13

77

BARD

FINAL REPORT

PROJECT NO. IS-1075-86

הספיקה להספקים
למ.י.י.י.י.י.י.
ג'י.י.י.י.י.י.

The Role of the Plasma Membrane and Tonoplast in Salt-Tolerance: Characterization of the Atpases and Ion Transport Properties

G. Ben-Hayyim, F. DuPont, R. Lundin, J. Windle,
W. Hurkman

35

630.72
BAR/BEN
2nd copy

1991

000000229913

36906

Standard BARD Cover Page for Scientific Reports

Aug. 1991

Date: _____

BARD
P.O. BOX 6
Bet Dagan, ISRAEL

הספריה המרכזית
לפדגוגיה וחינוך
בית-17

I-1075-86

BARD project No. _____

Title

The role of plasma membrane and tonoplast in salt tolerance: characterization
of the ATPases and ion transport properties

Investigators' Names
(Principal listed first)

G. Ben-Hayyim

F. DuPont

R. Lundin

J. Windle

W. Hurkman

Investigators' Institutions

A.R.O. The Volcani Center

U.S.D.A. Western Research center

U.S.D.A. Western Research center

U.S.D.A. Western Research Center

U.S.D.A. Western Research Center

Project's starting date: 1.11.1987Type of Report: 1st Annual _____ 2nd Annual _____ Final x

G. Ben-Hayyim
Signature
Principal Investigator

Y. Kat
Signature
Institution's Authorizing Official

630.72
BAR/BEN
2nd copy

: 633.16 : 634.3 : 581.526.5 : 581.875 :
581.874 : 581.192.53

Table of Contents

	Page
Abstract.....	1
Citrus cells	
Objective.....	3
Summary.....	3
Fractionation and Characterization of membranes.....	4
Kinetic properties of plasma membrane and tonoplast	
ATPase.....	7
Proton transport in citrus cells.....	10
Literature cited.....	13
Tables.....	15
Figures.....	19
Barley roots	
Objectives.....	31
Summary.....	31
Multiple forms of proton translocating ATPase	
activities in plasma membrane fraction from	
barley roots.....	33
Published articles	

Abstract

Barley roots and citrus cells were the two experimental systems employed in this project. Well defined membrane preparations of barley roots and the existence of Na^+/H^+ antiporter in barley root tonoplast vesicles were the basis for the present study. Isolation of citrus plasma membrane and tonoplast enriched fractions basically followed the procedure developed for barley.

Plasma membrane ATPase of barley roots and citrus cells had the same V_{max} , K_m and pH optimum when grown in the presence or absence of NaCl. This is also true for ATP-dependent proton transport and membrane potential of barley roots. No proton transport activity could be detected with citrus plasma membrane vesicle at room temperature. Kinetic studies revealed salt-induced cooperativity of citrus plasma membrane ATPase.

Under conditions of excess Mg^{2+} , or ATP, cooperativity was no longer observed, but the K_m was significantly increased. Barley plasma membrane vesicles contained one or more protein kinases which were isolated along with the membranes and phosphorylated many plasma membrane polypeptides in vitro.

ATPase of citrus tonoplast vesicles was not affected by the presence of salt during growth. Tonoplast vesicles had both ATP and PPi -dependent proton transport activities. pH gradient was not collapsed by 100 mM NaCl unless EDTA was introduced together with the

salt. This result is the only indication that a Na^+/H^+ antiporter may be active in the tonoplast. In contrast, Na^+/H^+ antiporter activity was well established in barley root tonoplast as well as Ca^+/H^+ antiporter activity. Although several tonoplast polypeptides have been phosphorylated both in vivo and in vitro, no change in the pattern of phosphorylation was observed under condntion of Na^+/H^+ antiporter induction. Na^+/H^+ antiporter is distinct from Ca^+/H^+ antiporter in the magnitude of the gradient that was developed (2 to 5 fold for Na^+ and 1000 fold for Ca^+) and in its response to salt. Control and NaCl-grown roots had equal amount of Ca^+/H^+ antiporter while control roots had no Na^+/H^+ antiporter activity.

CITRUS CELLS

Objectives of the original research proposal

The objectives of the Israeli part of the proposal were to prepare purified fractions of plasma membranes and tonoplast from citrus salt-sensitive and salt-tolerant cells and study ATPase activities and properties of ion transport.

ATPase activities of plasma membrane and tonoplast were studied in salt-sensitive and salt-tolerant cell lines and the effect of NaCl both in vivo and in vitro were examined.

Proton transport, as measured by the fluorescence quench technique, was studied in salt-tolerant tonoplast membranes only, as no activity was found in plasma membrane. The collapse of the proton gradient formed by NaCl was used as an indicator for Na^+/H^+ antiporter activity.

Summary

The energy for transport of ions and solutes into the cytoplasm from the external medium or from storage inside vacuoles is derived from plasma membrane and tonoplast ATPases respectively. Enriched fractions of plasma membrane and tonoplast were characterized by the differential effect of specific inhibitors of the two ATPases, substrate specificity, and by immunoblots of membrane protein extracts with antibodies specific for either of the ATPases.

ATPases activities of enriched fractions of both plasma membrane and tonoplast were not affected by growing cells of either

salt-sensitive or salt tolerant cells in the presence of NaCl. Activities of both enzymes were inhibited by NaCl and other salts, such as KCl and choline chloride in vitro, and the effect on Vmax was more pronounced with tonoplast ATPase, measured at pH 7.5, than plasma membrane ATPase, measured at pH 6.3. In contrast, the apparent Km for ATPase was not affected by salt for the tonoplast enzyme, but was significantly increased for the plasma membrane one. Kinetic evidence showed that NaCl induced cooperativity in the plasma membrane ATPase and the data suggest the existence of more than one binding site for the substrate.

The sites interact in the presence of salt and act independently in its absence. In the presence of excess Mg^{2+} or ATP salt-induced cooperativity was no longer observed, but a significant increase in the apparent Km was obtained when compared to conditions of the assay consisted for Mg to ATP ratio of 1:1 ATP and pyrophosphate dependent proton transport was observed in tonoplast. The proton gradient was not inhibited by vanadate, was not formed in the presence of nitrate and completely collapsed by NH_4Cl . NaCl did not collapse the gradient, but in a combination with EDTA collapsed the gradient more than EDTA alone.

Fractionation and characterization of Membranes

Microsomal pellet was resuspended in buffer containing 0.25M sucrose, 1 mM DTT and 5 mM PIPES-KOH pH 7.0 and loaded on a 4 steps sucrose gradient consisted of 12, 19, 33 and 42% (w/w) for 2 1/2 h. The interfaces of 12/19 and 33/42 were collected and analyzed. For more details, see Ben-Hayyim and Ran (1990).

The protein recovery in the tonoplast fraction was very low, 0.2 to 2% of the total microsomal protein that was loaded on the sucrose gradient. Higher amounts of protein were recovered from salt grown salt-tolerant cells, 5 to 9%. This is in agreement with electron microscopic studies showing very few and small vacuoles in cells grown without salt and a dramatic increase in the vacuole size in salt-grown salt-tolerant cells (Ben-Hayyim and Kochba 1983). The yields of protein in plasma membrane fractions were similar in all types of cells analysed, and were in the range of 7 to 16% of the total microsomal protein.

As seen in Figure 1, the two fractions were identified by inhibitor studies of the ATPase activity as enriched fractions of plasma membrane (A) and tonoplast (B). The pH profiles of the ATPase activities in the two fractions are presented in Figure 2 and 3. An optimum pH of 6.3 was observed for the ATPase activity in enriched plasma membrane fraction, which was 75% inhibited by vanadate and very slightly inhibited by nitrate, especially at low pH. The pH optimum of the tonoplast enriched fraction was around 7.0, most likely because of contamination by other membrane fractions. The inhibition of vanadate at low pH indicates either the presence of plasma membrane ATPase, or a genuine inhibition of tonoplast ATPase as was suggested earlier (Henry and Pilet, 1988).

Both fractions show a relatively high optimal temperature for ATPase activity (Fig. 4). The activity is linear with time for about 40 min (Fig. 5) and thus, all the following experiments were carried for 30 min, at 45°C and at pH 6.3 and 7.5 for plasma membrane and tonoplast enriched fractions, respectively.

