, Phase contrast image of mature root, epidermal layer including base of root hair. D, Cells labeled with anti-PM-H^+-ATPase and a secondary antibody-FITC. Plasma membrane of root hair cell strongly labeled (arrow) (680×).

parenchyma, and endodermis (in order of decreasing labeling intensity) (Fig. 5). The central metaxylem element and the xylem parenchyma immediately surrounding it were not strongly labeled.

Control cryosections were treated identically except that nonimmune serum was substituted for specific antibodies. Alternatively, immunoglobulin incubation was replaced by incubation in buffer only. There was some dim autofluorescence in these control sections, but the bright yellow-green fluorescence of flouroscein was not present (Fig. 4, C and D). On sections treated with the anti-PM-H^+-ATPase antibody, there was binding to the cell wall region. This was apparent in cells that were slightly plasmolyzed, where both the plasma membrane and wall were labeled, and in the xylem vessels, where the protoplast was no longer present. To check if this cell wall binding was due to nonspecific immunoglobulin interactions with the wall, a high concentration of nonimmune serum was applied to the sections. This did not produce the nonspecific cell wall binding pattern, suggesting that there is a subset of these polyclonal antibodies that binds a common epitope in the cell wall. Cell wall binding was also observed by Hurley and Taiz in their immunocytochemical study of the vacuolar H^+-ATPase (12).

There were no differences in fluorescent labeling pattern or intensity between roots grown under conditions of high K or in K-deficient media (data not shown).

DISCUSSION

Earlier attempts to localize the PM-H^+-ATPase have suffered from several limitations. Transmission EM-cytochemical studies utilized lead precipitation of inorganic phosphate that was presumably released due to H^+-ATPase activity. However, fixation and lead treatments have been shown to destroy H^+-ATPase activity (14). More recently, immunohistochemical studies on paraffin or cryostat thick sections have had successful antibody binding but poor preservation, so individual cell types could not be resolved (20). For example, root hairs were not seen and peripheral binding at the root tip was interpreted as binding to root cap under mucilage. In this study, root hairs were preserved and peripheral binding

