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NaCl effects on root plasma membrane ATPase of salt tolerant wheat

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Abstract

Wheat seedlings of a salt tolerant cultivar were grown hydroponically in presence and absence of 100 mM NaCl. Roots were harvested, and the plasma membrane (PM) fraction was purified. PM ATPase required a divalent cations for activity (Mg > Mn > Ca > Co > Zn > Ni > Cu), and it was further stimulated by monovalent cations (K > Rb > NH_4 > Li > choline > Cs). The pH optima were 6.0 and 5.6 in absence and presence of 25 mM KCl, respectively. The enzyme was sensitive to vanadate and DCCD but insensitive to azide, oligomycine and nitrate. The enzyme displayed a high preference for ATP but was also able to hydrolyze other nucleotide tri- and diphosphates. The enzyme activity showed a simple Michaelis-Menten kinetics for the substrate Mg^{2+}-ATP in both control and salt exposed roots. The polypeptide patterns of control and salt stressed PM fractions, detected by SDS-PAGE, were very similar. NaCl substantially reduced the PM ATPase specific activity, whereas it had little effect on the apparent K_m for Mg^{2+}-ATP. Since the root PM ATPase of salt sensitive and resistant genotypes responded similarly to salinity stress, it seems unlikely that the mechanism of salt tolerance in wheat is primarily based on differences in PM ATPase characteristics.

Additional key words: lipids, polypeptide pattern, salt stress, *Triticum aestivum*.

Introduction

Control of ion translocation from root to shoot and ion accumulation in the vacuole is believed to be important for salt tolerance in higher plants (Yeo and Flowers 1986). While halophytes evolved mechanisms for controlled influx of Na^{2+} and/or Cl^- to be used for their benefit in a saline environment (*i.e.* osmotic adjustment), glycophytes show inadequate mechanisms to regulate influxes of these ions and hence they are unable to prevent toxification of their cytoplasm. Yeo and Flowers (1986) propose that this difference in response to salinity stress may result from different ion transport systems in the root cell plasma membrane (PM). The H^+-translocating ATPase creates a proton electrochemical gradient for solute and ion transport through cell membranes (Sze 1985), and it is to be expected that H^+-ATPase of the PM and tonoplast have a significant implication to salt adaptation of plants. Therefore, PM ATPase activity in combination with salinity stress has been the subject of several studies (Brüggemann and Janiesch 1988, Douglas and Walker 1984, Gronwald *et al.* 1990, Sanchez-Aguayo *et al.* 1991). However, the role of PM ATPase in salt tolerance is not clear and it is difficult to reach a general conclusion. Salt stress had no effect on PM ATPase properties of the halophyte *Plantago maritima* (Bruggemann and Janiesch 1988), it reduced PM ATPase specific activity of a salt tolerant tomato cultivar and a salt sensitive wheat cultivar (Gronwald *et al.* 1990, Mansour *et al.* 1998), and it stimulated PM ATPase in mung bean (Nakamura *et al.* 1992). Comparison of genotypes differing in salt tolerance may give clues in understanding the implication of PM ATPase in plant adaptation to salt stress.

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Abbreviations: DCCD - N,N'-dicyclohexylcarbodiimide; DDT - dithiotreitol; EDTA -ethylenediamine tetraacetic acid; IDPase - inosine diphosphatase; IM - intracellular membranes; PM - plasma membranes; PNPP - nitrophenylphosphate; PSMF - phenylmethylsulphonyl fluoride.
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The present investigation, therefore, was conducted to study the effects of NaCl stress on root PM ATPase of a salt tolerant wheat cultivar and to compare the response to that of sensitive wheat cultivar (Mansour *et al.* 1998). That is to find out whether PM ATPase is involved in differential salt tolerance of wheat cultivars.

Materials and methods

Plants: Salt tolerant wheat (*Triticum aestivum* L.) cultivar Sakha 8 was grown hydroponically. Caryopses were germinated for 5 d in dark and seedlings were established in 1/4-strength Hoagland solution in growth chamber for 5 d. Control plants were grown in 1/4-strength Hoagland solution and treated plants were grown in 1/4-strength Hoagland solution plus 100 mM NaCl (for further details see Mansour *et al.* 1998).

