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Proton Pumping in Growing Part of Maize Root: Its Correlation with 14-3-3 Protein Content and Changes in Response to Osmotic Stress

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Abstract—The spatial pattern of mitotic activity, cell elongation, rate of H^+ fluxes, and 14-3-3 protein content were determined in *Zea mays* roots. We found that the regions along the apical part of the growing root conversely differ in their proton pumping activity. Higher rate of H^+ efflux coincides with higher growth rate and correlates with increased 14-3-3 protein content in membrane preparations. The segment consisting of the root cap and the apical part of the meristem exerts net inward proton pumping, which can be inverted under fusicoccin treatment or osmotic stress. In the latter case, this inversion is accompanied by accumulation of 14-3-3 protein in plasma membranes. The results obtained highlight 14-3-3 protein as an obvious candidate for the fine regulation of plasma membrane H^+-ATPase in root apex.

Key words: *Zea mays*, root, elongation growth, plasma membrane H^+-ATPase, 14-3-3 protein, osmotic stress

It has been nearly thirty years since the concept that cell wall extensibility and thus growth is partly under the control of apoplastic pH was proposed [1]. Root growth also depends on extracellular pH [2], and the correlation between maximum intensity of elongation growth and acidic zones on the root surface has been shown [3-5]. Certainly, the plasma membrane (PM) H^+-ATPase plays a key role in the maintenance of optimal apoplastic pH. The enzyme activity can be modulated by different factors controlling plant physiology [6]. Activation of the H^+-ATPase causes a hyperpolarization of the PM and raises the ΔpH between the cytoplasm and the periplasm, thereby providing the driving force for transmembrane ion and metabolite transport [7]. In plants the PM-localized H^+-ATPase is a polypeptide of 100 kD containing ten transmembrane α-helices and four cytoplasmic domains, and the C-terminal polypeptide region is an auto-inhibitory domain [8]. It should be pointed out that a complex pattern of surface pH exists along the growing root apex [5] which is primarily created through variations in proton pumping activity of the H^+-ATPase and possibly by modulation of cation channel activity [9]. On the other hand, immunocytochemical studies have shown that H^+-ATPases are more or less equally abundant in all epidermal cells of the growing root apex, in phloem sieve tubes, and in companion cells of *Avena sativa*, *Pisum sativum*, and *Zea mays* [10, 11]. High concentrations of PM H^+-ATPase were also revealed in root caps (RC) of oat, pea, and garden cress [10, 12]. Taking these data together, it appears that fine regulation of H^+-ATPase occurs in growing root apex.

14-3-3 proteins are known as regulators of the H^+-ATPase. They are highly conserved hydrophilic proteins and widely present in animal and plant cells [13]. The C-terminal domain of H^+-ATPase possesses a specific sequence motif which acts as a binding site for 14-3-3 protein [8, 14-17]. Being phosphorylated on the threonine residue within this motif, 14-3-3 protein dimer interacts with H^+-ATPase leading to a marked increase in the enzyme activity [15-17]. Recently, 14-3-3 proteins have been implicated in PM H^+-ATPase regulation under cold or osmotic stresses in suspension-cultured sugar beet cells [18, 19] as well as under normal physiological conditions via blue light excitation in broad bean guard cells [20].

Since interaction of 14-3-3 proteins with the PM H^+-ATPase leads to an activation of the enzyme, the goal

Abbreviations: PM) plasma membrane; RC(s)) root cap(s); FC) fusicoccin; DES) diethyl stilbestrol.

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of the present work was to reveal the correlation between transport activity of the H^+-ATPase and content of 14-3-3 proteins in terminal portions of growing maize root under normal physiological conditions and under salt or osmotic stresses.

MATERIALS AND METHODS

Plant material. Maize (*Zea mays* L., cv. KOSS) seeds were thoroughly washed with tap water, placed on filter paper moistened with distilled water containing 20 μg/ml nystatin and germinated for 3 days in the dark at 25°C. All experiments were performed on 30-mm roots.