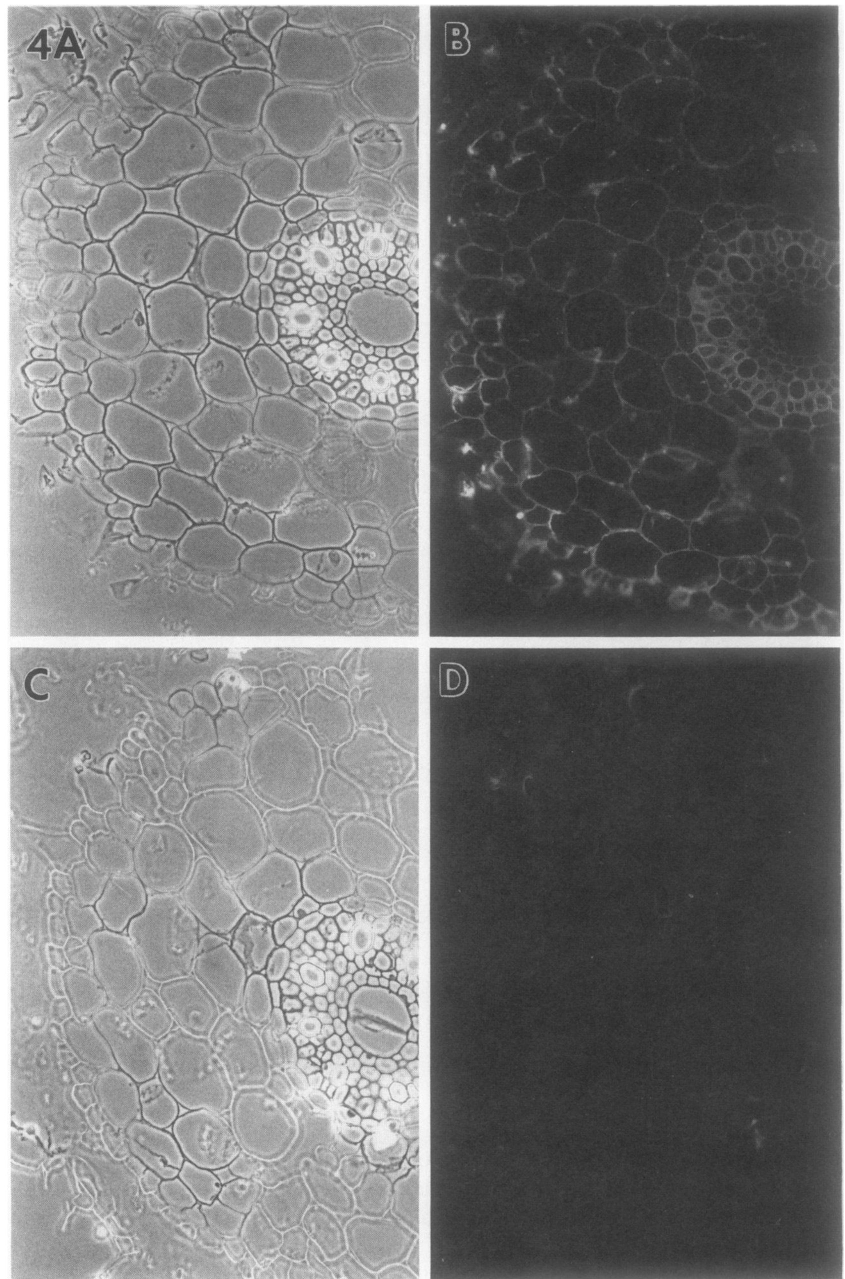

Figure 4. Cryosection of mature barley root. A, Phase contrast image of root cross-section. B, Root section labeled with anti-PM-H^+-ATPase and secondary antibody-FITC. Antibody labeling is most intense on stele and epidermal tissues (250×). C, Phase contrast image of control root cross-section. D, Control root section treated with nonimmune serum and secondary antibody-FITC, otherwise treated and photographed exactly the same as the section shown in B. (250×).

could be clearly resolved into protodermal as well as root cap components.

The corn PM-H^+-ATPase antibodies that were used in this study have been shown to cross-react with the 100-kD region of plasma membrane-enriched fractions from zucchini hypocotyls and barley roots (3, 7). Dupont *et al.* (3) used the corn anti-PM-H^+-ATPase antibody to define the plasma membrane fraction of barley root preparations. Fichmann *et al.* (7) demonstrated that the corn anti-PM-H^+-ATPase antibody cross-reacted with zucchini plasma membranes but not with purified coated vesicles. In these and the present studies, cross-reaction was also seen between the antibody and some minor bands on the western blots. It is not possible to know what contribution these minor bands make to the labeling patterns observed on the tissue sections, but the amount of reaction product in the minor bands is small relative to the abundant reaction at 100 kD. In addition, the general pattern found in this study, *i.e.* high binding in epidermis and stele, matches the pattern found by Parets-Soler *et al.* (20) in their immunohistochemical study, where they used a monoclonal antibody to the PM-H^+-ATPase.

Because the antigen employed to generate the antibodies to the PM-H^+-ATPase was the electrophoretically purified 100-kD band from corn roots (8, 9), the polyclonal antibodies may be recognizing several isoforms of the PM-H^+-ATPase. The strong cross-reaction with the phloem companion cells could indicate that a clonal subset of antibodies is recognizing an isoform analogous to the *Arabidopsis* H^+-ATPase-3 (26,

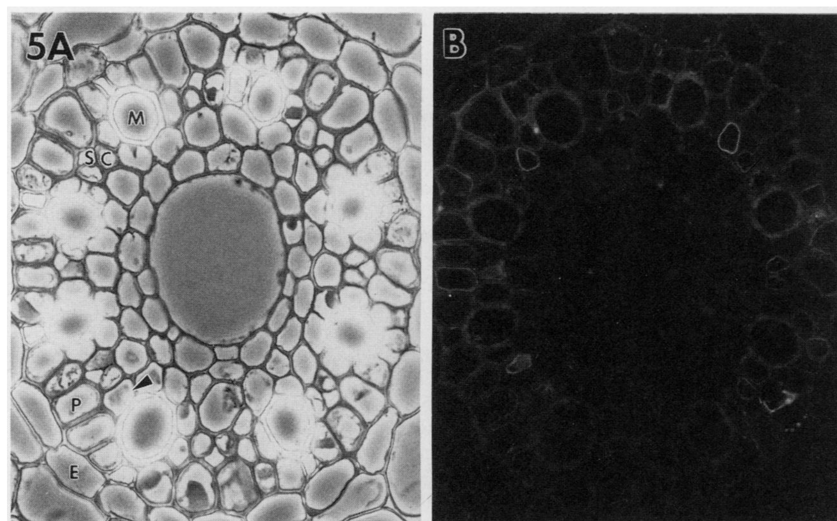

Figure 5. Stele of barley root in cross-section. A, Phase contrast image showing different cell types. (E, endodermis; P, pericycle; M, metaxylem element; C, companion cell of phloem; S, phloem sieve element; arrow, xylem parenchyma.) B, Cells treated with anti-PM-H^+-ATPase. Relative intensities of antibody labeling can be seen: companion cells of phloem have strongest labeling, followed by pericycle and xylem parenchyma. (590×).

