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Membrane Transport Adaptations Contributing to Salt Tolerance

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3. ABSTRACT

Salt tolerance involves the accumulation of ions for osmotic adjustment and vacuolar compartmentation of Na^+ and Cl^- . Salt adaptation also results in the capacity of cells to regulate intracellular uptake of Na^+ and K^+ such that uptake of Na^+ is reduced and K^+ is enhanced in environments with high external NaCl . Evidence has been provided indicating the existence of cation/ H^+ antiports in membrane vesicles of wild type and NaCl -adapted tobacco cell suspensions, roots of the halophyte *Atriplex nummularia* and the glycophyte *Gossypium hirsutum* and Na^+/H^+ antiporter activity is postulated to play a role in salt adaptation. Salt adaptation of tobacco results in an alteration to the tonoplast ATPase which enhances the capacity for ATP hydrolysis and H^+ transport.

Additional research indicates that adaptation to salinity involves a dependence on salinity for chlorophyll biosynthesis. ABA enhances osmotic adjustment during salt adaptation, however, the role of ABA in accelerating salt adaptation is not merely due to promoting osmotic adjustment.

4. DETAILED DESCRIPTION OF THE RESEARCH ACTIVITIES AND RESULTS

Results from the Hebrew University of Jerusalem

Studies on Membrane Mechanisms in
Membrane Vesicles Derived from
Halophytes and Non-Halophytes

Proton pumps and other membrane mechanisms may well have important roles in the adaptation of higher plants to salinity. We have, in fact, earlier brought evidence (9) suggesting that the proton pump may function both as detector and as effector in osmoregulation. In this Research Programme we have concentrated on monovalent cation/H⁺ antiporters as particularly likely candidates for mechanisms with roles to play in adaptation. We have been investigating their possible operation in the truly NaCl-tolerant plant Atriplex nummularia, in wild type and NaCl-adapted tobacco cell lines grown in suspension culture, and in cotton, which, while far more sensitive to NaCl than Atriplex, is nevertheless ranked among crop plants which can withstand mild saline stress.

Atriplex nummularia - Supplementary data
(Jerusalem Lab., Y.Braun)

Our published paper (see above) reported Na^+/H^+ exchange in Atriplex root membrane vesicles and brought evidence that this exchange was not attributable to electric coupling of the two fluxes, resulting from an electrical charge imbalance across the membrane. That the apparent dissipation of ΔpH observed was also not due to displacement of the cationic pH probe from anionic sites in the membrane vesicle, such as anionic lipids, is shown in Figure 1.1. In this experiment addition of NaMes 10 min after supply of ATP brought about partial loss of the MA which had accumulated during the 10 min period. In the absence of a ΔpH , however, i.e. in the absence of ATP, loss of MA was very slight. Displacement of MA from anionic lipids, or other anionic sites in the vesicle, thus appears to have played only a small part in the exchange phenomenon observed.

This conclusion is reinforced by Figure 1.2, in which the responses to external K^+ and Na^+ respectively are seen to differ. The most probable explanation of the evidence presented here and in the publication attached, therefore, is that the opposed H^+ and Na^+ fluxes are coupled by a membrane protein, a Na^+/H^+ antiporter.

Figure 1.2 also extends the range over which the dependence of antiport activity on Na^+ concentration is examined. In a similar experiment in the publication attached above, Na^+ was supplied as Na Mes, and technical difficulties resulting from its limited solubility did not allow investigation of concentrations over 180 mM. In the case of Figure 1.2 both Na^+ and K^+ were supplied as chlorides, and concentrations up to 400 mM were tested. A saturation curve relates ΔpH dissipation to cation concentration in both cases. The cation concentration at which the curves flatten is somewhat lower for K^+ than for Na^+ ; further, the plateau level for K antiport activity is only 60% as high as that for Na^+ . Figure 1.2 thus suggests that both K_m and V_{max} may be lower in the case of K^+/H^+ antiport than in that of Na^+/H^+ antiport.

Figure 1.1. Effect of addition of NaMes on level of MA accumulation in *Atriplex* root membrane vesicles in presence or absence of ATP. NaMes was added at the arrow to a final concentration of 100 mM. Medium as given in the Materials and Methods section of publication reproduced above. Points give means of 4 replicates $\pm$ SE.

Figure 1-2 Dependence of antiporter activity in Atriplex (salt grown) root membrane vesicles on the concentration of NaCl or KCl supplied. The Atriplex plants had been grown in the presence of 400 mM NaCl. The intravesicular MA level when NaCl or KCl was added (after 10 min of incubation with 5 mM ATP) was 24.4 ± 0.3 nmol MA/mg protein. The amount of MA displaced was measured one minute after salt addition. Points give means of 4 replicates $\pm$ SE.

Attempts to measure antiporter activity in the absence of concomitant H^+ -ATPase activity by halting the ATPase have not so far proved successful with this tissue, since treatment with EDTA (6) or apyrase to remove the ATP, rapidly cause the membrane vesicles to leak and the results are in consequence unreliable. However, experiments where an artificial ΔpH is imposed across the membrane by the "pH jump" technique have been initiated and an example is shown in Figure 1.4.

Plants grown in the absence of NaCl

The results presented above were obtained with membrane vesicles isolated from the roots of plants grown in the presence of 400 mM NaCl. Antiport activity is also observed in the case of plants grown in the absence of added NaCl. Curves relating antiport activity to ~~K^+~~ Na^+ concentration are shown in Figure 1.3. The curves suggest that V_{max} for antiport is well below that indicated for salt grown plants. The position with regard to K_m is less clear, but there are indications in this and other experiments that this parameter, too, may be somewhat lower.

II Tobacco cells in suspension culture (Jerusalem Lab., S. Cooper)

Sodium/proton antiporters, as noted above, have so far been reported in the halophytic species beetroot (2) and Atriplex (this Report and (4)), and in a salt-tolerant strain of barley grown in the presence of M NaCl.(6) The present investigation has produced evidence indicating Na^+/H^+ antiport activity in Tobacco cells grown in suspension culture, not only in the case of NaCl-adapted lines but also in that of the glycophytic wild type. This evidence has been obtained in several types of experiment, using both radio-isotopic and fluorescent probes.

Figure 1.3 Dependence of antiporter activity in Atriplex (non salt grown) root membrane vesicles on the concentration of NaMes supplied.

An artificial pH was imposed across the vesicle membrane by preparing the vesicles so as to contain a buffer at pH 5.5 and then diluting them into a medium buffered at pH 7.0 and containing labelled MA. NaMes was added to the medium 1.5 minutes after dilution (when the intravesicular MA content was 6.3 ± 0.4 nmol/mg protein) and the amount of MA displaced was measured after a further 10 minutes.

Points give means of 4 replicates $\pm$ SE

Figure 1-4 Comparison of antiporter activity in root membrane vesicles isolated from salt grown and non-salt grown *Atriplex* plants. Conditions as explained in the legend to Figure 3.

Material and Methods

The tobacco cell lines (*Nicotiana tabacum/gossii*) were developed and maintained as described in previous publications of our laboratory (11). Before disruption for vesicle preparation, salt-adapted cells were transferred in gradual stages (to avoid osmotic shock) from their high NaCl medium to standard medium. Membrane vesicle preparation followed essentially the same procedure as given above for Atriplex (see above).

Results and Discussion

The experiments described below have been carried out on a membrane fraction collected from the interface between 25 to 30, and 30 to 34 per cent (wt/v) sucrose in a stepped sucrose gradient.