In a further attempt to characterize these fractions, the substrate specificity of the ATPase was determined. As senn in Table 1, ATPase of the tonoplast enriched fraction was rather active with GTP and the membrane fraction had the characteristic pyrophosphatase activity. On the other hand GTPase and pyrophosphatase activities were very low in plasma membrane enriched fractions, similarly to observations made earlier in membranes of other plant sources (Sze 1985). Plasma membrane and tonoplast ATPase are consisted of different polypeptides. The former is consisted of one polypeptide having a molecular weight of 100 kD and the latter is consisted of many subunits, of which the more investigated are three subunits of about 70, 60 and 16 Kd. We have used specific antibodies raised against the Arabidopsis plasma membrane ATPase (kindly donated to us by Dr. R. Serrano) and the tonoplast ATPase holoenzyme of barley (kindly donated by Dr. F.M. duPont) to identify the presence of the enzymes in each of the membranes. As seen in Figure 6, the antibodies raised against the plasma membrane ATPase cross reacted with the plasma membrane fraction only, indicating a single band at the expected MW of about 100 kD. The antibodies against the tonoplast ATPase cross reacted with tonoplast fraction only, indicating a single band of a 68-70 kD polypeptide. It is evident that Citrus ATPases are immunological cross reactive with ATPases of other plant sources and that the two types of ATPases are separated in our fractions.

All the ATPase properties mentioned above were similar in salt-sensitive and salt-tolerant cells whether grown in the absence or presence of NaCl. The requirement for a relative high temperature to obtain good rates of ATPase activities in both fractions led us to

measure the Arrhenius energy of activation for the two enzymes. The estimated value for plasma membrane ATPase was 15.8 ± 2.7 kilocalories per mole of ATP hydrolysis and about 13.0 ± 1.9 for the tonoplast ATPase. We have not observed a significant difference between the two types of membranes. The value for the citrus plasma membrane ATPase is somewhat lower than the one observed in barley membrane but the value for the tonoplast ATPase is higher (DuPont 1989). Similar values were obtained using membranes derived from salt-sensitive and salt-tolerant cells grown in the presence and absence of salt.

Kinetic properties of plasma membrane and tonoplast ATPases

1. Plasma membrane ATPase

The enclosed article presents data concerning the kinetics of citrus plasma membrane ATPase as studied in the absence and presence of salt in vitro. In an attempt to determine whether results would change when activity was calculated vs. the substrate MgATP, rather than ATP, all the data were recalculated and Lineweaver-Burke plots of the dependence of ATPase activity on MgATP are presented. As seen in Figure 7, the recalculations did not change basically the data presented in the attached paper, namely the addition of NaCl to a plasma membrane enriched fraction at pH 6.3 resulted in an unreal value of V_{max} and extremely high value of apparent K_m for the substrate of ATPase. n values calculated from Hill analysis calculated for the substrate mgATP were somewhat lower than when calculated for ATP and were in the range of 1.4 to 1.45. It is interesting to note that when plasma membrane ATPase activity was measured at pH 7.5, the cooperativity induced by NaCl was no longer

observed (Fig. 7). The results presented in Table 2 show that the addition of NaCl increased the apparent K_m for the substrate at pH 6.3, but had no effect, or even slightly decreased the apparent K_m at pH 7.5.

The effect of NaCl on plasma membrane ATPase was concentration dependent. No effect on K_m and V_{max} and no induction of cooperativity was observed at 0.1 M NaCl (Fig. 8). Apparent K_m was increased at 0.2 M NaCl and even more so at 0.3 M NaCl and calculated n values by Hill analysis were 1.45 and 1.51, respectively.

The data presented in Figure 9 show that in the presence of excess Mg^{2+} , the NaCl-induced cooperativity was not observed. Lineweaver-Burke plots showed no effect of NaCl on V_{max} and the effect on K_m was variable. In about half of the experiments K_m was increased and in other cases it was hardly changed. As seen in Figure 10, in the presence of excess ATP no NaCl-induced cooperativity was observed. The addition of NaCl had no effect on V_{max} , but in contrast to excess Mg^{2+} a consistent increase in the apparent K_m value for MgATP was observed. Thus, the apparent K_m values in the absence and presence of NaCl were 0.83 mM and 1.74 mM, respectively. The data summarized in Table 3 point to the fact that the k_m value for MgATP is markedly lower than the value calculated for ATP when measured at equal Mg^{2+} and ATP concentrations. However, excess of Mg^{2+} or ATP increased the apparent K_m for MgATP and remained similar when calculated for ATP. Thus, the lowest K_m value was observed when Mg^{2+} and ATP were introduced at equal concentrations and the substrate was assumed to be MgATP rather than ATP. If this is indeed the case then it can be concluded that at free Mg^{2+} ions have an effect on the conformation of

the active site of the enzyme so they eventually interfere with the binding of ATP. Moreover, since free Mg^{2+} ions eliminate the Na^{+} ions induced cooperativity, it can be speculated that both of them interact with the enzyme at the same site.

NaCl-induced cooperativity was also abolished at pH 7.5, (Fig. 7) suggesting that either protons have a role in the interaction of Na^{+} with the plasma membrane ATPase, or else, an activity which may be attributed to another contaminating ATPase is determined at this pH. The second possibility is much more likely to be the case, since unlike the situation with excess Mg^{2+} or ATP, V_{max} was very much reduced while apparent K_m for MgATP was unchanged, or even decreased (Table 2).

We were interested to determine the effects of salt both in vivo and in vitro on plasma membrane and tonoplast ATPases. Thus, the isolation of both membranes was carried out by a sucrose gradient. The unexpected results of NaCl-induced cooperativity of plasma membrane ATPase led us to isolate this membrane by the two phase separation method (Palmgren et al 1990), which is supposed to yield the best purified preparation. We collected the upper phase which contains mainly plasma membrane at the right-side-out orientation, washed it twice and kept it frozen at $-80^{\circ}C$. Freezing and thawing of the membranes resulted mainly in inside-out vesicles. ATPase activity was only slightly stimulated by Triton X-100, similar to what was observed with the gradient isolated enriched fraction (Fig. 11). Most of the two-phase separated membrane fractions had higher activities than the gradient separated fractions, (40 to 80 $\mu mol. Pi mg protein^{-1}. h^{-1}$). The two-phase separated plasma membrane ATPase was slightly more

sensitive to vanadate than the sucrose gradient isolated fraction, the I_{50} being 10 and 30 μM , respectively (Fig. 12). Salt-induced cooperativity in ATPase activity was observed in the two-phase isolated membrane fractions and the n values of the Hill analysis were similar to those observed in ATPase of sucrose gradient isolated fractions. It should be noted plasma membrane vesicles isolated from melon roots by the two-phase separation technique did not show the salt-induced cooperativity, when measured under identical conditions.

2. Tonoplast ATPase

The kinetic parameters of tonoplast ATPase are summarized in Figure 7 and Table 2. Apparent K_m values for MgATP of tonoplast ATPase were lower than those observed for plasma membrane ATPase. At pH 6.3 and 7.5 V_{max} was decreased by NaCl and the apparent K_m for MgATP was either unchanged (7.5), or decreased (6.3). NaCl did not induce cooperativity in ATPase activity of tonoplast even at pH 6.3. Excess of Mg^{2+} ions had no effect on the V_{max} of the enzyme, and markedly increased the K_m , when measured at the characteristic optimal pH of 7.5. As seen in Table 4, the increase in the apparent K_m was observed in the presence and absence of 0.2 M NaCl, and whether calculated for ATP, or MgATP, as substrates.