Determination of meristem and enhanced cell elongation zone. Lengths of metaxylem cells along the root axis were measured on the longitudinal median sections. The place where cell lengths began to increase very sharply was regarded as the region of cell transition to elongation [21]. To determine the boundaries of the meristem, longitudinal median root sections were fixed with ethanol–glacial acetic acid (3 : 1 v/v). Fixed sections were washed with 70% ethanol and water. Photomicrographs of sequential fields from a root tip were taken using a epifluorescence microscope after staining sections with 1 μg/ml 4,6-diamidino-2-phenylindole in 2 mM MES (pH 6.0) containing 2 mM EDTA and 10 mM NaCl. For each experiment, the number of mitotic figures in successive 126-μm zones was counted and presented as a sum for six roots.

Proton flux measurements and salt and osmotic treatments. The roots were washed with distilled water and cut with a razor blade block into 800-μm successive segments. Equally distant from a root tip segments from 10-12 different roots were incubated in a thermostatted cuvette at 25°C in 1 ml of air-bubbled buffer A (0.5 mM Mes-Bistris, pH 6.2, 5 mM K_2SO_4) for 30 min, then the buffer was replaced with 1 ml of either fresh buffer A or buffer A supplemented with salt (150 mM NaCl or 5 mM LiCl) or with 0.3 M sorbitol under constant aeration. After 30-min incubation, the pH of the medium was recorded continuously and linear parts of the pH curves were used to calculate the proton flux rates. The consumption of titrant (1 mM HCl or 1 mM KOH) was monitored and converted into change in proton concentration. To examine the changes in proton flux under salt stress (1.5 and 2 h), intact roots were preincubated for 0.5 and 1 h, respectively, in buffer A with 150 mM NaCl. The roots were then gently blotted dry and cut into 800-μm successive segments. All the subsequent procedures were carried out as described above but 150 mM NaCl was included into all buffer solutions. In separate experiments, intact maize roots were preincubated for 3 h in buffer A with 1 μM fusicoccin (FC) or with 0.1 mM diethyl stilbestrol (DES) before proton flux measurements. The net proton flux was expressed in pmol H^+ per mg root segment protein per second. Protein concentration of root segments was measured using the method described by Lowry et al. [22] with BSA as a standard.

Membrane isolation, SDS-PAGE, and immunodetection of 14-3-3 proteins. The roots of 10-15 untreated seedlings or seedlings after 30-min or 1.5-h incubations in buffer A with 150 mM NaCl were gently blotted dry and cut into 800-μm successive segments. Segments of the same number starting from the root tip were placed into an Eppendorf tube in 100 μl of 50 mM Mes-Tris buffer (pH 6.2) containing 0.4 M sucrose, 10% glycine (w/v), 10 mM NaF, 30 mM β-glycerophosphate, 10 mM EDTA, 5 mM EGTA, 2 mM DTT, and 1 mM PMSF with polyvinyl polypyrrolidone to 10% of the tissue mass. Root segments were ground with a Teflon pestle and sonicated three times for 10 sec at 0°C using an MSE unit with immersed probe at medium setting. The homogenate was centrifuged at 10,000*g* for 10 min and the supernatant was then centrifuged at 100,000*g* for 30 min at 4°C. The microsomal pellet was resuspended in a medium containing 0.33 M sucrose, 3 mM KCl, and 5 mM KH_2PO_4 (pH 7.8). The plasma membranes were purified by aqueous two-phase partitioning in Dextran T500–PEG 3350, 6.2% each [23]. The microsomal or plasma membranes were resuspended in 20 μl SDS-PAGE sample buffer, heated at 95°C for 3 min, and loaded onto an SDS gel. Electrophoresis was performed in 12.5% polyacrylamide gel according to Laemmli using a Bio-Rad Modular Mini Electrophoresis System (Bio-Rad, USA). One part of the gel was stained with 0.2% Coomassie R-250. Polypeptides from the second part of the gel were electrophoretically transferred to a Hybond-C Super transfer membrane (Amersham Pharmacia Biotechnology, England). The membrane was blocked in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20, and 5% (w/v) non-fat dry milk overnight at 4°C and then incubated with the first antibodies at 1 : 50,000 dilution for 1 h at room temperature. The first rabbit antibodies were raised against purified 14-3-3 proteins from suspension-cultured sugar beet cells (antibodies obtained by Dr. O. I. Klychnikov, unpublished data). The specificity of the first antibodies to 14-3-3 proteins was similar to that of polyclonal antibodies against the *BMH1* gene translation product encoding a 14-3-3 homolog in *Saccharomyces cerevisiae* (BMH1 antibodies were kindly provided by Dr. G. P. H. van Heusden from Leiden University, The Netherlands). Immunodecoration was performed with goat anti-rabbit IgG conjugated with horseradish peroxidase (Promega, USA) using an enhanced chemiluminescence system (ECL system; Amersham Pharmacia Biotech). Total protein content was estimated by scanning individual lanes on the gels stained with Coomassie Brilliant Blue R-250 and 14-3-3 proteins were quantified by scanning of the bands on the chemiluminescence-sensitive film. Images obtained were analyzed with the ONE-Dscan 1.3 program (Scananalitics Inc., USA). The 14-3-3 protein con-