The following criteria indicate that this is a tonoplast-enriched fraction with little plasmalemma, mitochondrial or E.R. contamination:

(1) NO_3^- at 100 mM inhibits ΔpH formation by the H^+ -ATPase by 90%.

(2) Formation of ΔpH is also sensitive to NEM, as expected for tonoplast H^+ -ATPase activity (12).

(3) No inhibition by oligomycin or vanadate is detectable.

(4) GTP cannot replace ATP as substrate for the H^+ -ATPase indicating that E.R. ATPase is not involved to a detectable extent.

The establishment of a ΔpH across the vesicular membrane after supply of ATP has been observed both by following the accumulation of ^{14}C -MA and by following the quenching of quinacrine fluorescence. It is of interest that when MA accumulation is measured over very brief time intervals, (10 seconds) 2 phases of uptake can be distinguished, the first lasting for about 1 min. (Fig. 2.1)

Figure 2.1 Time course for MA accumulation within membrane vesicles isolated from wild type tobacco cells and incubated without or with 5 mM ATP. In this and following Figures, bar indicates SE of mean of 3 replicates.

Figure 2.2 shows that the presence of NaCl in the medium in concentrations ranging from 5 to 50 mM depressed the rate of MA accumulation. Inhibition was detectible in this experiment within 2 min (in the case of NaCl concentrations of 5 mM and over), and was statistically significant within 8 min. On the other hand when SCN^- accumulation within the vesicles (as a measure of $\Delta\psi$) was followed with time subsequent to ATP supply, these NaCl concentrations had no detectible inhibitory effect for the first 6 min (Fig. 2.3) and the very slight inhibitory effect suggested after 12 min was not statistically significant. Thus NaCl did not appreciably depress the activity of the H^+ -translocating ATPase within this period. Depression of ΔpH formation without a corresponding depression of $\Delta\psi$ formation suggests H^+/Na^+ exchange.

As pointed out in the section on Atriplex, several bases for this exchange effect might be envisaged: - (1) Flux of one solute might create or diminish an electrical charge imbalance across the membrane, i.e. it might set up a diffusion potential. The opposed flux of the second ion would then result from the change in $\Delta\psi$ and the coupling would not be specific. The possibility might be raised that Na^+ supply had created a Na^+ diffusion potential. However, in the experiments such as that shown in Figure 2.2 above Cl^- was added in sufficient amount (100 mM) for total depolarization of the membrane potential, as checked experimentally. Electric coupling is therefore a very unlikely explanation for the putative Na^+/H^+ exchange.

(2) Na^+ might compete with, and thus displace, the positively-charged probe (MA) from anionic sites on the membrane, e.g. anionic lipids (1). Figure 2.4 shows, however, that in the absence of a ΔpH , i.e. in the absence of ATP, the presence of NaCl did not detectibly depress MA uptake suggesting that competition for anionic sites played a very small part in the phenomenon observed.

It may therefore be concluded that coupling of opposed H^+ and Na^+ fluxes by a transport protein in the membrane is the most probable explanation of the results presented.

Figure 2.2 The effect of the presence of NaMes at various concentrations on the time course for MA accumulation within membrane vesicles isolated from wild type tobacco cells. ATP concentration was 5 mM.

Figure 2.3 The effect of the presence of NaMes at various concentrations on the time course for SCN⁻ accumulation within membrane vesicles isolated from wild type tobacco cells. ATP concentration was 5 mM.

Figure 2.4 The effect of NaCl on MA uptake into vesicles isolated from wild type tobacco cells, in the presence and absence of 5 mM ATP.

Evidence for antiport using a fluorimetric technique

Supply of Na^+ brings about immediate dissipation of a previously established ΔpH , as is shown by the tracings presented in Figure 2.5. It may be seen in these tracings (A, B and C) that addition of vesicles to the reaction mixture did not cause quenching of the fluorescent probe quinacrine. Subsequent addition of ATP, however, initiated quenching (after a short time-lag), indicating generation of a ΔpH (interior acid). After this proton gradient had been allowed to form, NaCl (Tracing A) or Na gluconate (Tracing B) was added. In the case of Tracing A NO_3^- was added 0.5 min before the Na^+ in order to inhibit the H^+ -ATPase. Tracing C shows that NO_3^- addition indeed inhibits the ATPase - when added before ATP it prevented ΔpH formation. As may be seen in tracings A and B, Na^+ addition brought about immediate partial recovery of fluorescence (Tracings A and B) indicating dissipation of the ΔpH which had been formed. Full recovery of fluorescence was obtained by subsequent addition of nigericin (an ionophore which exchanges H^+ for K^+ or Na^+) or (tracing B) gramicidin (an H^+ channel former), indicating that fluorescence quenching had indeed been due to the formation of a ΔpH .

In this experiment, as in the radioactive one described above, the most likely explanation of the observed H^+/Na^+ exchange is the operation of an antiporter mechanism in the membrane. Mere electrical coupling of opposed H^+ and Na^+ fluxes via a membrane potential can be discounted since choline chloride at 150 mM had been loaded into the vesicles and was also present in the medium; no membrane potential will have been formed under these conditions.

Competition between probe and Na^+ for non-specific binding sites can also be discounted as a sufficient explanation of the effect since no quenching was observed in the absence of ATP (Fig. 2.5).

Amiloride, an antidiuretic substance which is known as a blocker of Na^+ channels and an inhibitor of Na^+/H^+ antiport in animal cells, did not inhibit Na^+/H^+ exchange in our system (Fig. 2.6).

Figure 2.5 Fluorimeter tracings showing H^+ -gradient dependent quenching of Quinacrine fluorescence in membrane vesicles isolated from wild type tobacco cells, and its reversal by Na^+ . At first arrow choline chloride was added to the reaction mixture to a final concentration of 150 mM; at the second arrow the membrane vesicles (which were also loaded with 150 mM chloride) were added. For details of further additions see text.

Figure 2.6 Effect of amiloride on pH formation, and on its dissipation by addition of Na⁺, in membrane vesicles isolated from wild type tobacco cells.

BTP-Chloride at 100 mM was present in the medium.

Antiport was not specific for Na^+ but could be elicited by K^+ as well. This behaviour recalls the cation - proton antiport system in E. coli (the " K^+/H^+ antiporter") the substrates of which include K^+ , Na^+ , Li^+ , Rb^+ and Tl^+ (5).

Comparison of cell lines

The membrane transport phenomena described above have been observed both for wild type and for NaCl-adapted cell lines. This is of particular interest, since as pointed in the Introduction, Na^+/H^+ antiport has hitherto only been reported for halophytes. Work is in progress to compare the transport activities on a quantitative basis (which will require the development of special techniques and the correct approach to the problem) and to estimate the proton motive force generated in the cell lines in media of varying salinity.

III Cotton

(Jerusalem Lab., M. Hassidim)

Our comparative studies of membrane transport mechanisms in plants of varying resistance to salinity have included cotton, a glycophytic crop which can withstand mild saline stress. We have previously reported on H^+ -translocating performance (7). More recent experiments have provided evidence for K^+/H^+ antiport in the membrane fraction under investigation (collected from the interface between 34 - 38% and 38 - 45% sucrose (weight/volume)).

Evidence that this is a plasmalemma enriched fraction may be summarized as follows:

(1) Formation of ΔpH , after ATP supply, is not inhibited by NO_3^- up to 100 mM.

(2) Formation of $\Delta \Psi$ is sensitive to vanadate (an inhibitor of plasmalemma ATPase), 40% inhibition being produced by 170 mM vanadate.

(3) Formation of $\Delta\psi$ is not sensitive to oligomycin, an inhibitor of mitochondrial F_1F_0 ATPase.