27), and/or the antibodies may be recognizing a common domain on all isoforms present. The strong labeling of phloem companion cells would then reflect a higher concentration of PM-H^+-ATPase in the plasma membrane of these cells.

The expression of PM-H^+-ATPase differed greatly among cell types of the root. This heterogeneity must be considered when interpreting biochemical and molecular studies on root tissue, which is a composite of cell types.

The high proton pump expression in the plasma membrane is evident early in the development of the epidermal cells. In contrast, the cells of the stele, which show high PM-H^+-ATPase expression in their differentiated state, have low expression in the procambial state.

There are two possible routes across the cortex for ions to travel upon entering the root from the surrounding solution: the apoplast and the symplast. The strong labeling of PM-H^+-ATPase in epidermal cells, including root hairs, is consistent with the theory that the epidermis is an important site of loading of ions into the symplast (22). It has been suggested that when the concentration of ions in the surrounding solution is low (less than 1 mM), ion absorption by epidermal cells so reduces the concentration of ions moving through the apoplast that there are insufficient amounts to contribute significantly to ion uptake by cortical cells. At higher concentrations, ions would exceed the uptake capacity of the epidermis and proceed across the root apoplastically, with loading at the cortex and endodermis (1). In the present study, where the ion concentrations were always less than 1 mM, the epidermal cell layer had much higher levels of PM-H^+-ATPase expression than the cortex and endodermis.

It has been suggested that ions are released from the symplasm into the xylem by secretion from the xylem parenchyma (17) or by loading of living, developing metaxylem elements that release a "slug" of concentrated ions into the xylem stream upon rupturing during development (13, 19). In this study, the xylem parenchyma cells were labeled throughout the root, including mature areas where the peripheral metaxylem elements lacked living protoplasts. This may indicate a high level of ion pumping activity in such cells, in accordance with their ultrastructural characteristics (16). The pericycle cells also had high antibody labeling; these cells may play a previously unrecognized role in secretion of ions from symplasm to xylem. Alternatively, the high PM-H^+-ATPase expression of these cells may reflect their potential for renewed meristematic activity. The low labeling level in the central metaxylem and its surrounding xylem parenchyma cells suggests that this area of the root may not play a major role in secreting ions into the transpiration stream. Alternatively, ion secretion by this tissue may not involve activity of the proton pump.

There were no differences in PM-H^+-ATPase levels between roots grown in high K or low K media, as detected by these polyclonal antibodies on western blots or cryosections. Therefore, the large increases in K influx seen under conditions of low K appear not to be due to an increase in PM-H^+-ATPase content of the membranes.

These results are consistent with earlier physiological studies that suggested that the regulation of Cl^- and K^+ influx is due to changes in the activity of ion carriers rather than to changes in the electrochemical driving forces for solute cotransport (24). For example, plants deprived of Cl^- or K^+ show large increases in Cl^- or K^+ fluxes, but these cannot be attributed to changes in the electrochemical potential gradients for these ions (2, 10, 11). It is only when the proton pump is inhibited or ATP supplies are limited by metabolic poisons that the secondary transport systems show evidence of their quantitative dependence on a free energy source (21). Although this is consistent with the concept that the amount of the PM-H^+-ATPase is tightly regulated and insensitive to nutritional variations (25), it is possible that the amount of one or more specific isoforms of PM-H^+-ATPase may be changing. Recent studies in barley in which [^{35}S]methionine was used to label plasma membranes (6) indicate that under conditions where K^+ influx is increasing most rapidly there is no incorporation of label in the 100-kD (PM-H^+-ATPase) region of the gel. Rather, several lower mol wt peptides become heavily labeled. In summary, the evidence indicates that the regulation of K^+ influx does not involve increased

PM-H^+-ATPase activity but the generation of specific secondary transporters for K^+.

ACKNOWLEDGMENTS

Specific antibodies (polyclonal antibodies against corn PM-H^+-ATPase) were the kind gift of Drs. R.T. Leonard and S.R. Gallagher, University of California, Riverside. The technical assistance of Jarnail Mehroke is gratefully acknowledged.

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