tent is presented as a ratio of ECL signal to protein content in the gel.

Reagents. Dextran T500 was from Pharmacia (Sweden) and all the other chemicals were purchased from Sigma (USA). FC was from the Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences (Moscow).

RESULTS

The cytological analysis indicated that roots of 3-day-old maize seedlings had a root cap as long as 370-420 μm (Fig. 1a). The elevation in mitotic activity started at 400 μm above the root tip; the bulk of mitoses was situated at 400 to 1600 μm although single mitotic figures could be revealed up to 1900 μm (Fig. 1b). The transition of cells to elongation was observed at 1650-1800 μm above the root tip and the cell lengths began to increase very sharply at 2000 μm (Fig. 1b). The region between 2000 and 5000 μm was paramount in cell elongation (Fig. 3d) and this process ceased at 6.5 to 8 mm above the root tip (data not shown). For further investigations successive segments of 800 μm were cut: 0-800 μm (1st segment), RC with an apical part of meristem; 800-1600 μm (2nd segment), bulk of meristem; 1600-2400 μm (3rd segment), region occupied with the elongating cells; 4-8th segments (up to 6400 μm), the rest of the elongating cells and the beginning of the differentiation zone.

In preliminary experiments we determined the rates of proton extrusion from whole 4.8 mm long root apex and from the sum of its 800-μm successive segments. The rate values for sum of root segments (3.74 ± 0.97 pmol/sec per mg protein) and for whole root apex (3.66 ± 0.19 pmol/sec per mg protein) did not differ significantly ($p > 0.05$). This fact enabled us to use individual 800-μm root segments for further research.

Figure 1c shows that cells of the 1st segment clearly alkalized the incubation medium, the 2nd segment acidified the incubation medium, and the rate of H^+ efflux from the 3rd segment was higher. The 5th segment displayed the maximum rate of cell exterior acidification and beyond it the rate began to decline (Fig. 3d).

To ascertain what part of the 1st segment determined the alkalization of the cell exterior, we removed the first 400 μm, which is merely an RC. As shown in Fig. 1d, the isolated RCs alkalized the incubation medium even more than the 1st segments, while the residual segments (400-1200 and 1200-2000 μm) demonstrated active proton extrusion.

Treatment of maize roots with DES, a not entirely specific but reasonable inhibitor of the PM H^+-ATPase [24], led to a drop in the proton efflux rate from the 2nd and 3rd segments by 4-5-fold (Fig. 1e) while the treatment of the roots with FC, a specific activator of the proton pump [6, 15], resulted in an increase in the proton efflux rate in all segments (Fig. 1f), the net inward proton pumping of the 1st segment being changed to an outward one of the same amplitude. Therefore, we link proton efflux to PM H^+-ATPase activity.