(4) Ppiase is not detectible in this fraction, though it is in a lighter fraction.

(5) The H^+ -translocating ATPase in this fraction shows high specificity to ATP and cannot utilize GTP or IDP.

Among the experiments which indicate that this fraction possesses K^+/H^+ antiport activity are the following:

(1) If an artificial ΔpH is imposed across the vesicle membrane by preparing the vesicles so that the intravesicular pH is 5.5, and the vesicles are subsequently diluted into a medium at pH 7.0, MA enters the vesicles in response to this ΔpH (Fig. 3.1A). Subsequent addition of K^+ causes displacement of MA (Fig. 3.1A). On the other hand if the vesicles are prepared with an interior pH 7.0, and subsequently diluted into a medium at pH 7.0 (i.e. when no transmembrane ΔpH is imposed) MA is taken up to a far lesser extent (presumably it only equilibrates with the intravesicular volume). Addition of K^+ under these conditions causes negligible MA displacement (Fig. 3.1B). The latter result indicates that the displacement of MA observed in Fig. 3.1A was not due to competition between K^+ and the positively charged ions of MA for non-specific anionic sites, such as anionic membrane lipids. The same conclusion may be drawn from the fact that there is negligible displacement of MA in the case of minus ATP controls in experiments where a ΔpH is generated by supply of ATP to the H^+ -ATPase (not shown here, but cf. Figs. 1.1 and 2.4).

(2) Two kinds of experiments were carried out to investigate whether mere electric coupling of opposed K^+ and H^+ fluxes, due to the existence of a $\Delta\psi$ across the vesicle membrane, could be ruled out.

Figure 3.1. H^+ -gradient-dependent MA uptake and release in membrane vesicles isolated from cotton roots.

A. Vesicles prepared so as to contain BTP-Mes buffer at pH 5.5, and diluted at time 0 into buffer at pH 7.0.

B. Vesicles prepared so as to contain BTP-Mes buffer at pH 7.0, and diluted at time 0 into buffer at pH 7.0.

In both figures arrow indicates addition of K-Mes to final concentration of 20 mM. Medium contained 20 mM BTP-Chloride in addition to other constituents given under Methods.

Points give the mean of 4 replicates $\pm$ SE.

(a) In one type of experiment the vesicles were prepared so as to contain 40 mM choline chloride. The presence of the rapidly permeating chloride ion within and surrounding the vesicles prevented formation of any membrane potential. ATP was supplied to initiate Δ pH generation. (Fig 3.2 A) After 10 min KMes was added and brought about rapid partial dissipation of the Δ pH which had been formed. To evaluate the contribution of antiporter possibly present in tonoplast vesicles contaminating this plasmalemma-enriched fraction, 20mM NO_3^- was added together with ATP in the experiment shown in Figure 3.2B. Generation of Δ pH was only inhibited to a slight extent (cf. Fig. 3.2A with Fig. 3.2B). Addition of KMes caused dissipation of the Δ pH as in Fig. 3.2A, showing that the K^+/H^+ exchange was occurring in membrane vesicles possessing a NO_3^- resistant ATPase (i.e. a membrane other than the tonoplast and highly likely to be the plasmalemma). The rise in the curve observed after its initial fall (Fig. 3.2B) might be connected with NO_3^-/H^+ symport but this requires further investigation.

(b) The formation of a membrane potential has also been prevented by a second stratagem - preparing vesicles so as to contain 5mM K^+ , and a buffer at pH 5.4, and then diluting them into a buffer at pH 8.0 again containing 5 mM K^+ together with valinomycin. The ionophore allows ready transmembrane K^+ flux and therefore clamps membrane potential near zero. Under these conditions K^+ concentration will be equal on the two sides of the vesicle membrane and $\Delta\mu_{\text{K}}$ will thus contribute nothing towards the driving force for the postulated K^+/H^+ antiporter, which would be driven exclusively by $\Delta\mu_{\text{H}^+}$. Nevertheless, one might expect to see the effect of raising the K^+ concentration on dissipation of Δ pH since occupancy of K^+ sites on the antiporter would be expected to be obligatory for the operation of the antiporter and would be a function of K^+ concentration as reflected by the $K_m(\text{K}^+)$ for antiport.

Figure 3.3 shows an experiment carried out as described in the previous paragraph.

Figure 3.2. ATP-dependent MA uptake and release in membrane vesicles isolated from cotton roots.

ATP (final concentration 5 mM) was supplied at time 0, either without (Fig 3.2.A) or with (Fig 3.2.B) 20 mM BTP-NO₃.

Arrow indicates addition of K-Mes to a final concentration of 20 mM. Choline-chloride (40 mM) was present both within the vesicles and in external medium.

Points give the mean of 4 replicates $\pm$ SE.

Accumulation of MA in response to the imposed Δ pH reached a peak after 2 min and then declined (Fig. 3.3). After 1 min KCl, LiCl or Choline Cl was added to a final concentration of 15 mM, with the results shown in the inset, which magnifies the scale between 1 and 2 min. The addition of KCl accelerated the dissipation of Δ pH under these conditions, where the membrane was depolarized and electric coupling of K^+ and H^+ fluxes thus excluded. Mediation of K^+ and H^+ exchange by a membrane constituent is the most likely explanation. Li^+ also appeared to accelerate H^+ efflux, but to a lesser extent (not statistically significant). Choline chloride actually slowed H^+ efflux relative to the control. There are several possible explanations for this effect which may merit further investigation in future work.

Fig. 3.4 shows that addition of $(NH_4)_2SO_4$ at various intervals after the "pH jump" brings about virtually total displacement of MA from vesicles, indicating that MA accumulation was indeed reflecting Δ pH.

Na^+ antiport activity has also been observed in these membrane vesicles, but at a somewhat lower level than K^+ antiport for the same cation concentration. Thus, if one antiporter is responsible for the flux of both cations, it would appear to resemble the K^+/H^+ antiporter in bacteria, which as pointed out above, can use K^+ , Na^+ , Li^+ , Rb^+ and Tl^+ .

Figure 3.3. H^+ gradient-dependent uptake and release of MA in membrane vesicles isolated from cotton roots. Vesicles were prepared so as to contain BTP-Mes buffer at pH 5.5, plus 5 mM K-Mes; they were diluted at time 0 into medium at pH 8.0, containing 5 mM KMes plus valinomycin. After 1 min (at arrow) 15 mM K^+ , Li^+ , or $choline^+$ were added as chloride salts. The inset enlarges the scale between 1 and 2 min. Points give the mean of 4 replicates $\pm$ SE.

Figure 3.4. H^+ -gradient-dependent MA uptake and release in membrane vesicles isolated from cotton roots. An artificial pH gradient was imposed across the vesicle membrane as described in legend to Figure 3.1. Ammonium sulphate was added after 2 min or after 2.5 min as indicated by arrows. Points give the mean of 4 replicates $\pm$ SE.

General Conclusions

Both K^+ and Na^+ antiport activity can be observed, as in bacteria, in all three plant systems investigated. In the case of Atriplex and cotton the activity appeared to be present in plasmalemma-enriched fractions. Whether one or more antiporters are involved is not yet known. It has been customary to regard plasmalemma H^+ antiporters as means of exploiting the $\Delta \mu H$ established by the H^+ extrusion pump in order to drive monovalent cations out of the cell. However, antiporters should perhaps be regarded in a different light, that of devices for controlling the re-entry of extruded protons into the cell and thus contributing substantially to internal pH control (see literature for bacterial cells in this respect, e.g. (8)). In this case one would expect the K^+ antiporter to be under tight control so that the cell would not expend excessive energy circulating K^+ . Booth (3) has suggested that in the whole cell the cation/ H^+ antiporters involved in pH homeostasis are regulated via binding of low molecular weight ligands, or alternatively, by covalent protein modification. Such control mechanisms are probably lost during the isolation of membrane vesicles. While the work we report here has contributed evidence that such antiport mechanisms operate in plant cell membranes, the manner in which they are regulated in the intact plant cell must await further investigation.

