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Plasma membrane H^+-ATPase activity in salt-tolerant and salt-sensitive lines of spring wheat (*Triticum aestivum* L.)

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SUMMARY

Relationships between the primary H^+-ATPases on the plasma membrane (PM-ATPase; EC 3.6.1.3) and vacuolar membrane (V-ATPase; EC 3.6.1.3) and the degree of salt tolerance were determined in spring wheat (*Triticum aestivum* L.), using fresh and dry weights and PM-ATPase and V-ATPase activities in shoots and roots from plants differing in salt tolerance. The salt-tolerant line, 'Selection line' (developed after mass screening of F_3 variable seed material from a cross of two salt-tolerant wheat cvs, LU26S and Kharchia) and the relatively salt-sensitive variety, 'Yecora Rojo', were subjected for 8 days to aerated solution culture containing 0 or 100 mmol/L NaCl in full strength Hoagland's nutrient solution after an initial growth of 22 days in normal non-saline conditions. The salt-tolerant line was superior to the salt-sensitive line in terms of shoot biomass production under saline conditions. The former line accumulated less Na^+ in both shoots and roots and maintained high K/Na ratio in the shoot compared to the latter, although the lines did not differ in root K/Na ratios. Selection line showed about 17% more activity of shoot PM-ATPase in the salt treatment compared to control, whereas a considerable decrease (about 45%) in shoot PM-ATPase activity due to salt was observed in Yecora Rojo. In contrast, in roots, an increase in PM-ATPase activity was observed in Yecora Rojo under saline substrate, whereas no influence of salt was observed on root PM-ATPase activity in Selection line. V-ATPase activities in shoots and roots were not significantly different among lines and were not affected by the salt treatment. From this study it appears that PM-ATPase activity in the shoot of tolerant plants was stimulated by salt, but PM-ATPase activity in the root was not. Both these conditions are

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Abbreviations: Bafilomycin A_1, 21-O-de(3-carboxy-1-oxo-2-propenyl)-2-demethyl-2-methoxy-21-methyl-hygrolidin; BTP, bis-tris propane or 1,3-bis[tris(hydroxymethyl)-methyl amino] propane; PVPP, polyvinylpoly-pyrrolidone.

postulated to be important components of salt tolerance in spring wheat.

Key-words: H^+-ATPases, plasma membrane, salt tolerance, spring wheat, *Triticum aestivum*, vacuolar membrane.

INTRODUCTION

Among the various responses of plants to salinity, regulation of ion transport is of prime importance. Unlike halophytes, glycophytes generally cannot utilize Na^+ and Cl^- as osmotica (Flowers *et al.* 1977; Greenway & Munns 1980). In glycophytes, the degree of salt tolerance and pattern of ion distribution vary between and within plant species. For instance, maintenance of a high K/Na ratio in the cytoplasm is suggested to be an important selection criterion in most non-halophytes as well as in halophytes (Greenway & Munns 1980; Wyn Jones 1981; Jeschke 1984), although the reverse is true in some others (Van Steveninck *et al.* 1982; Ashraf 1994). It has been observed that in closely related crop species, such as wheat and barley, the pattern of K^+ and Na^+ accumulation differs. In wheat, the degree of salt tolerance was correlated with a high K/Na ratio in the leaves (Wyn Jones *et al.* 1984; Schachtman *et al.* 1989; Gorham 1990a) indicating that exclusion of Na^+ from the leaves was a distinctive salt-tolerant trait. In contrast, in barley, discrimination between Na^+ and K^+ in transport to the leaves was found to be low (Gorham 1990b).