Proton transport in Citrus cells

The microsomal fraction of Citrus cells is capable of proton transport. ATP-dependent proton transport was measured by fluorescence quench of acridine orange (Blumwald and Poole, 1985). All experiments were performed with fractions derived from

salt-tolerant cells grown in the presence of 0.2 M NaCl, conditions that seemed best suited for detection of Na^+/H^+ exchange activity, if existed. As seen in Figure 13, ATP-dependent fluorescence quench of acridin orange was observed, which was fully inhibited by nitrate but not affected by vanadate. Fluorescence quench was fully reversible upon addition of NH_4Cl suggesting that H^+ transport activity was observed. It can be concluded that under the conditions chosen for the experiment most if not all, of the H^+ transport activity can be related to vacuolar type membranes such as tonoplast, rather than to plasma membrane. Results presented in Figure 14 indicate that higher rates of proton uptake were observed at pH 7.5 or 8.0 than at pH 6.3, while the steady state proton gradient was higher at pH 6.3. This phenomenon probably results from two reactions occurring at opposite directions, i.e proton uptake having a pH optimum of about 7.5, which is characteristic for tonoplast vesicles, and a secondary reaction causing the collapse of the gradient whose rate is increase with pH. Proton uptake is not dependent on the presence of a cation, but its rate is increased upon addition of either KCl or NaCl (Fig. 15). Tonoplast vesicles exhibit an additional H^+ pump activity which is PPi-dependent (Rea and Sanders 1987). As seen in Figure 16, citrus microsomal pellet exhibit this activity, with initial rates of proton transport very similar to those observed with ATP. The reason for the fast collapse of the gradient is not clear.

Acridine orange fluorescence quench experiments were performed with purified plasma membranes and tonoplast vesicles. No activity was observed with plasma membrane fractions at room temperature, while proton transport activities were observed with tonoplast fractions.

The very small fraction of tonoplast that could be obtained from citrus cells limited our experiments a great deal. ATP-dependent proton gradient was slightly higher with K^+ than choline, although initial rates were similar (Fig. 17). High concentrations of choline chloride or NaCl decreased the steady state proton gradient, but high concentration of NaCl increased the initial rate of proton transport (Fig. 18). KCl had similar effect as NaCl (data not shown). The addition of high concentrations of NaCl had no effect on the proton gradient after it was formed, irrespective whether ATP, or PPi, energized the vesicles (Fig. 19). However, the addition of NaCl together with EDTA seemed to accelerate the collapse of the proton gradient (Fig. 20). EDTA was used at a concentration which almost eliminated the proton gradient due to chelation of Mg^{2+} (Fig. 15). This is the only data that might give an indication for Na^+/H^+ exchange activity in tonoplast of citrus cells. It should be noted that such activity could not be detected while the membrane was in energized state (no EDTA), and that the effect of NaCl in the presence of EDTA was rather small. No effect was detected with low NaCl concentrations (10 to 50 mM) and LiCl did not compete with NaCl (data now shown). More experiments are needed to evaluate these results in relation to its magnitude and specificity to Na^+ .

Literature Cited

Ben-Hayyim G. and Kochba J. 1983 Aspects of salt tolerance in NaCl-selected stable cell line of Citrus sinensis. Plant Physiol. 72: 685-690.

Ben-Hayyim G. and Ran U. 1990 Salt-induced cooperativity in ATPase activity of plasma membrane-enriched fractions from cultured Citrus cells: kinetic evidence. Physiol. Plant 80: 210-216.

Blumwald E. and Poole R.J. 1985 Nitrate storage and retrieval in Beta vulgaris: effect of nitrate and chloride on proton gradients in tonoplast vesicles. Proc. Natl. Acad. Sci. USA 82: 3683-3687.

Bush D.S., Sticher L., Van Huystee R., Wagner D. and Jones R.L. 1989 The calcium requirement for stability and enzymatic activity of the two isoforms of barley aleurone α -amylase. J. Biol. Chem. 264: 19392-19398.

DuPont F.M. 1989 Effect of temperature on the plasma membrane and tonoplast ATPase of barley roots. Plant Physiol. 89: 1401-1412.

Henry H. and Pilet P-E. 1988 Inhibition by vanadate of the H^+ translocating ATPase of Rubus cells. Plant sci. 56: 149-154.

Laemmli U.K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Palmgren MG., Askerland P., Fredrikson K., Widell S., Sommarin M. and Larsson C. 1990 Sealed inside-out and right-side-out plasma membrane vesicles. Plant Physiol. 92: 871-880.

Rea P.A. and Sanders D. 1987 Tonoplast energization: Two H^+ pumps, one membrane. Physiol. Plant 71: 131-141.

Sze H. 1985 H^+ translocating ATPases: Advances using membrane vesicles. Ann. Rev. Plant Physiol. 36: 175-208.

Towbin H., Staehelin T. and Gordon J. 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

Table 1. Substrate specificity for ATPase activities in plasma membrane and tonoplast enriched fractions.

ATPase activity was determined as described in Fig. 1. All substrates were introduced at a concentration of 3 mM. ATPases of plasma membrane and tonoplast were measured at pH 6.3 and 7.5, respectively.

Substrate	ATPase Activity	
	Plasma membrane	tonoplast
	$\mu\text{mol Pi.mg protein}^{-1}.\text{h}^{-1}$	
ATP	21.8	16.5
GTP	3.4	11.1
UTP	2.9	7.2
PPi	2.8	16.6
ADP	3.9	0.6
p-nitrophenyl phosphate	0.5	0.9

Table 2. The effect of pH and NaCl on the kinetic parameters of plasma membrane and tonoplast ATPases. ATPase activity was measured as described in Fig. 1. Vmax and Km were calculated from the data presented in Fig. 7.

membrane	pH	Km		Vmax	
		mM		$\mu\text{mol Pi.mg protein}^{-1}.\text{h}^{-1}$	
		-NaCl	+NaCl	-NaCl	+NaCl
plasma membrane	6.3	0.49	0.89 ^a	41.7	41.7 ^b
plasma membrane	7.5	0.42	0.30	11.8	5.9
tonoplast	6.3	0.20	0.13	11.8	7.4
tonoplast	7.5	0.26	0.29	13.2	9.1

^a calculated by Hill analysis

^b assumed value

Table 3. Apparent K_m values for ATP and MgATP for plasma membrane ATPase in various combinations of $MgSO_4$ and ATP concentrations. All details as described in Fig. 1. Variable concentrations were in the range of 0.2 to 3.0 mM. Data calculated from Figs. 9 and 10.

ATP	Mg^{2+}	K_m	
		MgATP	ATP
		mM	
variable	variable ^a	0.4	0.85
variable	3 mM	0.73	0.76
3 mM	variable	0.83	0.9

^a varied 1:1 with ATP

Table 4. Apparent K_m values for ATP and MgATP for tonoplast ATPase determined in equal Mg^{2+} and ATP concentrations and in excess of Mg^{2+} ions. Variable concentrations of ATP and $MgSO_4$ were in the range of 0.2 to 3.0 mM and the experiment was carried out at pH 7.5

ATP	Mg ²⁺	Km			
		MgATP		ATP	
		-NaCl	+NaCl	-NaCl	+NaCl
		mM			
variable	variable ^a	0.23	0.18	0.35	0.34
variable	3 mM	0.77	0.78	0.72	0.78

^a varied 1:1 with ATP

Figure 1. Characterization of ATPases in plasma membrane and tonoplast enriched fractions isolated on a sucrose gradient. (A) plasma membrane (B) tonoplast. ATPase activity of plasma membrane and tonoplast were measured at pH 6.3 and 7.5 respectively. Control activities of (A) and (B) were 25 and 15 $\mu\text{mol Pi. mg protein}^{-1} \cdot \text{h}^{-1}$. Routine reaction mixture contained 30 mM buffer, 50 mM KCl or KNO_3 where indicated, 0.12 mM ammonium molybdate, 3 mM MgSO_4 , 3mM ATP, 200 μM sodium vanadate where indicated, and 8 to 20 μg protein of membrane vesicles in total volume of 150 μl . All other details are given in Ben-Hayyim and Ran (1990). (a) control, no addition; (b) 50 mM nitrate replaced KCl; (c) 5 μM diethyl stilbestrol, (d) 100 μM vanadate; (e) 1 mM azide; (f) 3 mM Ca^{2+} replaced Mg^{2+} .

Figure 2. Effect of pH on ATPase activity in plasma membrane enriched fraction. Tris-MES buffer was used for the range of 5.0 to 6.3; Tris-HEPES buffer for the range of 6.8 to 7.5 and tris-Iricine for the of range 8.0 and above. All other details as described in Fig. 1.

Figure 3. Effect of pH on ATPase activity in tonoplast enriched fraction. Details as described in Figs. 2.