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Achievements

Up till now monovalent cation/ H^+ antiport has only been documented in a very limited number of higher plant cells (2, 6, 10). We have produced evidence for the operation of such antiporters in isolated membrane vesicles prepared from three plant sources: (a) Wild type and NaCl-adapted tobacco cells in suspension culture;

(b) Roots of the halophyte Atriplex
nummularia.

(c) Roots of the glycophyte Gossypium
hirsutum.

In the case of the root vesicles, this antiport activity has been identified in plasmalemma -enriched fractions and is the first report of such activity for higher plant cells.

The Na^+/H^+ antiporter activity in the halophyte Atriplex has been shown to be modulated by saline conditions during growth and may thus play a role in the adaptation of this plant to salinity.

We have also made the interesting observation that our NaCl-adapted tobacco cell line has, during the course of adaptation to salinity, developed a dependence on salinity for chlorophyll biosynthesis (see reproduction of publication below).

Results from Purdue University

1. H⁺- and K⁺-Transport Adaptations Contributing to Salinity Tolerance of Cultured Cells. - A. A. Watad, P. M. Hasegawa and R. A. Bressan, abstract submitted to the XIV International Botanical Congress.

Maintenance of intracellular (int) K⁺ levels in the presence of high levels of external (ext) Na⁺ is an adaptive characteristic of NaCl-tolerant cells (stable in the absence of the stress). These cells exhibited a 1.5-fold to 3.0-fold higher net rate of K⁺ (⁸⁶Rb⁺) influx compared to intolerant cells due primarily to increased K⁺ influx and not to decreased K⁺ efflux. Increased net K⁺ influx was accompanied by a 50% higher rate of H⁺ efflux for NaCl-tolerant than for intolerant cells at 10 mM [KCl]_{ext}. H⁺-translocation was inhibited completely by vanadate although only 55-60% of net K⁺ influx was inhibited. Net K⁺ influx in the presence and absence of vanadate was greater for NaCl-tolerant cells. At high [K⁺ or Na⁺]_{ext} (100 mM), higher rates of net K⁺ influx into salt tolerant cells were mediated also by a K⁺/H⁺ antiporter.

2. Intracellular Compartmentation of Ions in Salt Adapted Tobacco Cells. - M. L. Binzel, F. D. Hess, R. A. Bressan and P. M. Hasegawa. 1988. Plant Physiol. 86:607-614. Reprint enclosed

3. Abscissic Acid Stimulated Osmotic Adjustment and Its Involvement in Adaptation of Tobacco Cells to NaCl. - P. C. LaRosa, P. M. Hasegawa, D. Rhodes, J. M. Clithero, A. A. Watad and R. A. Bressan. 1987. Plant Physiol. 85:174-181. Reprint enclosed

4. Mechanisms of Adaptation to Salinity in Cultured Glycophyte Cells. - M. L. Binzel, F. D. Hess, R. A. Bressan and P. M. Hasegawa. 1989. In: J. H. Cherry, ed., Biochemical and Physiological Mechanisms Associated with Environmental Stress Tolerance. Springer-Verlag, Berlin. In Press. Draft enclosed

5. Enhanced H⁺ Transport Capacity and ATP Hydrolysis Activity of the Tonoplast ATPase after Adaptation to NaCl. - M. Reuveni, A. B. Bennett, R. A. Bressan and P. M. Hasegawa. Manuscript in preparation. Draft enclosed

Running Head: H^+ TRANSPORT AND TONOPLAST ATPASE ACTIVITY AFTER ADAPTATION TO
NACL

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ENHANCED H^+ TRANSPORT CAPACITY AND ATP HYDROLYSIS ACTIVITY OF THE TONOPLAST
 H^+ -ATPASE AFTER ADAPTATION TO $NaCl$ ¹

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- (4) Abbreviations

BTP - Bis tris propane (1,3-bis[tris(hydroxymethyl)-methylamino]propane)

SDS-PAGE - sodium dodecylsulfate polyacrylamide gel electrophoresis

ABSTRACT

Tonoplast enriched membrane vesicle fractions were isolated from unadapted and NaCl (428 mM) adapted tobacco cells (*Nicotiana tabacum* L. var. Wisconsin 38). ATP dependent H^+ transport was inhibited by KNO_3 (50 mM), virtually insensitive to Na_3VO_4 (0.3 mM) and insensitive to oligomycin (25 μ g/ml). ATP hydrolysis was inhibited to a greater extent than H^+ transport by Na_3VO_4 . Polypeptides from the tonoplast enriched vesicle fractions were separated by SDS-PAGE⁴ and analyzed by Western blots using antibodies to the 70 kDa subunit of the tonoplast ATPase of red beet. The anti-70 kDa subunit antibodies cross reacted exclusively to tobacco polypeptide(s) of an apparent molecular weight of 69 kDa. Antibodies against the red beet 70 kDa subunit inhibited ATP dependent, NO_3^- sensitive H^+ transport into vesicles in tonoplast enriched membrane fractions from both tobacco cell lines. The H^+ transport capacity per amount of immunoblotted 69 kDa subunit of the tonoplast ATPase of vesicles from adapted cells was four-fold greater than that observed for vesicles from unadapted cells. This increase in specific H^+ transport capacity after adaptation was also observed for ATP hydrolysis activity.

Introduction

The ability of plants to survive and grow in a saline environment involves an integration of many intracellular and multicellular adaptive mechanisms (22, 33). Although it is problematic, if not impossible, to assess accurately the relative contribution of either type of mechanism to salt tolerance of plants, it is generally hypothesized that intracellular mechanisms must be integral components of salt tolerance (12, 23). Of critical importance are those cellular adaptations which facilitate osmotic adjustment by mediating ion accumulation and intracellular compartmentation (4, 41). Utilization of the ions which comprise the saline environment for osmotic adjustment seemingly would be an efficient way to counteract the water deficit imposed by high extracellular concentrations of ions. However, there is implication that adaptation to salt does not result in enhanced capacity of cytosolic physiological and biochemical processes to function more effectively at high ion concentrations. As a consequence the ability to use ions for osmotic adjustment must involve mechanisms which facilitate ion accumulation into the vacuole while at the same time regulate the concentration of the ions in the cytosol (3,4).

Cell expansion and cytomorphological adaptations to NaCl have been observed which ostensibly would facilitate ion accumulation for osmotic adjustment (2,4). These include reduced rates of cell expansion and final cell volumes which would lower the total ion flux through the cytosol and also diminish the absolute ion content per cell. Increased cytoplasm to vacuole volume ratios would further reduce the amount of ion accumulation needed per cell to establish osmotic adjustment. The enhancement of the membrane surface area to volume and especially an increased surface area of the tonoplast would

increase the ability of cells to maintain ion pools during growth (4). These changes in geometry would not by themselves uniquely favor influx or efflux but would decrease the time necessary to load or unload a compartment (based on the direction of net flux) and facilitate the maintenance of gradients under steady state.