Purification of the plasma membrane: Roots were harvested, washed in cold deionized water and homogenized in a buffer solution (1 g fresh mass per 5 cm^3 buffer solution) containing 10 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 0.1 mM DTT and 1 mM PSMF for 3 × 20 s in a *Braun* mixer at maxium speed. The homogenate was filtered through two layers of miracloth and the cell debris was again homogenized in the previous homogenization medium and filtered. The two filtrates were combined together and centrifuged at 9 000 *g* for 20 min. The supernatant was then centrifuged at 50 000 *g* for 60 min. The pellet was suspended in 9 cm^3 of 5 mM potassium phosphate (pH 7.8), 250 mM sucrose, 4 mM KCl, 0.1 mM DTT and 5 mM EDTA. A purified PM fraction was obtained by partitioning in a dextran-polyethyleneglycol two phase system, using a three patch procedure (Mansour *et al.* 1998).

Enzyme and protein assays: ATPase activity was measured as the release of Pi from ATP after 30 min at 30 °C in 0.5 cm^3 reaction volume (Mansour *et al.* 1998). Cytochrome *c* oxidase was determined according to the oxidation of reduced cytochrome *c* measured by the change in absorbance at 550 nm as described by Hodges and Leonard (1974). The reduction of cytochrome *c* was also assayed in presence of antimycine A to determine antimycine A insensitive NADH-cytochrome *c* reductase. Latent IDPase activity was assayed according to Chanson *et al.* (1984), but 0.1 % (m/v) Triton was used instead of digitonin. Protein content was determined by Bio-Rad microassay, using bovine serum albumine as the standard.

Electrophoresis: SDS-PAGE was carried out in 4 % (m/v) stacking gel and 7.5 % (m/v) running (degree of crosslinking was 2.7 %) at 4 °C with a current of 200 V for 50 min as detailed elsewhere (Mansour *et al.* 1998). Change in the bands staining intensity was measured by scanning with a laser densitometer, *Ultrascan XL* (*Pharmacia*, Uppsala, Sweden).

Statistics: Three independent PM isolations were carried out on roots of control and NaCl exposed wheat plants. Standard deviations were between 1 - 12 % among the three experiments. Duplicate analyses within an experiment deviated only 1-5 % from the mean value. A single representative experiment is presented in the results.

Results and discussion

Activities of mitochondria, Golgi apparatus and endoplasmic reticulum marker enzymes in PM fraction were negligible (Table 1). NaCl stress had little or no effect on sensitivity to inhibitors (Table 2), activation by divalent and monovalent cations (Tables 3, 4), pH optimum (Fig. 1), and substrate specificity (Table 5). Significant inhibition was only found with Na_2VO_4 and DCCD, in the presence and absence of K^+ (Table 2).

Table 1. Activities of marker enzymes [μmol(substrate) mg^{-1}(protein) h^{-1}] in plasma membrane (PM) and intracellular membranes (IM) isolated from roots of wheat plants grown in absence and presence of 100 mM NaCl.

	Control		NaCl	
	PM	IM	PM	IM
Cytochrome *c* oxidase	0.03 ± 0.003	0.48 ± 0.01	0.01 ± 0.001	0.27 ± 0.005
Cytochrome *c* reductase	0.05 ± 0.003	0.99 ± 0.05	0.06 ± 0.004	1.06 ± 0.008
IDPase	0.80 ± 0.020	13.80 ± 0.91	0.60 ± 0.006	12.30 ± 1.010

Mg^{2+} was the most effective divalent cation in activation of ATPase (Table 3), and K^+ among other monovalent cations greatly further stimulated Mg^{2+}-ATPase (Table 4). PM ATPase had a pH optimum of 6.0 in the presence of Mg^{2+}-ATP, and addition of 25 mM KCl shifted the optimum to 5.6 (Fig. 1). Although PM ATPase had highest affinity to ATP as the substrate, it could also hydrolyze other nucleotides (Table 5).