Figure 4. Time course of ATPase activity of plasma membrane and tonoplast enriched fractions. Details as described in Fig. 1.

Figure 5. Effect of temperature on ATPase activity of plasma membrane and tonoplast enriched fractions. Details as described in Fig. 1 except Tris-MES buffer at pH 6.3 and Tris-HEPES buffer at pH 7.5 were employed for plasma membrane and tonoplast, respectively. (—) plasma membrane; (--) tonoplast.

Figure 6. Western blot analysis of ATPases in plasma membrane and tonoplast enriched fractions. Membrane proteins were solubilized in buffer containing 62.5 mM Tris HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM p-chloro mercurybenzoate and 10 μ M leupeptin. Modified Laemmli (1970) procedure was used for 1-D electrophoresis. Separation of proteins was performed on a 10% polyacrylamide mini gel. Western blots were performed following the procedure of Toblin et al (1970), and proteins were detected by horseradish peroxidase conjugated rabbit anti goat IgG. Other details are given in the text. P- plasma membrane; T- tonoplast. (a) immunoblot with anti plasma membrane ATPase. (b) immunoblot with anti tonoplast ATPase.

Figure 7. Lineweaver-Burke plots of the dependence of ATPase activity on MgATP in plasma membrane and tonoplast enriched fractions. ATPase activities were measured in the presence of 50 mM KCl as control medium (●), or with the addition of 0.2 M NaCl on top of KCl (○). All other details are described in Fig. 1. MgSO_4 and ATP concentrations were varied in 1:1 ratio and MgATP concentrations at the two pH values and in the absence and presence of NaCl were calculated according to a computer program devised by Bush et al (1989).

Figure 8. Effect of NaCl concentration on ATPase activity in plasma membrane enriched fraction. Details as described in Figs. 1 and 7(●). Control with 50 mM KCl; (○) addition of 0.1 M NaCl; (▲) 0.2 M NaCl; (△) 0.3 M NaCl.

Figure 9. Lineweaver-Burke plots of the dependence of ATPase₂ activity on MgATP in plasma membrane enriched fraction with excess Mg^{2+} and in the presence and absence of NaCl. Conditions as described in Fig. 7 except that 3 mM MgSO_4 were added for each ATP concentration. (▲) control; (Δ) 0.2 M NaCl; (●) MgSO_4 and ATP concentrations were varied in 1:1 ratio.

Figure 10. Lineweaver-Burke plots of the dependence of ATPase activity on MgATP in plasma membrane enriched fraction with excess ATP and in the presence and absence of NaCl. Conditions as described in Fig. 7 except that 3 mM ATP were added for each MgSO_4 concentration. (▲) control; (Δ) 0.2 M NaCl.

Figure 11. Effect of Triton X-100 on ATPase activity in plasma membrane enriched fraction. Conditions as described in Fig. 1.

Figure 12. Effect of vanadate on ATPase activity in plasma membrane fractions obtained by separation on sucrose gradient and with the two-phase separation method. Conditions as described in Fig. 1. 100% activity for the preparation purified on sucrose gradient and the preparation purified by the two-phase technique were 50.5 and 33.6 $\mu\text{mol Pi}\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$, respectively.

Figure 13. Effect of vanadate and nitrate on ATP-dependent H^+ pump of the microsomal fraction. 121 μ g protein of membrane vesicles were incubated in 0.6 ml of 15 mM Bis Tris Propane buffer pH 7.5, 50 mM KCl, 250 mM sucrose, 2 mM $MgSO_4$, 1 mM EGTA and 2 μ M acridine orange. Decrease of fluorescence was measured at room temperature with a Shimadzu spectrofluorimeter model RF-540 at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit-width of 5 nm for both. The concentrations of Tris-ATP, vanadate and NH_4Cl were 2, 0.2 and 10 mM, respectively. Where indicated, 50 mM KNO_3 replaced KCl.

Figure 14. Effect of pH on ATP-dependent H^+ pump of the microsomal fraction. Conditions as described in Fig. 13, except that Tris-MES and Tris-HEPES were used as buffers for pH 6.3 and 8.0, respectively.

Figure 15. Effect of cations and EDTA on ATP-dependent H^+ pump of the microsomal fraction. Fluorescence quench was measured in the absence of KCl or in the presence of 50 mM KCl, or NaCl. 4 mM EDTA were added to the control containing 50 mM KCl. All other details as described in Fig. 13.

Figure 16. PPI-dependent H^+ pump of the microsomal fraction. Conditions as described in Fig. 13, except that 100 μ M PPI replaced ATP. Decrease of fluorescence was measured with 50 mM KCl (control) and 200 mM KCl (KCl).

Figure 17. ATP-dependent H^+ pump of tonoplast. Conditions as described in Fig. 13, except that 28.4 μ g protein of tonoplast vesicles and 50 mM of either KCl, or choline-Cl were employed.

Figure 18. Effect of high salt concentration on ATP-dependent H^+ pump of tonoplast. Conditions as described in Fig. 13, except that 23 μ g protein of membrane vesicles were employed. 100 mM of choline-Cl, or NaCl were added to the control containing 50 mM KCl.

Figure 19. Effect of NaCl on ATP and PPi-dependent H⁺ pump of tonoplast. Conditions as described in Fig. 13, except that 31 μ g protein of membrane vesicles and either 2 mM ATP (a) or 100 μ M PPi (b) were employed. 100 mM NaCl was introduced 3.5 min after energization of the membrane.

Figure 20. Effect of NaCl and EDTA on ATP-dependent H⁺ pump of tonoplast. Conditions as described in Fig. 13, except that 34.5 μ g protein of membrane vesicles were employed and 50 mM choline-Cl replaced KCl. The additions were made 4 min after energization. The concentrations of NaCl and EDTA were 100 and 4 mM, respectively.

BARD Final Report: F. M. DuPont

The objectives of the proposal were to characterize the plasma membrane and tonoplast ATPases, examine the effects of *in vitro* and *in vivo* salt treatments on the ATPases and seek for regulation of ATPase activity in response to salt. In addition, the transport properties of the tonoplast vesicles were to be characterized, with an emphasis on elucidation of the mechanisms by which the vacuole sequesters Na^+ and Cl^- .

BARLEY ROOTS

Barley roots were chosen as one experimental system, in order to examine the transport properties of membranes from organized tissues, utilizing one of the most salt tolerant crop plants.

In vitro transport activities of the plasma membrane ATPase. The plasma membrane ATPase was characterized in detail and the ATPase from control and salt-grown plants was compared. ATP hydrolysis, formation of a membrane potential and formation of a pH gradient were compared, using sealed, inside-out vesicles. Growth in 100 mM NaCl did not appear to alter K_m , V_{max} , or pH optimum for any of the parameters measured. However, the data suggest that there was more than one form of ATPase in the plasma membrane preparation. No Na^+/H^+ antiporter activity was detected in the plasma membrane fraction.

The role of protein kinases in the activation of the Na^+/H^+ antiporter. A Na^+/H^+ antiporter in the tonoplast vesicles was activated by exposing roots to salt for 10 to 30 min. Protein synthesis was not required. The role of protein kinases in the activation of the antiporter was explored, and this led to a detailed study of *in vivo* and *in vitro* protein kinase activity in the tonoplast and plasma membrane from barley roots. Phosphorylation of several tonoplast polypeptides was observed when roots were exposed to $^{32}\text{P}_i$ for *in vivo* phosphorylation. In addition, *in vivo* phosphorylation of several low mol wt polypeptides in a supernatant fraction was observed after a short exposure to 150 mM NaCl. This may be a salt-shock response. No increase or decrease in phosphorylation was observed in response to the 100 mM NaCl treatment that activated the Na^+/H^+ antiporter, however. Many polypeptides in the plasma membrane fraction were phosphorylated *in vitro*, indicating that one or more protein kinases were isolated along with the plasma membrane. These include one or more calcium-dependent protein kinases that may alter membrane transport properties during ATPase assays.