It is, however, also likely that salt tolerance involves adaptations of the membranes which affect permeability and facilitate transport of ions. Identification of Na^+/H^+ exchange activity in isolated vesicle preparations driven by pH gradients (ΔpH) has been interpreted to indicate the presence of a Na^+/H^+ antiporter located in the tonoplast (6, 16). Presumably, this antiporter is primarily responsible for facilitated uptake of Na^+ from the cytosol across the tonoplast into the vacuole. Cl^- uptake into the vacuole follows the membrane potential gradient ($\Delta\psi$) generated by H^+ translocation via a uniport (7, 13), although the presence of a H^+/anion antiport has been proposed (13).

Regardless of the exact mechanism responsible for facilitated Na^+ and Cl^- influx into the vacuole, it is clear that the primary driving force for this transport is the $\Delta\mu_{\text{H}^+}$, generated by H^+ translocation across the tonoplast. The primary H^+ pump in the tonoplast is the H^+ -ATPase (7, 36, 39), which is a vacuolar-type ATPase (31). These ATPases, which have been categorized as a result of analogous biochemical properties, are localized in the endomembranes of all eukaryotic cells (31). More recently, confirmation of the similarity of vacuolar-type ATPases has been obtained by sequence analysis of cDNA and genomic clones encoding for ATPase polypeptides (8, 9, 24, 26, 30, 42).

The tonoplast ATPase is composed of at least 3 subunits of apparent molecular weights of 16-18, 58-60 and 68-70 kDa (31). The largest subunit (68-70 kDa) has been shown to contain a nucleotide binding site (25, 28) and analysis of cDNA (42) and genomic (9) clones encoding this subunit indicate sequence homology with the β subunit of F_0F_1 ATPases in regions which have been implicated to have catalytic function (15). The 58-60 kDa subunit also possesses a nucleotide binding site and analyses of cDNA (26) and genomic (8) clones indicate significant structural similarity to the α subunit of F_0F_1 ATPases. The 16-18 kDa subunit is a proteolipid proposed to be involved in H^+ transport (24, 34)

In this report, evidence is presented that a component of NaCl adaptation of a glycophyte involves altered regulation of the tonoplast ATPase. This is reflected in enhanced capacity of the enzyme for ATP hydrolysis and H^+ transport. In these experiments, ATP hydrolysis and H^+ transport data were normalized based on the cross reactivity of an antibody to the 68-70 kDa subunit in order to compensate for differences in the composition of tonoplast enriched vesicle preparations and in membrane composition between cell lines.

MATERIALS AND METHODS

Cell Growth and Isolation of Membrane Vesicles. Liquid suspensions of tobacco cells (*Nicotiana tabacum* L. Var. Wisconsin 38) were grown in the presence of 428 mM NaCl (NaCl adapted) or the absence of NaCl (unadapted), as described (2, 3). Cell lines were grown in the dark and collected at fresh weight stationary phase for membrane vesicle preparation (23-26 days for unadapted cells and 36-40 days for NaCl adapted cells). It was noted that fresh weight growth rate of both unadapted and NaCl adapted cells was substantially slower in the dark.

Membrane vesicles were prepared from unadapted and NaCl adapted cells utilizing previous protocols that were modified for use on cell suspensions (7, 10, 18). Eighty to 100 grams (fresh weight) of cells were gravity filtered on a 0.1 mm mesh nylon screen and then incubated in homogenization medium (2 ml of medium per gram fresh weight of cells) containing 250 mM sorbitol, 100 mM ascorbic acid, 10 mM MgSO_4 , 2 mM EGTA, 5 mM Mes, 0.03% PMSF, 2 mM DTT, 0.5 mM Dibucain and 4% PVP (insoluble) adjusted to pH 7.2 (with BTP). Cells were homogenized on ice in an Omni-mixer (Du Pont) for 45 sec and the slurry filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 13,000g for 10 min, the pellet discarded and the supernatant recentrifuged at 80,000g for 30 min. The supernatant was decanted and the microsomal pellet resuspended, using a 26 gauge syringe needle, in suspension buffer (250 mM sorbitol, 1 mM MgSO_4 and 1 mM Mes, pH 7.2). The suspension was overlayed onto a discontinuous sucrose gradient (22%, 27%, 36%, and 45% (w/v) sucrose, 1 mM Mes and 1 mM MgSO_4 , pH 7.2) and centrifuged for 2 h at 100,000g. All centrifugation steps were carried out at 5°C. The 0/22% interface was collected and frozen at -80°C. Storage of the

vesicle preparations for up to 90 days did not result in detectable loss of ATP hydrolysis or H^+ transport activities. Protein content in the vesicle fractions was determined by the Bio Rad reagent based on the method of Bradford.

Assays for Phosphate Hydrolysis and H^+ Transport. Vesicles from the 0/20% sucrose interface, either freshly prepared or stored at $-80^{\circ}C$ and thawed prior to use, were diluted with 2 volumes of suspension medium and centrifuged at 150,000g for 30 min. The membrane pellet was resuspended as described above in suspension medium supplemented with 20 mM KCl and used for analysis.

The phosphate hydrolysis assay, using ATP, IDP or PPi as a substrate, was initiated by adding 50 μ l of vesicle suspension (5 to 20 μ g protein) to 450 μ l of reaction solution (15 mM Mes-BTP, 50 mM KCl, 3 mM MgATP, 0.2 mM $Na_2M_2O_4$, 0.05% Triton X-100 and 0.1% BSA, pH 8.0) at $24^{\circ}C$. The reaction was terminated after 1 h by the addition of an equal volume of 15% ice cold TCA. After 15 min of incubation at $4^{\circ}C$, the mixture was centrifuged in a Beckman microfuge for 5 min at full speed and the Pi in the supernatant was determined (40). IDPase and PPiase were assayed in the reaction solution at pH 6.5 and by replacing MgATP with MgIDP or MgPPi, respectively.

Proton Transport Assay. Net H^+ transport into vesicles was measured as a function of [^{14}C]-methylamine (MeA) uptake or the rate of quinacrine fluorescence quenching (1, 7, 10, 13, 18). For measurement of ΔpH formation in vesicles, 50 μ l of membrane vesicles (10 to 50 μ g of protein) were added to 10 μ l of assay medium with the final solution containing: 250 mM sorbitol, 100 mM glycerol, 0.1% BSA, 25 μ g/ml oligomycin, 5 μ M ^{14}C -MeA (30,000 cpm), 5 mM Mes-BTP, pH 7.2 and either 20 mM KCl and 0.1 μ g/ml valinomycin. ATP dependent H^+

transport was determined using 2.5 mM MgATP. Membrane vesicles were separated from the assay medium by rapid filtration (10, 18) and the radioactivity was determined in a Beckman L-6800 liquid scintillation spectrometer. ^{14}C -MeA binding in the absence of ATP was subtracted from all uptake data.

For fluorometric measurements of ΔpH formation, 100 μl of membrane vesicles preparation (100 to 270 μg of protein) were suspended in 2850 μl of the following solution: 250 mM sorbitol, 200 mM glycerol, 46 mM choline chloride, 3 mM MgSO_4 , 5 mM Mes at pH 7.2 and 3 μM quinacrine. The reaction was initiated by the addition of tris-ATP (1.2 mM final concentration) and monitored in a LS-5 Perkin Elmer fluorometer at 445 and 502 nm excitation and emission wavelengths, respectively.