Table 2. Effect of inhibitors on activity of plasma membrane ATPase [μmol(Pi) mg^{-1}(protein) h^{-1}], in presence and absence of 25 mM KCl, isolated from roots of wheat plants grown with or without 100 mM NaCl. Activities were assayed as described in Materials and methods at pH 6, and expressed as percentage of assay without inhibitor given in parentheses [μmol(Pi) mg^{-1}(protein) h^{-1}].

	Control		NaCl	
	$-K^+$	$+K^+$	$-K^+$	$+K^+$
Without inhibitor	100 (34.4)	100 (40.8)	100 (21.9)	100 (25.8)
Na_2VO_4 (0.1 mM)	20	25	18	22
DCCD (0.1 mM)	33	40	30	29
Oligomycin (10 μg cm^{-3})	98	99	97	98
NaN_3 (1 mM)	99	98	99	100
Na_2MoO_4 (1 mM)	97	96	94	96
KNO_3 (25 mM)	99	101	105	103

PM preparations showed no significant contamination by other membrane systems (Tables 1, 2), and the measured ATPase activity was essentially PM ATPase activity. The pH optimum and nucleotide specificity observed in this study are similar to those previously reported in other wheat genotypes (Sommarin *et al.* 1985, Kuiper *et al.* 1991, Mansour *et al.* 1998). The shift in the pH optimum observed here upon addition of 25 mM KCl was also found for PM ATPase from maize (Gronwald *et al.* 1990) and wheat (Kuiper *et al.* 1991, Mansour *et al.* 1998). PM ATPase in our preparations was completely inhibited by PNPP and AMP but not by molybdate indicating that PM preparations are free from non specific phosphatases.

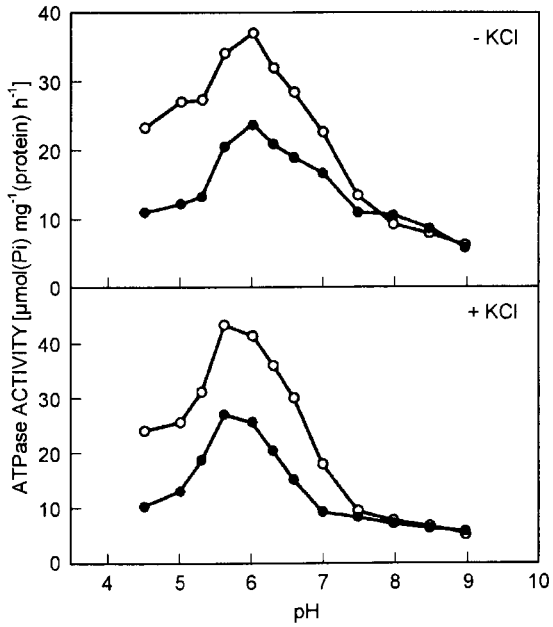

Fig. 1. Effect of pH on ATPase specific activity, measured in absence and presence of 25 mM KCl, of plasma membrane preparations isolated from roots of control (*open circles*) and NaCl treated (*closed circles*) plants.

Table 3. Effect of divalent cations (1 mM) on activity of plasma membrane ATPase [μmol(Pi) mg^{-1}(protein) h^{-1}], in presence and absence of 25 mM KCl, isolated from roots of wheat plants grown with or without 100 mM NaCl.

	Control		NaCl	
	$-K^+$	$+K^+$	$-K^+$	$+K^+$
None	6.4 ± 0.70	20.1 ± 1.11	3.7 ± 0.17	10.1 ± 1.02
$MgSO_4$	36.7 ± 1.31	43.5 ± 3.02	16.9 ± 1.17	20.6 ± 3.01
$MnSO_4$	35.1 ± 2.66	41.4 ± 2.73	14.8 ± 1.63	19.1 ± 2.66
$CaSO_4$	29.6 ± 2.09	29.5 ± 2.12	14.1 ± 2.00	13.1 ± 2.00
$CoSO_4$	25.8 ± 3.01	27.8 ± 0.89	9.7 ± 1.05	11.3 ± 1.23
$ZnSO_4$	24.3 ± 1.91	25.1 ± 1.91	6.3 ± 0.09	5.6 ± 1.21
$NiSO_4$	19.5 ± 1.67	19.3 ± 0.97	5.7 ± 0.71	5.1 ± 1.11
$CuSO_4$	17.8 ± 0.90	17.9 ± 0.90	2.2 ± 0.02	1.6 ± 0.02