Antiport activity in the tonoplast vesicles: detailed characterization of a Ca^+/H^+ antiporter. For contrast and comparison with the Na^+/H^+ antiporter, a Ca^+/H^+ antiporter in the tonoplast was characterized. Vesicle volumes were determined using an electron spin resonance technique, and the intravesicular concentrations of Ca^+ were determined. The K_m for Ca^+ uptake was very low (2 μM), in contrast to a K_m for the Na^+/H^+ antiporter of 9 mM. The Ca^+/H^+ antiporter developed 1000 fold

concentration gradients, rapidly accumulating Ca^{+} to a concentration of 1 mM or greater within the vesicles. In contrast, Na^{+} gradients of only 2 to 5 fold have been measured so far for the barley membranes. The $\text{Ca}^{+}/\text{H}^{+}$ antiporter was detected in equal amounts in membranes from control and salt-grown roots, whereas the $\text{Na}^{+}/\text{H}^{+}$ antiporter was not detected in control roots. The $\text{Ca}^{+}/\text{H}^{+}$ antiporter and the $\text{Na}^{+}/\text{H}^{+}$ antiporter are distinct, independently regulated systems. The ability of the vacuole to rapidly take up large quantities of Ca^{+} from the cytoplasm may play an important role in conditions of salt stress.

TITLE: Multiple forms of proton translocating ATPase activities in a plasma membrane fraction from barley roots.

Frances M. DuPont, USDA Agricultural Research Service, Western Regional Research Center, Albany, Ca 94710

Gozal Ben Hayyim, Institute of Horticulture, ARO, the Volcani Center, Bet Dagan 50250, Israel

Abstract: Plasma membranes were isolated from barley (*H. vulgare* cv CM72) roots using sucrose density gradients or phase partitioning. On sucrose gradients the plasma membrane formed a single, well defined peak of proton translocating ATPase which was not inhibited by nitrate and had optimal activity at 38° C. However, the peak consisted of a vanadate-inhibited and vanadate-insensitive component. The ATPase was characterized in terms of ATP hydrolysis and for proton transport using acridine orange and oxonol V. Similar but not identical results were obtained for the three measures of enzyme activity. Quench of oxonol fluorescence had a sharp pH optimum between pH 6.5 and 7.0, and a K_m for ATP:Mg of approximately 0.25 mM. Quench of acridine orange fluorescence had a sharp pH optimum between pH 7 to 7.5, had non-linear kinetics for ATP:Mg if purified from a sucrose gradient, and a K_m of approximately 0.9 mM if purified by phase partitioning. The K_m for ATP hydrolysis was variable, depending on the concentrations of ions used in the assay medium, and ranged from 0.25 to 1 mM. ATP hydrolysis and formation of a membrane potential were inhibited 60% by 100 μ M vanadate but proton transport was inhibited only 12%. All three parameters were inhibited 60% above 250 μ M vanadate. It is suggested that the barley plasma membrane fractions that were obtained from sucrose gradients or by phase partitioning had multiple forms of proton translocating ATPase activity with differential sensitivity to vanadate. No significant differences in membranes from control and salt grown roots was observed.

INTRODUCTION

The plasma membrane H^+ ATPase creates the large electrochemical potential for protons that is the principal driving force for solute transport across the plasma membrane of plant cells (31). Plants have multiple genes for the plasma membrane ATPase and differences in tissue specificity have been reported (13,16) but it is not known whether the ATPase isozymes have different characteristics in terms of activity. There is general agreement about many of the characteristics of the plasma membrane ATPase, however. The ATPase is usually reported to have a narrow pH optimum with a peak between pH 6.5 and 7.0, inhibition by vanadate, and insensitivity to anions (2-4,15,19,24,29,31). There is considerable variability in reports of other characteristics, such as the percent stimulation by K^+ (2-4,24,29,31) or the K_i for inhibition by vanadate (3,4,19,20,28,30,31). Differential activation of the ATPase by phospholipids and proteolysis have been reported (20,25,26,27). Because plants have many ATP hydrolyzing activities that can be confused with the activity of the plasma membrane ATPase, there has been an emphasis on defining characteristics that can be used to identify the plasma membrane ATPase. However, differences in the ATPase from different plants and tissues may be of significance with respect to differential expression and regulation of ATPase isozymes.

The plasma membrane fraction obtained from barley roots contains a proton translocating ATPase of high specific activity (10,11). In this paper transport and ATP hydrolysis by the ATPase are characterized in detail. Significant differences in the effects of vanadate on ATP hydrolysis, formation of a membrane potential and formation of a pH gradient were observed. The ATPase from control and salt-grown roots was compared, as a part of the search for key elements in membrane transport that are regulated in response to salt. No differences related to the salt treatment were detected.

METHODS

Plant Material Barley (*Hordeum vulgare* cv CM72) seeds were germinated and grown on cheesecloth laid over stainless steel mesh supports suspended over plastic trays that contained 7 liters of aerated nutrient medium (12), plus 100 mM NaCl for salt-grown roots. Trays were kept in the dark at 22° C. The pH of the nutrient medium was tested daily after the seeds germinated, and adjusted to pH 5.6 with Ca(OH)₂. After 7 days 70 to 100 gram fresh weight of roots were obtained per tray, from 80 g fresh weight of dry seeds.

Membrane Preparation Roots were excised, briefly washed in cold distilled water and ground in a mortar and pestle. The homogenization buffer consisted of 40 mM Tris, 8 mM EDTA, 0.25 M sucrose, 0.2 or 5 mM phenyl methyl sulphonyl flouride (PMSF) and 4 mM EDTA. Roots were ground in 80 ml of homogenization buffer, the homogenate was poured off and filtered through cheesecloth, fresh buffer was added to the roots, and the procedure was repeated four times. The final volume of homogenate was adjusted to 400 ml. Differential and gradient centrifugation were as described previously (11). It was demonstrated that the distribution of marker enzymes for ER, tonoplast and plasma membrane on continuous sucrose gradients was similar for membranes from control and salt-grown roots, as was the protein composition of the membrane fractions from step gradients (18). To prepare the plasma membrane enriched fraction, the interface between the 34% and 40% (w/w) sucrose layers (approx 5 ml) was collected with a syringe, added to 60 ml of suspension buffer consisting of 0.25 M sorbitol in 5 mM Tris-Mes, pH 7.2 and centrifuged in a Beckman 45Ti rotor for 35 min at 35,000 rpm. An alternative procedure was to wash the membranes in 0.25 M sucrose, 150 mM KCl and 5 mM Pipes KOH, pH 7.2. The pellet was resuspended in approximately 1 ml of suspension buffer and frozen at -70° C until use. Sucrose was substituted for sorbitol in the final suspension buffer if the membranes were used for ATPase assays.

Phase Partitioning Plasma membranes were prepared by phase partitioning following the procedure developed by Korner *et al.* for barley roots (21).

Optical Assays of Proton Transport

Formation of a pH gradient was measured by following quench of fluorescence or absorbance of acridine orange or quinacrine. Vesicles were added to 3.0 ml of fluorescence assay buffer in plastic, disposable fluorescence cuvettes maintained at 36° C in a water jacketed cuvette holder in a Farrand System 3 spectrofluorimeter. The standard fluorescence assay buffer

consisted of 2 μ M acridine orange, 0.25 M sorbitol or sucrose, 50 mM Choline Cl, 50 mM KNO_3 , 1 μ M valinomycin and 3 mM Tris ATP in 10 mM Pipes Tris, pH 6.8. Additions and alterations are indicated in text or figure legends. Transport was initiated by addition of 4 mM MgCl_2 . Fluorescence was measured with an excitation wavelength of 495 nm and an emission wavelength of 525 nm for acridine orange. Relative rates of proton transport were estimated by measuring the initial rate of quench of fluorescence, %Q/min, upon addition of ATP.

Formation of a membrane potential was measured by following quench of oxonol V fluorescence. Membranes were added to 3.0 ml of assay buffer consisting of 0.25 M sorbitol, 25mM K_2SO_4 , 4 mM MgSO_4 , 2 μ M oxonol V and 25 mM BTP-MES pH 6.75, at 28°, C. Excitation was measured at 580 nm and emission at 630 nm. Transport was initiated by addition of indicated amount of Tris-ATP, pH 6.8. The fluorescence intensity of Oxonol V was low, compared to acridine orange and the noise level of the recordings was high. The initial fluorescence, prior to addition of ATP, increased with the amount of protein added, since the fluorescence originated from oxonol bound to the membranes. However, the rate of quench of Oxonol V was independent of protein concentration and the sensitivity of the assay was not increased by using larger amounts of protein in assay.