Western Blot Analysis. Acetone powders of proteins (37) from membrane fractions were solubilized in sample buffer containing 4% SDS, 20% glycerol, 0.25% mercaptoethanol and 100 mM tris, pH 6.8 and separated by SDS-PAGE on a Bio Rad mini Protean II. Protein was determined according to the method of Bradford using the Bio Rad reagent after removal of SDS. Except where noted, 5 to 10 μg of protein were used for immunoblotting. After electrophoresis, proteins were transferred to nitrocellulose paper, using the semi-dry procedure (5), at 1.2 mA per cm^2 for 1 hr. The blots were incubated with 1:5000 dilution of antibodies against the 70 kDa subunit of the red beet root tonoplast ATPase (14) for 2 hr and the bands were visualized using a goat anti-rabbit alkaline phosphatase conjugate (Bio Rad # 170-6460).

Quantitation of the Western blots was accomplished with a DU-8 Beckman spectrophotometer Slab Gel Compuset module. In each experiment, equal amounts of

protein (within the linear-range of detection) from unadapted and NaCl adapted cells were separated on the same blot.

RESULTS

Tonoplast Enriched Vesicle Fractions. The composition of the membrane vesicles, based on sensitivity of ATPase activity to NO_3^- , vanadate and oligomycin (7, 39), in the 0/22% sucrose gradient interface from unadapted and NaCl adapted cells was comparable (Table I). This fraction was enriched with membrane vesicles possessing a vacuolar-type ATPase as indicated by the NO_3^- sensitive, oligomycin insensitive ATP hydrolysis (Table I). The observed vanadate sensitive ATPase hydrolysis is likely due to ER membrane vesicles (17) since the 0/22% interface contained very little glucan synthase II activity relative to the microsomal pellet and contained antimycin A insensitive NADH-Cyt c reductase activity (data not shown). All ATP-dependent H^+ transport activity was NO_3^- sensitive in the vesicle fractions obtained from unadapted cells while over 80% of the H^+ transport activity in NaCl adapted cell vesicles was inhibited by NO_3^- (Table II, Fig. 1).

NO_3^- sensitive ATPase activity and H^+ transport were indicative of the enrichment of the 0/22% sucrose interface with tonoplast vesicles (Table I, II, Fig. 1). This fraction was enriched relative to the microsomal pellet for pyrophosphatase activity, 2.1 ± 0.2 to 1.2 ± 0.07 $\mu\text{mol Pi/mg protein/hr}$ and 2.5 ± 0.2 to 1.7 ± 0.4 $\mu\text{mol Pi/mg protein/hr}$, respectively for unadapted and NaCl adapted cells. IDPase activity in the 80,000g membrane pellet was 4.5 ± 1.1 and 4.4 ± 1.4 $\mu\text{mol Pi/mg protein/hr}$ and in the 0/22% sucrose interface was 1.7 ± 0.2 and 1.6 ± 0.3 $\mu\text{mol Pi/mg protein/hr}$, respectively for unadapted and NaCl adapted cells. These data further substantiate that the 0/22% sucrose gradient interface was tonoplast enriched.

Further evidence that the 0/22% interface was enriched with tonoplast vesicles was demonstrated using Western blot analysis (Fig. 2). Polyclonal antibodies to the 70 kDa subunit of the red beet tonoplast ATPase cross reacted to a polypeptide with an apparent molecular weight of 69 kDa in these fractions. There was approximately a 6 fold enrichment in this polypeptide in the 0/22% sucrose interface relative to the microsomal pellet when the blot was scanned (Fig. 2). The antibodies to the red beet 70 kDa ATPase subunit inhibited H^+ transport of vesicles in tonoplast enriched fractions (Fig. 6).

Characterization of H^+ Transport Activity. Vesicles in tonoplast enriched membrane fractions from unadapted and NaCl adapted cells exhibited MgATP dependent H^+ pumping which was inhibited by NO_3^- (Fig. 1, Table II). ATP-dependent net uptake of ^{14}C -MeA was linear over a 10 min time course (Fig. 3). ATP was the most effective nucleoside triphosphate substrate for H^+ transport (Table III). Leakage of preloaded MeA from vesicles, isolated from unadapted and NaCl adapted cells was similar (Fig. 4). Similar results were obtained for quinacrine leakage after H^+ pumping was inhibited with NO_3^- or terminated by scavenging ATP using hexokinase and glucose (data not shown).

Specificity of Red Beet Antibodies to the Tobacco Tonoplast ATPase.

Polyclonal antibodies to the 70 kDa subunit of the red beet tonoplast ATPase cross reacted to a 69 kDa polypeptide(s) in tonoplast enriched vesicle fractions from unadapted and NaCl adapted cells (Fig. 2). Two dimensional immunoblots revealed the presence of 2 cross reactive 69 kDa polypeptides (data not shown). Similar results have been reported for barley (14). The degree of reactivity to the polypeptides from the different cell types was similar and linear to about 15 μ g of membrane protein (Fig. 3).

Antibodies against the red beet ATPase exhibited specificity to the tonoplast ATPase as indicated by the inhibition of the NO_3^- sensitive ATP-dependent H^+ transport in tonoplast enriched vesicle fractions (Fig. 6). The same differential effects between antibodies to the analogous subunit and pre-immune sera on H^+ transport have been interpreted to be indicative of immunological specificity for the tonoplast ATPase (35). The antibodies to the 70 kDa subunit exhibited similar inhibition affinities to the tonoplast ATPase from unadapted and NaCl adapted cells (Fig. 7). Inhibition of the ATPase of unadapted cells required greater antibody protein to membrane protein ratio. The data are indicative that the red beet antibodies have similar cross reactivities to the tonoplast ATPase from unadapted and NaCl adapted cells and that the tonoplast enriched vesicle fraction from unadapted cells contains greater amounts of this ATPase compared with NaCl adapted cells.

ATPase Activity and H^+ Transport in Vesicles from Tonoplast Enriched Sucrose Gradient Fractions. In view of the differences in membrane composition of the 0/22% sucrose gradient interface between unadapted and NaCl adapted cells, comparison of the tonoplast ATPases as a function of total protein in the fraction would be obscured. As a consequence, NO_3^- sensitive ATPase activity and H^+ transport data were normalized to the content of 69 kDa subunit of the tonoplast ATPase which was determined by immunoblots (Fig. 2). In each experiment, the vesicle proteins (5-10 μg , see Fig. 5) from unadapted and NaCl adapted cells were compared on the same immunoblot. The cross reactivity of the red beet antibodies to the 69 kDa subunit from unadapted and NaCl adapted cells was similar based on immunoblots and H^+ transport activity inhibition affinity (Figs. 5, 6, 7). Direct comparisons of net ^{14}C -MeA uptake and quinacrine

fluorescence quenching between vesicle preparations of the 2 cell types were not complicated by significant differences in leakage of the pH gradient indicators (Fig. 4) from the vesicles of unadapted and NaCl adapted cells.

The tonoplast ATPase from NaCl adapted cells exhibited a 4 fold higher H^+ pumping capacity than the ATPase from unadapted cells (Table IV). This difference in the tonoplast ATPase as a function of salt adaptation was verified by comparison of the ATP hydrolysis activities which indicated a 3 fold (Table V) higher activity for the tonoplast ATPase of NaCl adapted cells. This eliminates the possibility that differential sidedness of the tonoplast vesicles from the different cell types caused the observed change in H^+ transport capacity after adaptation.