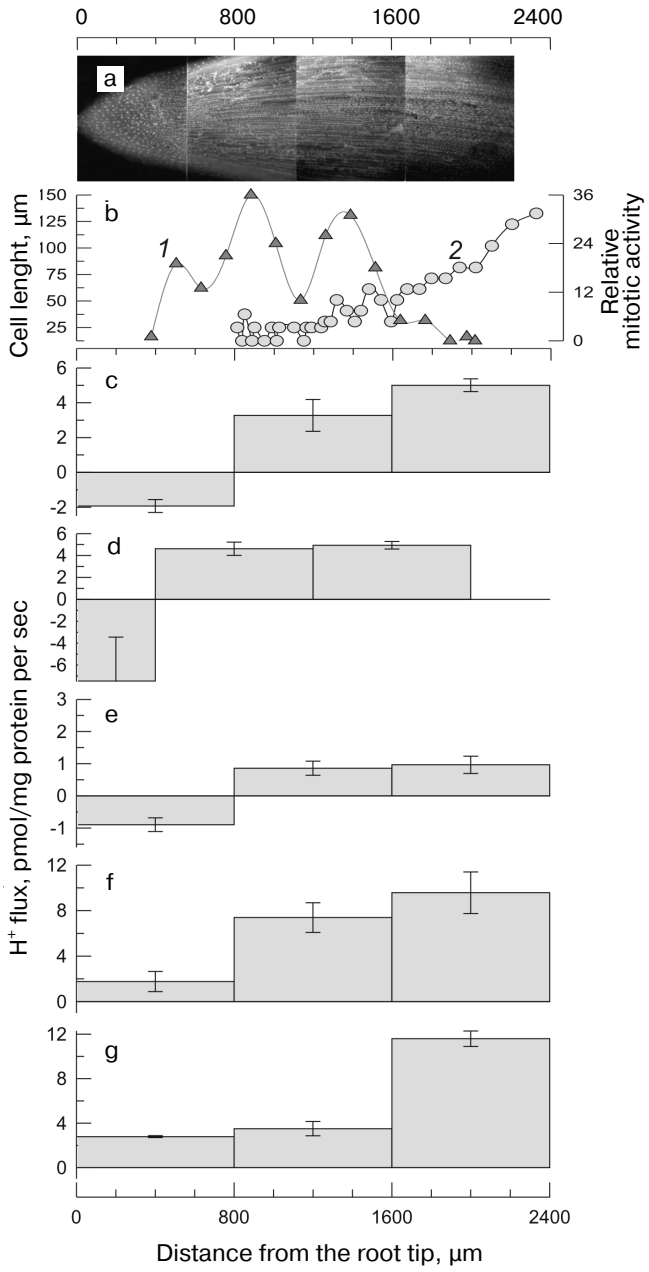

Fig. 1. Growth parameters and rates of proton fluxes along a maize root apex: a) DAPI-stained longitudinal median section of a root apex of 3-day-old maize seedling; b) mitotic activity ((*1*) sum for six roots) and the lengths of metaxylem cells along 2400-μm root apex of a typical root (*2*). Rates of proton fluxes in: c) the successive 800-μm root segments; d) the root cap (first 400 μm) and 400-1200 and 1200-2000 μm segments; e) the successive 800-μm root segments after 3-h treatment of seedlings with 0.1 mM DES; f) 1 μM FC treatment; g) 2-h treatment with 150 mM NaCl. Values in (c)-(g) are means and standard errors of three experiments.

The typical pattern of H^+ fluxes depicted in Fig. 1c was altered under salt stress (2-h treatment with 150 mM NaCl): instead of alkalifying the external medium, the 1st segments acidified the cell exterior at an approximately equal rate; proton efflux rates from the 2nd segments remained unchanged and from the 3rd ones became 2.3-fold higher (Fig. 1g).