ATPase Assays ATP hydrolysis was measured by release of Pi, using the same assay solutions as were used for proton transport, minus the fluorescent dye. Sucrose was used as osmoticum; sorbitol could not be used because it interferes with determination of Pi. ATPase was assayed for 15 or 20 min at 25° C, and Pi was determined by a modification of the method of Lebel et. al (22).

Calculation of ATP:Mg Concentrations: Concentrations of ATP:Mg were calculated from the concentrations of added ATP, Mg, salts, pH and temperature, using stability constants for the components such as HATP, MgHATP , KATP, etc (23, 32) and a computer program that was a gift from Douglas Bush (6).

RESULTS AND DISCUSSION

Effect of temperature on proton transport. Two peaks of proton-translocating ATPase were detected when microsomal membranes from barley roots were separated on a continuous sucrose gradient (Fig. 1). Under optimal conditions for measurement of the tonoplast ATPase the activity of the plasma membrane ATPase appeared insignificant, and vice versa. The highest proton transport activity of the plasma membrane ATPase exceeded the highest tonoplast ATPase activity. Manipulation of three variables permitted a complete differentiation between the two ATPase peaks; these variables were temperature, choice of potassium salt and use of valinomycin in the assay (11,7,8). The assay used to emphasize plasma membrane was conducted at a higher temperature and included KNO_3 , which inhibited the tonoplast but not the plasma membrane ATPase, and valinomycin, which enhanced transport by the plasma membrane ATPase but for unknown reasons collapsed the pH gradient formed by the tonoplast ATPase from barley roots (8). The assay used to emphasize tonoplast was conducted at a lower temperature and included KCl.

Temperature was useful as a criterion to distinguish between the two ATPases only when transport was measured by observing quench of acridine orange fluorescence. For some reason, maximal rates of quench of acridine orange fluorescence for the tonoplast ATPase were achieved between 18 and 25 C, although quench of quinacrine fluorescence and ATP hydrolysis by the tonoplast ATPase reached a maximum between 36 and 40 C (7). The rate of quench of acridine orange fluorescence for the plasma membrane ATPase paralleled the rate of ATP hydrolysis, reaching a maximum near 40 C. There was a high energy of activation for the plasma membrane ATPase, and it was difficult to measure transport below 25 C (7). Proton transport is often measured using a fluorimeter or spectrophotometer that is not equipped with a temperature controlled cuvette. If the barley membranes were assayed at room temperature it would be assumed that transport rates by the plasma membrane ATPase were low, compared to those for the tonoplast ATPase. The conclusion might be that most of the plasma membrane vesicles were of the wrong sidedness or not sealed. The transport activity of both ATPases would vary with changes in room temperature and degree of heating by the spectrophotometer lamp. Without the ability to achieve a controlled temperature between 30 and 40 C, it is easy to miss the very active proton translocating

ATPase in the barley plasma membrane fraction.

To simplify collection of the two ATPase peaks, membranes were collected on sucrose step gradients. The fraction at the 22/30% (w/w) interface was highly enriched in a vacuolar type ATPase and the fraction at the 34/40% (w/w) interface was highly enriched in plasma membrane ATPase (11). The fraction at the 30/34% (w/w) interface contained both types of membrane and was discarded. In previous papers it was demonstrated that the plasma membrane fraction had little cross contamination with tonoplast ATPase activity or subunits (11), was highly enriched in free sterols and sterol glycosides compared to the other membrane fractions (5), and was highly enriched in endogenous protein kinase activity that was absent from the other membrane fractions (14). Immunoblots of 1D and 2D gels of the plasma membrane fraction demonstrated that it was highly enriched in antigens that cross react to antibodies against the *Neurospora* plasma membrane ATPase (DuPont, unpublished), a partially purified ATPase from corn root plasma membrane (11) and an arabinogalactan protein found on the surface of tobacco protoplasts (11).

Effects of vanadate on proton transport and ATP hydrolysis. High concentrations of vanadate inhibited proton transport by the ATPase in the plasma membrane fraction (Fig. 1). However, preliminary observations indicated that ATP hydrolysis was inhibited by lower concentrations of vanadate than was proton transport. The effects of vanadate on ATP hydrolysis, formation of a pH gradient, and formation of a membrane potential were compared (Fig. 2). Nearly identical results were obtained, whether membranes were obtained by 2-phase partitioning or from a sucrose step gradient. Formation of a membrane potential and ATP hydrolysis were both inhibited to a similar extent by vanadate. Both ATP hydrolysis and membrane potential were inhibited by 60 to 70% by 100 μ M vanadate with little additional inhibition up to 500 μ M vanadate. Inhibition of proton transport followed a completely different pattern, with a linear decrease in proton transport from 5 to 500 μ M vanadate. To determine if differences in assay conditions for ATPase and proton transport might affect the result, ATP hydrolysis and proton transport was measured for the same reactions (Fig. 2) as well as separately (not shown). The results suggest that there was more than one component to the proton-translocating ATPase activity in the barley plasma membrane fraction. One component generated a membrane potential that was detected with oxonol V fluorescence,

and had K_i of 25 μM or lower for vanadate. A second component generated a pH gradient and had a much higher K_i for vanadate.

Effect of various substances on formation of membrane potential. Since the extent of inhibition of quench of oxonol V fluorescence by vanadate was very different from the inhibition of quench of acridine orange fluorescence, the effect of other compounds on the rate of quench of oxonol fluorescence was tested (Table I). It was conceivable that formation of the membrane potential was caused by a different cation translocating ATPase. For example, Ca^{2+} concentrations in the assay buffer were approximately 2 μM , high enough to support a Ca^{2+} -ATPase (8). However, similar rates of quench were observed in the presence or absence of EGTA. Addition of gramicidin to collapse the pH gradient eliminated oxonol quench. Transport also was inhibited up to 80% by valinomycin, FCCP, and nigericin (not shown). Therefore, the membrane potential appeared to be created by a proton translocating ATPase. In case vanadate acted by collapsing the membrane potential via an anion transporter, the effect of 100 μM Pi was tested, but Pi did not substitute for vanadate. Both quench of acridine orange fluorescence and quench of oxonol fluorescence appeared to measure the activity of a proton translocating ATPase, although formation of a membrane potential was much more sensitive to vanadate than was formation of the pH gradient.

pH dependence of proton transport. Quench of acridine orange fluorescence and oxonol fluorescence were measured as a function of assay pH, to compare the pH optima for formation of the pH gradient and the membrane potential (Figs. 3 and 4). Membranes from control and salt-grown roots were compared. Rate of membrane potential formation had a pH optimum between pH 6.0 and 7.0 (Fig. 3). Rate of pH gradient formation had a sharp pH optimum of pH 7.0 to 7.5 (Fig. 4). In these experiments, virtually identical rates were obtained for membranes from control or salt-grown roots. This is in contrast to the tonoplast ATPase, which had a broad pH optimum from pH 7.0 to 9.0 and had twice the rate of proton transport for membranes from salt-grown compared to control roots (Dupont and Morrissey, submitted).

Kinetics of transport and ATP hydrolysis. ATP hydrolysis, quench of acridine orange fluorescence and quench of oxonol fluorescence were compared as a function of ATP:Mg concentration. Mg^{++} was held constant and ATP was varied in all of the transport assays,

whereas Mg^{2+} also was varied for some of the ATP hydrolysis assays. The rate of quench of oxonol fluorescence showed saturable kinetics, with a K_m of approximately 0.25 mM ATP:Mg (Fig. 5) for membranes from control or salt-grown roots. V_{max} was identical for membranes from control or salt-grown roots. ATP hydrolysis also gave saturable kinetics (Fig. 6). The K_m for the experiment shown in Fig. 6 was approx 0.4 mM ATP:Mg, with equal amounts of ATP and Mg added to each assay. The kinetics for proton transport, measured with acridine orange, were more complex. At concentrations of ATP:Mg above 0.5 mM, the results for proton transport were similar to those for ATP hydrolysis (Fig 6). However, transport rates at concentrations of ATP:Mg below 0.5 mM were low, compared to ATPase, and did not fall on a straight line for any of the linear transformations of the Michaelis Menton equation. Figure 6 is a representative example for proton transport and ATP hydrolysis from plasma membranes collected from a sucrose gradient. Non-linear kinetics were consistently obtained for proton transport when the membranes were obtained from a sucrose gradient.