DISCUSSION

Evidence has been presented which indicates that salt adaptation results in a an alteration of the primary H^+ pump in the vacuolar membrane, the tonoplast ATPase. Relative to the ATPase from unadapted cells, the capacity for ATP hydrolysis and H^+ transport is greater for the tonoplast ATPase of salt adapted cells when the content of this ATPase in the tonoplast vesicle enriched fraction is determined by immuno-detection of the 69 kDa subunit (Tables IV and V). Differential cross reactivities of the polyclonal antibodies to the 70 kDa subunit of the tonoplast ATPase from red beet to the 69 kDa subunits from unadapted and NaCl adapted cells could theoretically obscure the above interpretation. Such an occurrence would indicate differences in the catalytic subunit which affect the antigenicity and therefore are likely to affect the function of the ATPase. However, this is unlikely considering that no differences in the binding affinities of the red beet antibodies to the 69 kDa subunits from unadapted and NaCl adapted cells were evident based on immunoblots or inhibition of H^+ transport (Figs. 5, 6, and 7).

Although the capacity of the tonoplast ATPase is enhanced as a function of salt adaptation, the immunoblot data indicate that there has been a reduction in the amount of ATPase in the vacuolar membrane of adapted cells. The interpretation of these data are complicated by the differential composition of the vesicle fractions from the different cell types, the protein to lipid ratio of various types of membrane vesicles which the fractions contain and the protein to lipid ratio of specific membranes which result from salt adaptation. However, it does not seem likely that these possibilities can account totally for the several fold difference in ATPase content observed from the immunoblot

analysis (Fig. 2). Thus it appears that salt adaptation has resulted in a reduction in the content of the H^+ ATPase in the tonoplast which is compensated for by an increased capacity of the enzyme for ATP hydrolysis activity and for H^+ transport. Clear confirmation awaits further examination involving perhaps cytochemical techniques or utilization of purified vacuole preparations.

The significance of a decrease in the amount of ATPase protein in the tonoplast after salt adaptation is presently unclear. It is also not clear if the increased specific H^+ transport capacity of the tonoplast ATPase has resulted in enhanced net H^+ transport capacity across the tonoplast. Enhanced capacity for unidirectional transport of protons into the vacuole would lead not only to a greater accumulation of Na^+ and Cl^- into the vacuole for the purpose of osmotic adjustment but would affect the transport of many other solutes also. There may be no net changes in ΔpH or $\Delta \psi$ as a result of enhanced H^+ transport assuming that there is the utilization of a Na^+/H^+ antiporter and a Cl^- uniporter for uptake of these ions into the vacuole. This of course is a somewhat simplistic generalization involving maybe one use of the H^+ gradient for facilitated solute uptake and achieving H^+ electrochemical balance. Nevertheless, the fact that adaptation to salt has resulted in both a decrease in the amount of tonoplast H^+ ATPase and an increase in the specific H^+ transport capacity indicates that the H^+ transport capacity of the tonoplast is highly regulated. It is possible that during initial exposure to NaCl an increased amount of a more active ATPase is made and after ion equilibration is reached the amount of this, more active, ATPase in the membrane is reduced.

It has been shown that facultative CAM plants, *Mesembryanthemum crystallinum* and *Kalanchoe blossfeldiana*, when exposed to conditions (high salt

exposure) which induce the transition to CAM metabolism exhibit a several fold increase in tonoplast ATPase activity (11, 38). The need for greater ATPase activity, upon exposure to NaCl which was used to induce CAM, was attributed to the requirement for protons to facilitate the accumulation of malic acid into the vacuole. It is possible that the response of facultative CAM plants to salt with respect to the tonoplast ATPase may be unique. However, it is quite plausible that enhanced tonoplast ATPase activity is a requisite for increased solute uptake into the vacuole, particularly under conditions of initial imposition of salt.

The biochemical basis for the increased ATP hydrolysis activity and H^+ transport capacity of the tonoplast ATPase which results from salt adaptation is not yet defined. Changes in lipid composition of membranes occur as a result of salt adaptation and the ability to mediate these alterations can be correlated to the salt tolerance of genotypes (20, 29). The lipid environment in membranes has been implicated to be involved in the regulation of ATPase activity (21, 32). Salt adaptation could involve post translational or allosteric regulation of the tonoplast ATPase affecting ATP hydrolysis and H^+ transport activities. Kinase mediated phosphorylation of the plasma membrane H^+ -ATPase may be involved in regulation of activity (27). It is possible that the altered level of tonoplast ATPase after adaptation is a result of transcriptional regulation. Synthesis of isozymes of the α subunit of the Na^+/K^+ ATPase is regulated through the expression of a family of genes (19). However, there is no evidence yet for the existence of multiple genes for any of the subunits of a vacuolar type ATPase (30).

The data in this report provide a clear indication that adaptation to salt involves both qualitative and quantitative alterations in the tonoplast ATPase which exist in cells growing in a high saline environment. An adaptation to this primary tonoplast H^+ pump is consistent with the observed accumulation and compartmentation of solutes into the vacuole, particularly Na^+ and Cl^- , which occurs as a function of salt adaptation (4). The basis for the adaptation to the tonoplast ATPase which results in increased ATP hydrolysis activity and H^+ transport capacity and mode by which this adaptation is mediated are topics of ongoing research and are apparently critical components of osmotic adjustment under saline conditions.

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Table I. ATPase activity (ATP hydrolysis) of tonoplast enriched vesicle fractions isolated from unadapted and NaCl adapted cells (adapted to and growing in medium with 428 mM NaCl). Vesicles were collected from the 0/22% interface of a discontinuous sucrose gradient and ATP hydrolysis activity was measured in a buffer containing 15 mM Mes-BTP (pH 8.0), 50 mM KCl, 0.1% BSA, 0.05% Triton X-100, 0.2 mM Na_2MoO_4 and 3 mM MgATP as liberated Pi for one hour of incubation at 26°C. ATP hydrolysis is expressed in $\mu\text{mol Pi}$ per mg of total membrane protein in the fraction per hour after nonspecific ATP hydrolysis was subtracted from the data. Numbers in parentheses are percent of activity relative to the MgATP treatment. The data presented here are the mean $\pm$ S.E. of 3 experiments for unadapted and 5 experiments for NaCl adapted cells (duplicate samples in each experiment).

	Unadapted cells	Salt adapted cells
Treatment	$\mu\text{mol Pi (mg protein)}^{-1} \text{ h}^{-1}$	
MgATP (3 mM)	6.6 ± 1.7 (100)	6.5 ± 1.2 (100)
+KNO ₃ (50 mM)	2.9 ± 1.4 (44)	3.9 ± 1.1 (60)
+Na ₂ VO ₄ (0.2 mM)	5.6 ± 1.3 (85)	4.4 ± 1.6 (68)
+Oligomycin (25 $\mu\text{g/ml}$)	5.9 ± 2.2 (89)	5.6 ± 1.4 (86)

Table II. Generation of pH gradients in tonoplast enriched vesicle fractions from unadapted and NaCl (428 mM) adapted cells based on the uptake of ^{14}C -methylamine (MeA). The reaction buffer contained (final concentration) 200 mM glycerol, 20 mM KCl, 250 mM sorbitol, 25 $\mu\text{g/ml}$ oligomycin, 5 mM Mes-BTP, 0.1% BSA, 0.1 $\mu\text{g/ml}$ valinomycin and 5 μM ^{14}C -MeA, adjusted to pH 7.2. ATP dependent H^+ transport activity is expressed as rate of the uptake of ^{14}C -MeA as function of the total protein in the fraction ($\text{pmol MeA (mg protein)}^{-1} \text{ min}^{-1}$). MeA uptake was linear over the time period (10 min) of this experiment and MeA leakage from vesicle of both cell types was similar (Fig. 5). ATP dependent H^+ translocation was calculated after MeA binding in the absence of ATP was subtracted from the data. Numbers in parentheses are percent of MeA uptake relative to the MgATP treatment. Data represent means of triplicate samples $\pm$ S.E. MgATP (2.5 mM), KNO_3 (50 mM) and Na_2VO_4 (0.2 mM).