Then we ascertained which component of NaCl salt stress, namely the ion toxicity or hyperosmolarity of external medium, affected the rate of proton pumping. To impose osmotic stress 300 mM sorbitol was used. Toxicity of Na^+ was modeled by Li^+ because the cytotoxic effect of 5 mM LiCl is close to that of 150 mM NaCl [25]. Figure 2 shows that H^+ efflux from 1st segments of the maize roots was stimulated by 150 mM NaCl or 300 mM sorbitol to similar extent, whereas LiCl had no effect at least within the first 30 min.

As the regulatory role of the 14-3-3 proteins in PM H^+-ATPase functioning is widely accepted [8, 14-20], we studied the spatial distribution of 14-3-3 proteins in terminal portions of growing maize root. Figure 3b presents a western blot analysis of 14-3-3 proteins in the microsomal membrane preparations isolated from the successive 800-μm root segments. The amount of proteins at M_r ~ 29-31 kD detected by antibodies against 14-3-3 proteins was increased 4- and 10-fold in the 2nd and 3rd root segments, respectively, as compared to the 1st one (Figs. 3b and 3c). Figure 3c also shows that a rise in microsomal 14-3-3 proteins was paralleled by an increase in proton extrusion from successive root segments (Fig. 3d) and full blown in the 5-6th segments; beyond them the content of 14-3-3 proteins was significantly reduced. The low content of PM 14-3-3 proteins in the 1st segment could be raised by osmotic stress. Thus, an increase in osmolarity of the external medium up to 0.3 OsM not only excited H^+ extrusion from the 1st segment (Figs. 1g and 2), but was also paralleled by accumulation of PM 14-3-3 proteins (Fig. 4). The effect was clear after 30-min pretreatment of seedlings with 150 mM NaCl before plasma membrane isolation and when seedlings were pretreated for 1.5 h the amount of 14-3-3 proteins increased 2.5 times (Figs. 4b and 4c).

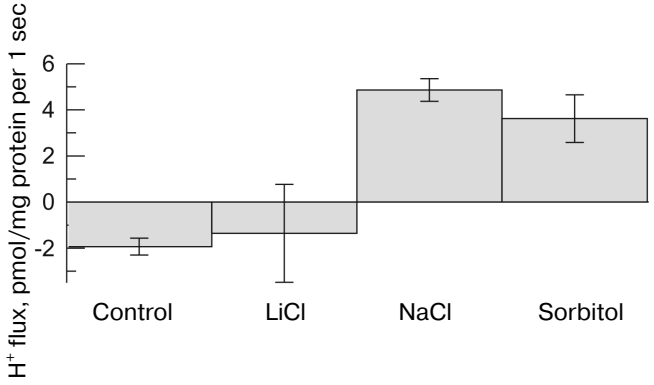

Fig. 2. Rates of proton fluxes in the first 800-μm root segment under 30-min treatment with 5 mM LiCl or under hyperosmotic treatments with 150 mM NaCl or 300 mM sorbitol. The means and standard errors of three experiments are presented.