In a long-term study in sand culture salinized with varying concentrations of NaCl, a salt-tolerant 'Selection line' performed well in terms of biomass production and seed yield and yield components as compared to a salt-sensitive line 'Yecora Rojo' (Ashraf & O'Leary 1996). The salt-tolerant line was developed after mass screening of the F_3 variable seed material produced from the cross, LU 26S × Kharchia (salt-tolerant lines from Pakistan and India, respectively). The salt-tolerant line showed enhanced discrimination between Na^+ and K^+ in transport to shoots as compared to salt-sensitive Yecora Rojo. Such differences in ion uptake in two lines of wheat differing in salt tolerance could be due to many factors, such as differences in genes for specific ion transport proteins, regulation of gene expression, or regulation of transport activity (Dupont 1992). It is now evident that plasma membrane H^+-ATPase (PM-ATPase) plays a primary role in providing metabolic energy for ion transport at the plasma membrane of plant cells (Sze 1985; Serrano 1985; Briskin 1990). This energy generating process is thought to be central to a number of physiological processes in plants including nutrient uptake by plant roots from soil and nutrient distribution in different plant organs (Leonard & Hotchkiss 1976; Taiz 1984; Sze 1985; Marschner 1986; Sussman & Surowy 1987; Sanders 1990). In addition, the vacuolar membrane H^+-ATPase (V-ATPase) also plays an important role in the growth and development of plant cells by providing the driving force for secondary transport of numerous ions and metabolites (Sze *et al.* 1992).

In relation to salt stress, there are contrasting reports of the activity of H^+-ATPases in glycophytic crops. For instance, in cotton it was found that the degree of salinity during growth did not influence activity of H^+-ATPase in plasma membrane vesicles from roots (Hassidim *et al.* 1986). In contrast, a decrease in PM-ATPase activity was observed in salt treated roots of tomato (Gronwald *et al.* 1990; Sanchez-Aguayo *et al.* 1991) and buffalograss (Lin & Wu 1996). In another study, a low Na^+ accumulating line of *Trifolium alexandrinum* had higher ATPase activity than that of a high Na^+

accumulating line (Parihar *et al.* 1990). Working with cultured cells of *Pisum sativum*, Olmos *et al.* (1993) found that plasma membrane ATPase activity was increased due to salt stress. Reports concerning the effect of high salt on V-ATPase activity in glycophytes show stimulation in some (Matsumoto & Chung 1988; Reuveni *et al.* 1990; Dupont & Morrisey 1991; Nakamura *et al.* 1992) and no effect in others (Nakamura *et al.* 1992; Colombo & Cerana 1993). These contrasting reports about the activity of the H^+-ATPases in different crops led us to compare both PM- and V-ATPase activities in two lines of spring wheat differing in the degree of salt tolerance. Here we show that PM-ATPase activity is closely associated with salt tolerance in spring wheat.

MATERIALS AND METHODS

Plant material

Seed of the salt-tolerant line of spring wheat (*Triticum aestivum* L.) was developed after mass screening of F_3 seed material from a cross between two salt-tolerant varieties, 'LU26S' and 'Kharchia' from Pakistan and India, respectively. The seed of a salt-sensitive cv., Yecora Rojo, was obtained from Dr M.J. Ottman, Department of Plant Sciences, University of Arizona, Tucson. Both seed samples were surface sterilized in 5% sodium hypochlorite solution for 5 min before planting. The experiment was conducted in a naturally lit greenhouse in which PAR measured at noon ranged from 450 to 1350 μmol m^{-2} s^{-1}, relative humidity day/night from 42% to 80% and temperature from 27°C to 18°C.

About 300 seeds of each line were sown in normal potting compost in the greenhouse in November 1993. Each replicate consisted of 10 seedlings at the two leaf stage transplanted into a plastic pot (21·5 cm diameter and 16·0 cm deep) which contained 4·25 L of aerated full strength Hoagland's nutrient solution. The experiment was arranged in a completely randomized design with five replicates, two salt treatments and two lines. The NaCl treatments used were 0 and 100 mmol/L in full strength Hoagland's nutrient solution prepared in distilled water. Salt treatments were begun 22 days after the start of the experiment. The salt treatment was attained in increments of 50 mmol/L daily for 2 days. The level of solution was maintained daily with the addition of distilled water. The treatment solutions (pH=6·74) were replaced 4 days after the initiation of salt treatment.

Eight days after the final salt concentration was attained all the plants were harvested and separated into shoots and roots. The shoots were washed with distilled water whereas the roots were washed in cold $LiNO_3$ solution isotonic with the corresponding treatment in which the plants were growing. The $LiNO_3$ solution contained 1 mmol/L $Ca(NO_3)_2$. $4H_2O$ to maintain membrane integrity. After recording FW, shoots and roots of four randomly chosen plants were dried at 65°C to constant weight. The remaining plants from three replications were used for membrane preparation.