Transport and ATPase were also compared using plasma membranes obtained by phase partitioning (Fig. 7). In this example, the results for proton transport fit a linear transformation, but the K_m was 0.9 mM ATP:Mg, much higher than the K_m of 0.24 for ATP hydrolysis. As in the previous example, ATP hydrolysis was measured by adding equal amounts of ATP and Mg, while proton transport was assayed with 6.6 mM Mg^{2+} present throughout. Addition of excess Mg and excess KCl during the ATPase assay increased the K_m for ATP hydrolysis to 0.7 mM ATP:Mg (Fig. 8) and increased the similarity between ATP hydrolysis and proton transport.

Ben Hayyim reported that the concentration of monovalent and divalent cations included in the assay mixture greatly affected the kinetics of the plasma membrane ATPase from Citrus cells (1). Therefore, the effects of various salts on ATP hydrolysis and proton transport by the plasma membrane ATPase from barley was tested. There seems to be a complex relationship between excess Mg^{2+} , concentration of monovalent cations, very low concentrations of Ca^{2+} (8) and the proton transport activity of the plasma membrane ATPase. There are many potential effects of salts on ATPase activity, including effects on enzyme conformation, inhibition or stimulation of proteolysis, extraction of lysophospholipid from the membrane into the aqueous phase, and inhibition or stimulation of protein kinase activity.

Additional experimentation would be required to clarify effect of ions on the plasma membrane ATPase from barley roots.

Regulation of the plasma membrane ATPase in response to salt stress. Our laboratory is searching for key elements of membrane transport at the plasma membrane and tonoplast that are regulated in response to salt stress. The rate of proton transport by the tonoplast ATPase increased 2-fold when the plants were grown in 100 mM NaCl (Dupont and Morrissey, submitted for publication), and a sodium/proton antiporter in the tonoplast was activated by exposing roots to 100 mM NaCl (14). However, there was no evidence for a similar Na^+/H^+ antiporter in the plasma membrane (DuPont, unpublished) and no significant differences in transport by the plasma membrane ATPase were detected. It is highly unlikely that the plasma membrane does not play an important role in compartmentation of Na^+ , K^+ , and Cl^- in barley roots. It is possible that the modification of the tonoplast ATPase and activation of the tonoplast Na^+/H^+ antiporter were detected despite the destructive procedures required to prepare the membranes fractions, whereas the plasma membrane ATPase and antiporter are regulated by more labile factors or more susceptible to proteolysis. The bulk of the membranes probably originated from root cortical cells. It is possible that exposure to salt leads to changes in vacuolar transport in the cortical cells, whereas modifications in plasma membrane activity occur mainly in epidermal, root hair, or stelar cells and were not detected. Preliminary experiments in this laboratory have detected changes in message levels for the plasma membrane ATPase, but not for the tonoplast ATPase subunits (Berkelman and DuPont, unpublished). Therefore, the role for altered plasma membrane ATPase in salt tolerance is still of interest.

Multiple ATPase activities in the barley plasma membrane. The presence of several components of ATPase in the plasma membrane with differential sensitivity to vanadate might be explained by the presence of isozymes, multiple forms of the same ATPase, or several types of ATPase in the plasma membrane fraction. ATP hydrolysis and formation of a membrane potential were inhibited by relatively low concentrations of vanadate, and there was a sharp pH optimum around pH 6.5 to 7.0, indicating the presence of the typical plasma membrane ATPase. However, proton transport was not inhibited by low concentrations of vanadate, although there are now many reports for a low K_i for transport by the plasma

membrane ATPase from other plant tissues. A partial explanation might be a differential effect of vanadate on formation of the pH gradient compared to formation of the membrane potential. For example, vanadate may inhibit the leak as well as the pump (11,27), but this seems insufficient to explain the large difference in the effect on ATP hydrolysis and proton transport. Proteolysis increased proton pumping for the plasma membrane from oat roots (25). It is possible that using a high assay temperature to increase proton transport rates actually activated the barley ATPase via proteolysis, and the activated ATPase was less sensitive to vanadate. Antibodies against the plasma membrane ATPase from corn roots detected two antigens in immunoblots of 2D gels of the plasma membrane fraction (11). This could indicate the presence of two isozymes in the same membrane fraction, or two types of ATPase modified by proteolysis or phosphorylation. Purification and reconstitution of the ATPase from barley roots would be very helpful in resolving some of these questions.

Acknowledgements: Many thanks for the technical help of Melissa Kimball. Thanks to Douglas Bush and Gozal Ben Hayyim for working out the phase partitioning procedures, and to Gozal Ben Hayyim for many assays of ATP hydrolysis.

REFERENCES

1. Ben-Hayyim G. and Ran U. Salt-induced cooperativity in ATPase activity of plasma membrane-enriched fractions from cultured Citrus cells: kinetic evidence. *Physiol. Plant.* 80:210-216 (1990).
2. Blein J. P., Suty L., Allard M. R. and Scalla R. Characterization of a proton pump from *Acer pseudoplatanus* cell microsomes *Plant Science* 40:25-33 (1985).
3. Brauer D. T., S., Hsu, A., Thomas, C.E. Kinetic analysis of proton transport by the vanadate-sensitive ATPase from Maize root microsomes. *Plant Physiol.* 89:464-471 (1989).
4. Briskin D. P. The plasma membrane H^+ -ATPase of higher plant cells: biochemistry and transport function. *Biochim. Biophys. Acta* 1019:95-109 (1990).
5. Brown D. J. and Dupont F. M. Lipid composition of plasma membranes and endomembranes prepared from roots of barley (*Hordeum vulgare* L.) *Plant Physiol.* 90:955-97 (1989).
6. Bush D. S., Sticher L., Van Huystee R., Wagner D. and Jones R. L. The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone α -amylase *J. Biol. Chem.* XXX (1989).
7. DuPont F. M. Effect of temperature on the plasma membrane and tonoplast ATPases of barley roots. *Plant Physiol.* 89:1401-1412 (1989).
8. DuPont F. M. Variable effects of nitrate on ATP-dependent proton transport by barley root membranes. *Plant Physiol.* 84:526-534 (1987).
9. DuPont F. M., Bush D. S., Windle J. J. and Jones R. L. Calcium and proton transport in membrane vesicles from barley roots. *Plant Physiol.* 94:179-188 (1990).
10. DuPont F. M. and Hurkman W. J. Separation of the Mg^{2+} -ATPases from the Ca^{2+} -phosphatase activity of microsomal membranes prepared from barley roots. *Plant Physiol.* 77:857-862 (1985).
11. DuPont F. M., Tanaka C. K. and Hurkman W. J. Separation and immunological characterization of membrane fractions from barley roots. *Plant Physiol.* 86:717-724 (1988).
12. Epstein E. and Norlyn J. D. Seawater-based crop production: a feasibility study. *Science* 197:249-251 (1977).
13. Ewing N. N., Wimmers L. E., Meyer D. J., Chetelat R. T. and Bennett A. B. Molecular cloning of tomato plasma membrane H^+ -ATPase *Plant Physiol.* 94:1874-1881 (1990).
14. Garbarino J. E., Hurkman W. J., Tanaka C. K. and DuPont F. M. In vitro and in vivo phosphorylation of polypeptides in plasma membrane and tonoplast-enriched fractions from barley roots *Plant Physiol.* 95:1219-1228 (1991).
15. Giannini J. L., Gildensoph L. H. and Briskin D. P. Selective production of sealed plasma membrane vesicles from red beet (*Beta vulgaris* L.) storage tissue. *Arch. Biochem. Biophys.* 254:621-630 (1987).
16. Harper, J.F., Manney, L., DeWitt, N.D., Yoo, M.H., Sussman, M.R. The *Arabidopsis thaliana* plasma membrane H^+ -ATPase multigene family. *J. Biol. Chem.* 265:13601-13608 (1990).
17. Hsu A.-F., Brauer D. and Tu S.-I. Characterization of reconstituted plasma membrane H^+ -ATPase from maize roots. *Physiol. Plant.* 76:544-550 (1989).
18. Hurkman W. J., Tanaka C. K. and DuPont F. M. The effects of salt stress on polypeptides in membrane fractions from barley roots *Plant Physiol.*