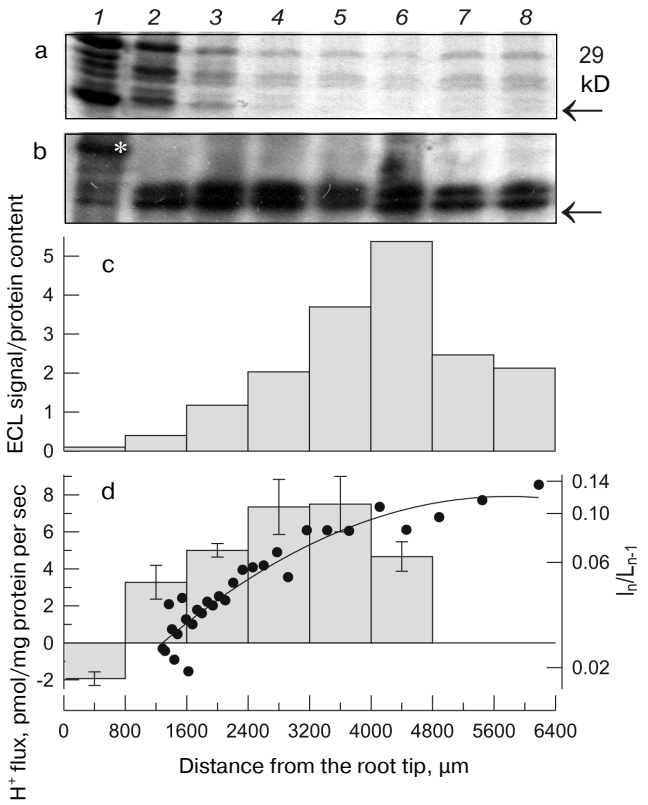

Fig. 3. Correlation between 14-3-3 protein content and rate of proton pumping and cell lengthening along the maize root apex. Microsomes were isolated from the successive 800-μm root segments and electrophoresed in SDS-polyacrylamide gel. a) Fragment of one part of the gel stained with Coomassie R-250; b) western blot of the second identical part of the gel probed with antibodies to 14-3-3 protein; c) relative amounts of 14-3-3 protein presented as the ratio of western blot ECL signal to protein content in the gel (see "Materials and Methods"). Lanes *1-8* in (a), (b), and (c) correspond to successive (from 1th to 8th) 800-μm root segments. The asterisk in (b), lane *1* shows the polypeptide(s) at ~35 kD, which is probably a phosphorylated form of 14-3-3 protein. The experiment presented in (a)-(c) was repeated two times with similar results. d) Rates of proton fluxes in the successive (from 1st to 6th) 800-μm segments (bars, left ordinate) and the ratio of the cell length (l_n) to the length of the root without this cell (L_{n-1}) (curve, right ordinate).

DISCUSSION

Our results on the spatial pattern of mitotic activity along growing maize root apex and transition of cells to elongation as well as the localization of the maximum

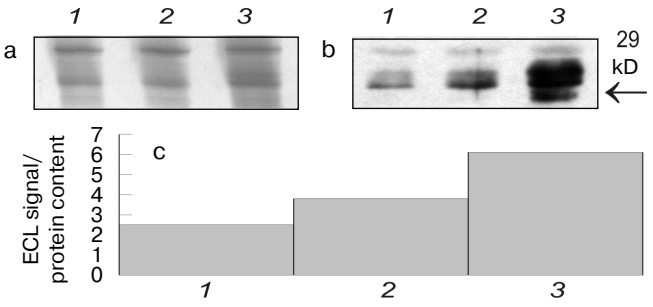

Fig. 4. Effect of salt stress on 14-3-3 protein content in plasma membrane of the first 800-μm root segment. Plasma membranes were isolated from the 1st root segments of untreated seedlings and ones after 30-min or 1.5-h pretreatment with 150 mM NaCl and electrophoresed in SDS polyacrylamide gel. a)Fragment of one part of the gel stained with Coomassie R-250; b) western blot of the second identical part of the gel probed with antibodies to 14-3-3 proteins; c) relative amounts of 14-3-3 proteins presented as the ratio of western blot ECL signal to protein content in the gel. Lane numbers in (a) and (b) and column numbers in (c) correspond to untreated seedlings (*1*) and 30-min (*2*) or 1.5-h (*3*) salt treatments. Similar results were obtained in three independent experiments.

elongation are in good accordance with the previous data presented on maize roots [3, 5, 21, 26].