Preparation of membrane vesicles

All extraction procedures were carried out at 4°C. Seven g fresh leaves or roots of both lines of wheat were homogenized with a mortar and pestle in a buffer containing 250 mmol/L sorbitol, 25 mmol/L HEPES-BTP (pH 7·4), 3 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L iodoacetamide, 0·1 mmol/L phenylmethylsulphonyl fluoride, 0·01 mmol/L pepsatin A and 0·25 g PVPP (Sigma P-6755) per g fresh weight. Three mL

of homogenization buffer were used per g of plant material. Contamination of vacuolar and plasma membranes with thylakoids from broken chloroplasts was minimized by addition of 0·2% fatty acid-free BSA to the homogenization buffer. The homogenized material was filtered through cheesecloth, and the homogenate centrifuged for 15 min at 13 000 *g*. The 13 000 *g* supernatant was centrifuged for 30 min at 60 000 *g* and the resulting pellet was gently resuspended in a resuspension buffer containing 250 mmol/L sorbitol, 2·5 mmol/L HEPES-BTP (pH 7·4), and 1 mmol/L DTT. The resuspended microsomes (ER, Golgi, vacuolar and plasma membranes) were layered on a two-step gradient (6% and 12% dextran [average mol wt 73 000] made in resuspension buffer) and centrifuged at 70 000 *g* for 2 h. The 0–6% interface was collected and is referred to as vacuolar enriched vesicles; the 6–12% dextran interface was collected and is referred to as plasma membrane enriched vesicles.

Protein determination

Protein concentration was determined after precipitation with TCA by the Lowry method (Lowry *et al.* 1951) using BSA as the standard.

ATPase assays

ATPase activity, measured as release of inorganic phosphate from hydrolysis of ATP (Fiske & Subbarow 1925; Hodges & Leonard 1974; Schumaker & Sze 1986) was measured in a 0·5 mL volume. Reactions were initiated with the addition of membrane protein and conducted at 35°C for 60 min.

Plasma membrane ATPase activity (vanadate-sensitive) was measured in a medium containing 30 mmol/L HEPES (buffered with BTP to pH 6·7), 5 mmol/L $MgSO_4$, 5 mmol/L phosphoenol pyruvate, 4·5 units of pyruvate kinase (EC 2.7.1.40), 5 mmol/L ATP (buffered with BTP to pH 6·7), 0·01% triton X-100 and 10–15 μg of membrane protein, 50 mmol/L KCl to stimulate plasma membrane ATPase, 5 mmol/L Na azide to inhibit mitochondrial ATPases, and 0·1 μM Bafilomycin A_1 to inhibit vacuolar membrane ATPase. Vanadate-sensitive activity was calculated as the difference in activity in the absence and presence of 0·2 M sodium ortho-vanadate.

Vacuolar membrane ATPase activity (Bafilomycin A_1-sensitive) was measured in a medium containing 30 mmol/L HEPES (buffered with BTP to pH 7·5), 5 mmol/L $MgSO_4$, 5 mmol/L phosphoenol pyruvate, 4·5 units of pyruvate kinase, 5 mmol/L ATP (buffered with BTP to pH 7·5), 0·01% triton X-100 and 5–10 μg of membrane protein, 10 mmol/L Cl^--BTP to stimulate activity, 5 mmol/L Na azide to inhibit mitochondrial ATPases, and 0·2 mmol/L sodium ortho-vanadate to inhibit plasma membrane ATPase. Bafilomycin A_1-sensitive activity was calculated as the difference in activity in the absence and presence of 0·1 μM Bafilomycin A_1.

Determination of Na^+ and K^+

Shoot and root samples were dried at 65°C to constant weight. For the analysis of Na^+ and K^+, 25–50 mg dry leaves and young roots were digested in concentrated HNO_3 acid (2 mL for leaves and 3 mL for roots). Ion concentrations were determined by atomic absorption spectroscopy (Association of Official Analytical Chemists 1984).