- 88:1263-1273 (1988).
19. Kasamo K. Purification and properties of the plasma membrane H^+ -translocating adenosine triphosphatase of *Phaseolus mungo* L. roots. *Plant Physiol.* 88:818-824 (1986).
 20. Kasamo K. and Nouchi I. The role of phospholipids in plasma membrane ATPase activity in *Vigna radiata* L. (mung bean) roots and hypocotyls. *Plant Physiol.* 83:323-328 (1987).
 21. Korner L. E., Kjellbom P., Larsson C. and Moller J. M. Surface properties of right side-out plasma membrane vesicles isolated from barley roots and leaves. *Plant Physiol.* 79:72-79 (1985).
 22. LeBel D., Poirier G. G. and Beaudoin A. R. A convenient method for the ATPase assay. *Anal. Biochem.* 85:86-89 (1977).
 23. A. E. Martell and R. M. Smith Critical Stability Constants, Volumes 1-5, Plenum Press, New York, 1974-1982.
 24. Nagao T., Sasakawa H. and Sugiyama T. Purification of H^+ -ATPase from the plasma membrane of maize roots and preparation of its antibody. *Plant Cell Physiol.* 28:1181-1186 (1987).
 25. Palmgren M. G., Larsson C. and Sommarin M. Proteolytic activation of the plant plasma membrane H^+ -ATPase by removal of a terminal segment *J. Biol. Chem.* 265:13423-13426 (1990).
 26. Palmgren M. G. and Sommarin M. Lysophosphatidyl choline stimulates ATP dependent proton accumulation in isolated oat root plasma membrane vesicles *Plant Physiol.* 90:1009-1014 (1989).
 27. Palmgren M. G., Sommarin M., Ulvskov P. and Jorgensen P. L. Modulation of plasma membrane H^+ -ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A2 *Physiol. Plant.* 74:11-19 (1988).
 28. Rasi-Caldogno F., Pugliarello M. C. and De Michelis M. I. Electrogenic transport of protons driven by the plasma membrane ATPase in membrane vesicles from radish. *Plant Physiol.* 77:200-205 (1985).
 29. Sandstrom R. P., DeBoer A. H., Lomax R. L. and Cleland R. E. Latency of plasma membrane H^+ -ATPase in vesicles isolated by aqueous phase partitioning *Plant Physiol.* 85:693-698 (1987).
 30. Schaffner W. and Weissmann C. A rapid, sensitive, and specific method for the determination of protein in dilute solution *Anal. Biochem.* 56:502-514 (1973).
 31. R. Serrano, Plasma Membrane ATPase of Plants and Fungi, CRC Press, Inc., Boca Raton, Florida, 1985.
 32. Silen, L.G. and Martell, A.E. Stability Constants of Metal Ion Complexes, (Special Publication No. 17, the London Chemical Society, Burlington House, W1, London, 1964).

TABLE I. Effect of various compounds on rate of quench of oxonol fluorescence. Assayed as in Fig. 2.

Addition	Rate of Quench %Q/min ^a
None	26.8
1 mM EGTA	23.9
1 μ M Gramicidin in ethanol	1.4
Ethanol	27.5
100 μ M KPi, pH 7.0	27.0

FIGURE LEGENDS

Figure 1. Distribution of proton transport activities on a continuous sucrose gradient. A 10,000 to 100,000 g membrane fraction from barley roots was layered onto a 15 to 45% (w/w) sucrose gradient and centrifuged for 3 h. Densities increase from fraction to 18. For typical values see (8,9,10,18). Fractions were assayed for proton transport at 21°C with 50 mM KCl (□) or at 36°C with 50 mM KNO₃ and 1 μM valinomycin with (●) and without (○) 500 uM vanadate. PPIase was assayed at 21° C (▲).

Figure 2. ATP hydrolysis, formation of membrane potential and formation of pH gradient as a function of vanadate concentration. Proton transport and ATP hydrolysis were determined on identical samples. Proton transport was determined by measuring the initial rate of quench of acridine orange fluorescence (■). At 15 minutes 3 aliquots were taken from the fluorescence cuvette and assayed for Pi released (●). Results for a separate ATPase assay are included, done in the absence of acridine orange (○) and for two separate determinations of initial rate of quench of oxonol fluorescence (▲, Δ) as measures of formation of the membrane potential). The membranes were obtained by phase partitioning (■, ●), or from a discontinuous sucrose gradient (○, ▽, ▲). Similar results were found for proton transport obtained from sucrose gradients (not shown). ATPase activity was 1.4 μmol Pi/mg/min at 100% and proton transport was 600%Q/mg/min at 100%.

Figure 3. Formation of membrane potential as a function of assay pH. Formation of membrane potential was assayed as initial rate of quench of oxonol V fluorescence. Membranes were incubated in 25 mM BTP-Mes at the indicated pH, plus 3 mM ATP, 25 mM K₂SO₄, 0.25 M sorbitol, at 36°C. Quench was initiated by addition of 6.6 mM MgCl₂. Membranes were from control roots (●), or roots of plants grown in 100 mM NaCl (○).

Figure 4. Proton transport as a function of assay pH. Proton transport was assayed as initial rate of quench of acridine orange fluorescence. Membranes were incubated in 20 mM BTP-Mes at the indicated pH, plus 3 mM ATP, 50 mM KNO₃, 1 μM valinomycin, 0.25 M sorbitol, at 36°C. Quench was initiated by addition of 6.6 mM MgCl₂. Membranes were from control roots (○), or roots of plants grown in 100 mM NaCl (●).

Figure 5. A. Formation of membrane potential as a function of ATP:Mg concentration. Assayed at pH 6.5, with 6.6 mM MgCl₂ and various amounts of Tris-ATP. Membranes were from control roots (○) or roots of plants grown in 100 mM NaCl (●). B. Hanes-Woolf plot of data. Km for membranes from control roots was 0.22 mM and for membranes from salt-grown roots was 0.25 mM ATP:Mg. Membranes were obtained from a discontinuous sucrose gradient.

Figure 6. A. ATP hydrolysis (○) and proton transport (●) as a function of ATP:Mg concentration. To assay proton transport, membranes were incubated at pH 6.5 with 50 mM KNO₃, 1 uM valinomycin, 0.25 M sucrose, 2 μM acridine orange and various amounts of Tris-ATP. Quench was initiated by addition of 6.6 mM MgCl₂, and initial rate of quench of acridine orange fluorescence was determined. To assay ATPase, membranes added to the reaction mix containing equal concentrations of ATP and Mg, at pH 6.5 with 50 mM KCl. Pi was determined after 20 min. B. Hanes-Woolf plot of data. Km for ATP hydrolysis was 0.42 mM. Membranes were from control roots, and were obtained from a discontinuous sucrose

gradient. At Vmax ATPase was $1.7 \mu\text{mol Pi/mg/min}$.

Figure 7. A. ATP hydrolysis (○) and proton transport (●) as a function of ATP:Mg concentration. Proton transport and ATPase were assayed as in Fig. 6. B. Hanes-Woolf plot of data. K_m for ATPase was 0.24 mM and for proton transport was 0.9 mM. Membranes are from control roots, and were obtained by phase partitioning.

Figure 8. A. ATP hydrolysis (○) and proton transport (●) as a function of ATP:Mg concentration. Proton transport was assayed as in Fig. 6. ATPase was assayed in the presence of 200 mM KCl, with 3 mM MgCl_2 in all assays. Membranes are from control roots, and were obtained by phase partitioning.

Fig. 1

Fig. 2

Mem. Pot. ATPase I ATPase II
▲ ● ○
Proton Transport Mem. Pot.
■ △

Fig. 3

Fig. 4

Expt 42
salt grown

Expt 44

Fig. 5

Fig. 6

Transpt ATPase

Fig. 7

ATPase Transport

**2-Phase Prep
Transport & ATPase, Excess Mg**