A much debated question is the correlation of elongation growth with acidic zones on the root surface. Some of the reports in this area were contradictory because of limitations of the employed technique (discussed in [5]). Recently, using pH-sensitive microelectrodes a high-resolution profile of surface pH along maize primary root was determined: the distal acidic zone (about 430-1200 μm) is situated near the meristematic region and unrelated to growth, while the proximal acidic zone around the elongation region (about 2200-8000 μm) coincided with maximum growth rates [5]. It should be noted that the two mentioned acidic zones are separated by a zone of higher pH (about 1300-1800 μm).

In the present study we determined the rates of proton fluxes in the successive 800-μm maize root segments, because such cutting of the root, as already mentioned, did not influence the estimated parameter. The 3rd segment (1600-2400 μm), which is occupied by cells just switched to elongation and rapidly elongating cells (Fig. 1b), actively acidified the external medium (Fig. 1c). This segment corresponds to the beginning of proximal acidic zone that was detected by pH-microelectrode technique [5]. We also revealed spatial correlation between the maximum rate of proton efflux that was displayed by the 4-5th segments (2400-4000 μm, Fig. 3d) and the maximum increase in cell lengths (between 2 and 5 mm above the root tip, Fig. 3d). These data approach quite closely those of Peters and Felle [5]. According to them, the correlation of root surface pH and growth rate suggests a functional relationship only along proximal portions of the growing root apex. Certainly, root surface pH as well as root apoplastic pH depends not only on the proton transport but also on the bulk medium pH, medium flow rate, buffer capacitance, and interaction between cell wall ion exchange and cation channel activity [5, 9, 27]. Nevertheless, the PM proton pump is an essential factor in controlling the proximal acidic region which coincides with maximum elongation growth intensity. This is supported not only by our observations on proton efflux (from the corresponding root segments) which was modulated by H^+-ATPase inhibitor (DES) or specific activator (FC) (Figs. 1e and 1f), but also by other investigators [9, 27] in experiments with different proton pump inhibitors and FC.

The proposed pivotal role of the PM H^+-ATPase in the formation of root acidic zones and by this in the control of elongation growth has stimulated the search for factors involved in the regulation of the enzyme. It is known that in the PM, the H^+-ATPase interacts with a 14-3-3-protein dimer and the activity of the enzyme markedly increases [8, 15-17]. Our results from immunodetection experiments indicate that in the maize root there is a complex longitudinal profile of 14-3-3 proteins which ranges from minimum 14-3-3 protein amount in microsomes from the 1st 800-μm root segment to 50-fold higher abundance in the 6th segment (Fig. 3c). These data have spatial correlation with the rate of proton efflux and the increase in cell lengths (Fig. 3d). Thus, we can suggest that the activation of the PM H^+ pump in the elongation growth zone involves 14-3-3 proteins. The explanation of the shift in maximums of proton pumping rate and 14-3-3 protein quantity in microsomes (Figs. 3c and 3d) requires further studies.

Let's look at the situation with proton fluxes in the most apical root segments. The 1st segment alkalized the incubation medium (Fig. 1c) and it was RC that determined this net proton influx (Fig. 1d). The residual segment 400-1200 μm displayed active proton extrusion and coincided with the already mentioned distal acidic zone [5].

The RC (stretching to ~60 μm in *Arabidopsis* or to ~400 μm in maize), a unique formation of a growing root, performs a number of physiological functions such as protection of the apical meristem, gravity sensing and polysaccharide secretion which are accompanied by prominent endomembrane movement [28]. It should be stressed that the tip of the RC, as have been shown with vertically or horizontally grown maize roots, had more alkaline pH than the remaining part of the growing root with the exception of a pH maximum separating proximal and distal acidic zones [5, 9]. We probably could not disclose this pH maximum because of the size of the root segments employed; the net proton efflux of segments curtained this narrow alkaline zone which is located

either at the end of the 2nd or at the beginning of the 3rd segment. Equally, in *Arabidopsis*, cell wall pH in RC around S2 and S3 columella cells was higher than in the meristematic region and the maximal surface acidification occurred in the elongation zone, but the second acidic zone around the meristem was not detected as well as the intermediate alkaline zone [29]. It has been also shown that the RC apoplast acidified from pH 5.5 to 4.5 with concomitant increase in cytoplasmic pH within 2 min of gravistimulation. These changes in RC pH are likely involved in the initial events of root gravitropism [29]. Perhaps the RC apoplast should be somewhat alkaline to be able to respond by rapid acidification under gravistimulation. In this case slightly depressed proton transport PM H^+-ATPase activity can be in line with the normal physiological strategy of the RC.