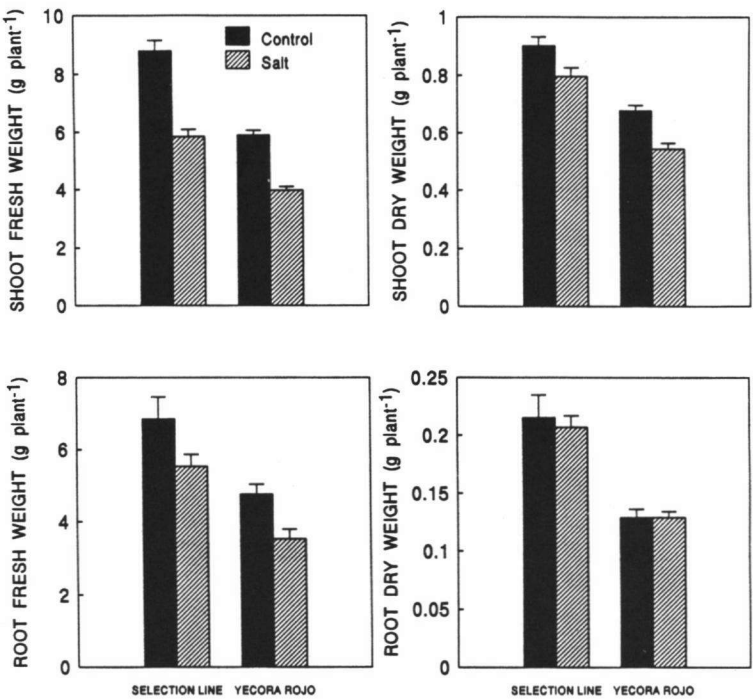

Fig. 1. Fresh and dry weights of shoots and roots of two lines of spring wheat when 22 day-old plants were subjected to 0 or 100 mmol/L NaCl in aerated solution culture for 8 days. Values represent means ± SE ($n=5$).

Statistical analysis

Analysis of variance of data for all the parameters was computed using the COSTAT computer package (CoHort Software, Berkeley, CA). The least significant difference between the mean values was calculated following Snedecor & Cochran (1980).

RESULTS

Data for fresh and dry weights of shoots and roots of two lines of spring wheat (Fig. 1) clearly show that 8 days of NaCl treatment had an inhibitory effect on the growth of both lines except in root DW. Selection line was superior to Yecora Rojo in all biomass parameters. Percentage reduction in shoot FW was almost similar in both lines at the salt treatment, but percentage reduction in shoot DW was higher in Yecora Rojo compared to Selection line (19·5% in Yecora Rojo and 11·2% in Selection line).

Na^+ and K^+ concentrations in the shoots and roots of the two lines presented on a tissue water basis (Fig. 2) show that Na^+ concentrations were significantly higher in the shoots and roots of Yecora Rojo compared to those of Selection line in the salt treatment. K^+ concentrations in the shoots of selection line increased in the salt treatment whereas those in Yecora Rojo decreased. Selection line had higher K^+ concentrations in the shoots than those of Yecora Rojo in the salt treatment. The reverse situation was observed in the roots since root K^+ concentrations decreased in Selection line in the salt treatment, whereas in Yecora Rojo they remained unaffected. Selection line had lower K^+ concentrations in the roots than Yecora Rojo in the saline treatment.