Simple Michaelis-Menten kinetic was found for ATPase isolated from control and salt stressed roots (Fig. 2). 100 mM NaCl added to the growth solution substantially reduced V_{max} of root PM ATPase both in absence and presence of K^+ (Fig. 2): 79 vs. 46 and 91 vs. 59 μmol(Pi) mg^{-1}(protein) h^{-1}, respectively. NaCl reduced the K_m values for Mg^{2+}-ATP in absence (1.1 vs. 0.86) and presence of K^+ (0.77 vs. 0.67). The reduction in V_{max} was,

however, substantially higher than that in K_m after NaCl exposure. Also, the effect of salinity on K_m for Mg^{2+}-ATP was not consistent: while NaCl decreased K_m values of

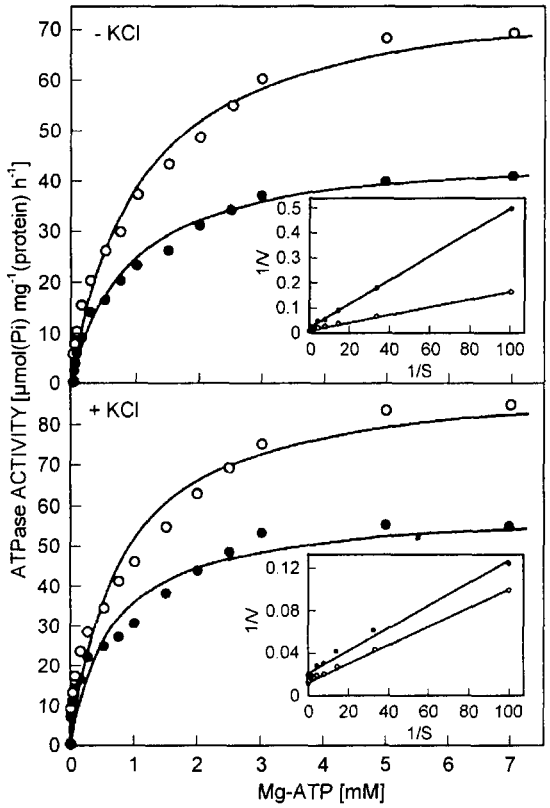

Fig. 2. Michaelis-Menten curves of plasma membrane ATPase specific activity, measured in the absence (*A*) and presence (*B*) of 25 mM KCl, in roots of control (*open circles*) and salt stressed (*closed circles*) plants as a function of increasing Mg^{2+}-ATP concentration. Insets: the 1/S versus 1/V plot of the data.

Table 4. Effect of monovalent cations (25 mM) on activity of plasma membrane ATPase [µmol(Pi) mg^{-1}(protein) h^{-1}] isolated from roots of wheat plants grown with or without 100 mM NaCl.

	Control activity	%	NaCl activity	%
None	35.8 ± 2.01	84	23.6 ± 2.20	87
KCl	42.6 ± 3.11	100	27.1 ± 2.21	100
RbCl	40.8 ± 4.02	96	25.2 ± 3.06	93
NH_4Cl	33.2 ± 4.06	78	11.4 ± 1.51	42
LiCl	30.8 ± 3.00	72	7.2 ± 0.51	27
CholineCl	23.4 ± 2.16	55	4.2 ± 0.35	16
CsCl	18.6 ± 1.66	44	3.0 ± 0.32	11

the salt tolerant cultivar used in this study, it increased K_m values in a salt sensitive cultivar (Mansour *et al.* 1998). Therefore, it is more likely that changes in K_m values were not correlated to salt treatment. Similar variations in K_m values under salt stress have been observed earlier and were considered not to be related to salt treatment (Brüggemann and Janiesch 1988, Gronwald *et al.* 1990, Mansour *et al.* 1998).