Sustained proton efflux catalyzed by the PM H^+-ATPase requires, in addition to ATP synthesis, metabolic conversion of sugars into acids (to prevent intracellular pH increase during H^+ efflux) and concomitant K^+ uptake (for electrical balance during H^+ efflux). Any of these systems may have little activity in the RC. On the contrary, K^+ inward transport may be enhanced, for example, due to an increase in protein synthesis that can lead to net alkalization of the cell exterior. When the dynamic equilibrium is disturbed, for example, through changes in pump or/and cation channel activity, a pH shift should be observed. Maybe the net inward direction of proton pumping in the RC (Fig. 1d) is conditioned not only by the higher needs for cations, which are co-transported with protons, but also by the low H^+-transporting ATPase activity. The latter is supported by the immunodetection of the 14-3-3 proteins in microsomes from the 1st segment where the abundance of these proteins was the least (Figs. 3b and 3c). Additionally, among the microsome proteins of the 1st root segment 14-3-3 protein antibodies recognized polypeptide(s) at ~35 kD (Fig. 3b, asterisk on lane *1*), which was defined as a phosphorylated form of 14-3-3 protein with low capacity for association with H^+-ATPase [30]. Note that hyperosmotic stress (0.3 OsM) resulted in recovery of the net outward proton pumping activity (Figs. 1g and 2) accompanied by accumulation of 14-3-3 proteins in plasma membranes (Fig. 4). Moreover, as well known, FC activates PM H^+-ATPase (Fig. 1f) by stabilizing the complex between H^+-ATPase and 14-3-3 proteins. Combined with our earlier finding that the amount of 14-3-3 proteins markedly increased in the plasma membranes isolated from osmotically stressed sugar beet cells and these proteins are involved in the osmotic regulation of H^+-ATPase [19], we conclude that the activity of the PM H^+ pump in the RC cells is also mediated by 14-3-3 proteins.

Since the RC is a place of active metabolism, it requires considerable energy and substrate expenses. For example, it has been shown that in the maize root segment consisting of the meristem and the RC ATP/AMP ratio was 1.41, but in the elongation zone this ratio was 4.03 [31]. Recently, data on the inhibitory effect of 5′-AMP on the association of 14-3-3 proteins with the C-terminal domain of the plant PM H^+-ATPase have been presented [32]. Perhaps H^+-ATPase activity in the PM of the RC cells is regulated by 14-3-3 proteins and their association with the enzyme depends not only on the phosphorylation of the 14-3-3 proteins as mentioned above, but also may be linked by 5′-AMP to the energy charge of the cell.

Thus, our results indicate that the regions along the apical part of a growing root conversely differ in their proton pumping activity. The higher rate of H^+ efflux coincides with maximum growth rate and correlates with the increased amount of the 14-3-3 proteins in the membrane preparations. The segment consisting of the RC and the apical part of meristem exerts the net inward direction of proton pumping, which can be inverted by FC or osmotic stress, and in the latter case this inversion is accompanied by accumulation of 14-3-3 proteins in plasma membranes. Our results highlight the 14-3-3 protein as an obvious candidate for the fine regulation of PM H^+-ATPase in the growing root apex.

Future work will be targeted to localizing more precisely along the root apex the regions differing in H^+-ATPase and 14-3-3 protein performance as well as to detect whether an endogenous metabolite(s) and environmental cues mediate interactions between H^+-ATPase and the 14-3-3 protein.

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