	Unadapted	Salt adapted
Treatment	$\text{pmol MeA (mg protein)}^{-1} \text{ min}^{-1}$	
MgATP	7.9 ± 0.7 (100)	8.8 ± 0.1 (100)
+ KNO_3	0 (0)	1.7 ± 0.3 (19)
+ Na_2VO_4	7.7 ± 0.2 (97)	8.9 ± 0.4 (101)

Table III. Nucleoside triphosphate (NTP) dependent H^+ transport into vesicles of tonoplast enriched fractions from unadapted and NaCl (428 mM) adapted cells. Vesicles were incubated for 10 min in assay medium with 2.5 mM NTP (see Table II and Materials and Methods). MeA binding in the absence of any NTP was subtracted from the results. Data are a mean of three replicates $\pm$ S.E.

Nucleotide	Unadapted cells	NaCl adapted cells
	pmoles MeA (mg protein) ⁻¹ min ⁻¹	
ATP	15.1 $\pm$ 0.6	15.8 $\pm$ 1.3
CTP	3.4 $\pm$ 1.0	1.0 $\pm$ 0.9
UTP	3.7 $\pm$ 0.5	0 $\pm$ 0.1
GTP	7.7 $\pm$ 3.8	4.4 $\pm$ 0.4

Table IV. NO_3^- sensitive H^+ transport activities of vesicles from tonoplast enriched fractions of unadapted and NaCl (428 mM) cells normalized to the content of the 69 kDa subunit of the tonoplast ATPase. The fractions were separated by SDS-PAGE and immunoblotted with the antibodies to the 70 kDa subunit of the red beet tonoplast ATPase (see Materials and Methods). H^+ transport was based on ^{14}C -MeA uptake (a) or quinacrine fluorescence quenching (b). Actual activity for the different membrane preparations in: a, pmol MeA $\text{area}^{-1} \text{min}^{-1}$ and b, % of fluorescence quench $\text{area}^{-1} \text{min}^{-1}$.

Exp. No.	Unadapted cells		NaCl adapted cells	
	H^+ transport activity		<u>NaCl adapted cells</u> unadapted cells	
1 ^a	20	66	3.2	
2 ^a	8	36	4.5	
3 ^a	5.6	29.7	5.3	
4 ^a	0.9	4.2	4.7	
5 ^a	2.7	6.8	2.5	
6 ^b	254	89	3.5	
Mean $\pm$ SE			4.0 $\pm$ 0.4	

Table V. Tonoplast ATPase activity (NO_3^- sensitive ATP hydrolysis) from tonoplast enriched vesicle fractions of unadapted and NaCl (428 mM) adapted cells as a function of the relative quantity of the 69 kDa subunit of the tonoplast ATPase. Polypeptides from tonoplast enriched vesicle fractions were separated on SDS-PAGE and Western blot analysis conducted. ATP hydrolysis (KNO_3 sensitive, oligomycin insensitive activity) is expressed as a function of the quantity of the kDa subunit based on the immunoprecipitated area of the Western blot.

Exp. No.	Unadapted cells		NaCl adapted cells	
	$\mu\text{mol Pi (area)}^{-1} \text{ h}^{-1}$		$\frac{\text{NaCl adapted cells}}{\text{unadapted cells}}$	
1	2.5	6.9	2.8	
2	2.1	8.5	4.0	
3	2.0	3.5	1.8	
Mean $\pm$ SE			2.9 $\pm$ 0.6	

Figure Legend

Figure 1. ATP dependent fluorescence quenching by vesicles in the 0/22% sucrose gradient fractions from unadapted (100 μ g protein) and NaCl adapted (270 μ g protein) tobacco cells. Tonoplast vesicles (100 μ L) were added to fluorescence assay buffer (2850 μ L) containing 250 mM sorbitol, 200 mM glycerol, 3 mM MgSO_4 , 46 mM choline chloride, 3 μ M quinacrine and 5 mM Mes (brought to pH 7.2 with powdered BTP) and ATP dependent quinacrine quenching was monitored at 445 nm excitation and 502 nm emission wavelength after addition of 1.5 mM tris-ATP. Uptake of quinacrine was stopped by addition of NO_3^- (final concentration 40 mM BTP- NO_3) and leakage of quinacrine was monitored. Reaction was terminated by adding 1.5 mM (final concentration) NH_4Cl .

Figure 2. Western blot of cellular membrane fractions from unadapted and NaCl adapted tobacco cells. Twenty four μ g of protein from microsomal membranes (lanes 1 and 2); 6 μ g of protein from the 0/22% sucrose gradient interface, tonoplast enriched (lanes 3 and 4) and 6 μ g of protein from the 36/45% sucrose gradient interface, plasma membrane enriched (lanes 5 and 6) were separated by SDS-PAGE and blotted with the antibodies to the red beet 70 kDa tonoplast ATPase subunit as described in the Materials and Methods. Lanes 1, 3, and 5; unadapted cells and lanes 2, 4 and 6; NaCl adapted cells.

Figure 3. Time course of ATP dependent ^{14}C -methylamine (MeA) uptake by vesicles in tonoplast enriched fractions from NaCl adapted and unadapted cells. Tonoplast enriched vesicles from A: unadapted cells (10 μ g protein) and B: NaCl adapted cells (30 μ g protein) were incubated with or without MgATP (as described in Table II).

Figure 4. ^{14}C -MeA leakage from vesicles of tonoplast enriched fractions prepared from unadapted and NaCl adapted cells preloaded for 10 min (as described in Table II). Leakage was measured after scavenging the ATP with Apyrase (0.13 units per assay). Each point is the mean of triplicate samples after MeA binding in the absence of ATP was subtracted from each point.

Figure 5. Binding of the antibodies to the 70 kDa tonoplast ATPase subunit as a function of increasing amount of membrane protein. SDS-PAGE of proteins from tonoplast enriched membrane vesicle fractions from unadapted (0) and NaCl adapted (0) cells were incubated with anti-70 kDa as described in Materials and Methods. Each lane was scanned twice and the area under the peak integrated and plotted as a function of the amount of protein loaded.

Figure 6. Inhibition of ATP dependent, NO_3^- sensitive ^{14}C -MeA uptake into vesicles of tonoplast enriched fractions by antibodies to the 70 kDa subunit of the red beet tonoplast ATPase (anti-70). Vesicles (50 μL) from unadapted (A) and NaCl adapted (B) cells were incubated with antibodies to the 70 kDa subunit or pre-immune (normal) rabbit serum for 12 min at room temperature before reaction buffer $\pm$ ATP (see Materials and Methods) was added. ^{14}C -MeA uptake was terminated after 10 min. Each point is the mean of triplicate measurements after MeA binding in the absence of ATP was subtracted.

Figure 7. Inhibition of ATP dependent, NO_3^- sensitive H^+ transport into vesicles of tonoplast enriched fractions by antibodies to the 70 kDa subunit of the red beet tonoplast ATPase (see Fig. 6) as a function of the ratio of serum

protein to membrane protein. Each symbol type represents a separate experiment, 5 for unadapted (closed symbols) and 5 for NaCl adapted (open symbols) cells. Line a is the best fit by linear regression analysis of the points from NaCl adapted cells; line b is the best fit by linear regression analysis of the points from unadapted cells; line c is the best fit by linear regression analysis of the points from unadapted cells up to the point where the line exceeds 100% activity and line d is the best fit by linear regression analysis of the points from unadapted cells between the point where line c crosses 100% and the ordinate.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

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