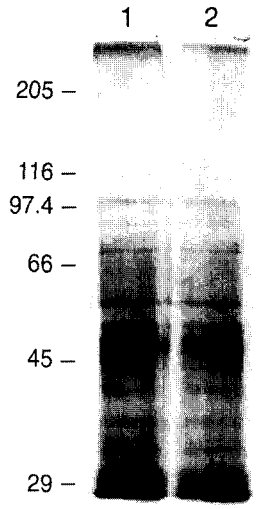

Fig. 3. SDS-PAGE of polypeptides from plasma membranes of wheat roots. *Lane 1* - preparation from control roots; *lane 2* - preparation from salt-treated roots. Molecular mass distribution in kDa is indicated on the left.

The control and salt treated PM fractions contained similar proteins with molecular masses of 27, 31, 40, 45, 52, 62, 75, 86, 102, and 108 kDa (Fig. 3). The bands at 102 kDa of both control and salt stressed PM fraction was identified as catalytic subunit of ATPase (Sze 1985). Staining intensity of bands from both fractions (not shown), measured with a densitometer, showed only minor differences. Polypeptide components of control and treated fractions were qualitatively and quantitatively similar, although NaCl caused a marked decrease in specific activity of PM ATPase. Therefore, the NaCl-induced reduction in ATPase activity may not be due to alterations in PM proteins. Alternatively, changes in lipids of the PM under salt stress (Kuiper 1984, Douglas and Walker 1984, Chung and Matsumato 1989, Mansour *et al.* 1994) could cause the reduced ATPase activity. It is interesting to note that in the previous studies, modification in PM specific activity was always associated with PM lipid alterations but not with alterations of PM polypeptides which supports the role of lipids in modulating ATPase activity under salt stress. Modifications in lipid composition of PM can modulate the kinetic properties of PM ATPase (Carrutherus and Melchoir 1986, Russell 1989, Lee 1991, Kasamo and Sakakibara 1995) through changing the membrane fluidity (Douglas and Walker 1984, Russell 1989), and through direct binding of lipids to the enzyme (Sandstrom

and Cleland 1989, Lee 1991, Cooke *et al.* 1994, Grandmougin-Ferjani *et al.* 1997). ATPase catalytic activity, however, is much more affected by boundary lipids than by the bulk lipid content of the membrane (Carrutherus and Melchoir 1986).

Table 5. Substrate (1 mM) specificity of plasma membrane ATPase, in presence and absence of 25 mM KCl, isolated from roots of wheat plants grown with or without 100 mM NaCl. Activities are expressed as percentage of ATP given in parentheses [μmol(Pi) mg^{-1}(protein) h^{-1}].

	Control		NaCl	
	$-K^+$	$+K^+$	$-K^+$	$+K^+$
ATP	100 (35.5)	100 (42.9)	100 (22.0)	100 (25.5)
UTP	44	50	37	44
GTP	33	41	25	37
ITP	37	44	25	35
CTP	11	16	11	13
ADP	39	45	29	34
UDP	17	19	10	14
GDP	13	17	10	12
IDP	11	13	9	10
CDP	9	10	7	10
AMP	0	0	0	0
PNPP	0	2	0	0

NaCl-induced reduction in root PM ATPase activity observed in the salt resistant wheat cultivar used in this study (42 %) was also previously found in a salt sensitive wheat genotype (40 %) (Mansour *et al.* 1998). Thus, root PM ATPase of salt sensitive and resistant genotypes of wheat respond similarly to salinity. It seems probable, therefore, that the behaviour of root PM ATPase under NaCl stress is not correlated with salt tolerance in wheat. Other parameters might be crucial for salt tolerance of wheat, as, *e.g.*, Na^+/H^+ antiport of tonoplast which was found in salt tolerant *Plantago* species but not in salt sensitive species under NaCl stress (Staal *et al.* 1991). Salinity stimulated tonoplast ATPase activity of tomato roots despite a reduction in PM ATPase activity (Sanchez-Aguayo *et al.* 1991). Stimulating the tonoplast ATPase is essential for providing H^+ gradient necessary for Na^+/H^+ antiport. Tonoplast ATPase and Na^+/H^+ antiport are obviously a potential area for future research in order to clarify the differential response of wheat genotypes to salinity.

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