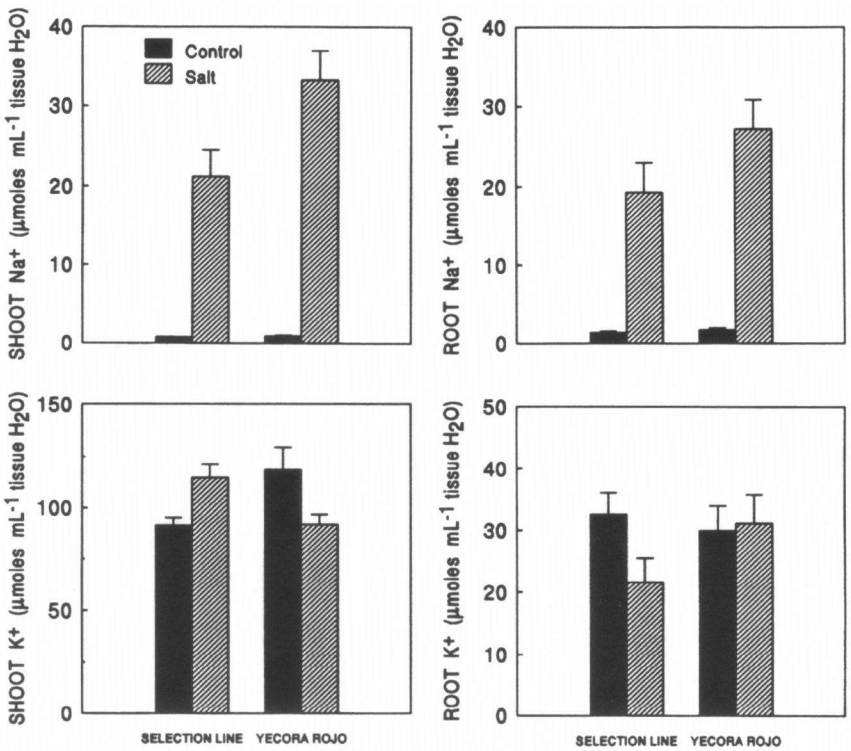

Fig. 2. Na^+ and K^+ concentrations (μmoles mL^{-1} tissue water) of shoots and roots of two lines of spring wheat when 22 day-old plants were subjected to 0 or 100 mmol/L NaCl in aerated solution culture for 8 days. Values represent means ± SE ($n=5$).

Table 1. K/Na ratios of shoots and roots of two lines of spring wheat when 22 day-old plants were subjected to aerated solution culture containing 0 or 100 mmol/L NaCl in full strength Hoagland's nutrient solution for 8 days. Values represent means ± SE ($n=5$). Means in the same column followed by the same letter are not significantly different ($P=0{\cdot}05$) according to LSD test

	mmol/L NaCl concentrations	
Lines	0	100
Shoot K/Na		
Selection line	114·2 ± 5·31a	6·00 ± 0·99a
Yecora Rojo	128·9 ± 6·34b	2·94 ± 0·42b
Root K/Na		
Selection line	26·12 ± 3·81a	1·17 ± 0·06a
Yecora Rojo	18·06 ± 2·52b	1·14 ± 0·03a

K/Na ratios of the shoots and roots of both lines differed significantly in the treatments without salt (Table 1). In the salt treatment, Selection line had significantly higher shoot K/Na ratio than Yecora Rojo, but did not differ in root K/Na ratio.

Table 2. Plasma membrane H^+-ATPase activity (μmoles P_i mg protein^{-1} h^{-1}) of shoots and roots of two lines of spring wheat when 22 day-old plants were subjected to aerated solution culture containing 0 or 100 mmol/L NaCl in full strength Hoagland's nutrient solution for 8 days. Values represent means ± SE (n=3)

Lines	mmol/L NaCl concentrations		Relative activity (%) $activity_{100}/activity_0$
	0	100	
Shoot H^+-ATPase activity			
Selection line	29·21 ± 1·36	34·07 ± 2·47	116·97 ± 9·16
Yecora Rojo	37·56 ± 2·71	20·34 ± 1·46	55·12 ± 7·18
Root H^+-ATPase activity			
Selection line	36·54 ± 8·56	32·12 ± 8·60	86·28 ± 9·48
Yecora Rojo	43·79 ± 5·51	56·37 ± 11·9	125·87 ± 11·63

PM-ATPase activity in the shoots (Table 2) was considerably reduced in Yecora Rojo in the salt treatment, whereas it was increased about 17% in Selection line compared to that in control. By contrast, PM-ATPase activity in the roots of Yecora Rojo was increased by about 25% in the salt treatment compared to the control, and in Selection line it was not significantly affected.

V-ATPase activity in shoots and roots was not significantly different among lines and was not affected by the salt treatment (data not shown). In addition, V-ATPase activity was negligible (less than 1·8 μmoles Pi mg protein^{-1} h^{-1}) in shoots but relatively higher in roots (about 4 μmoles Pi mg protein^{-1} h^{-1}).

DISCUSSION

From the results for Na^+ and K^+ concentrations of the two lines, it is evident that Selection line had enhanced discrimination between Na^+ and K^+ in transport to the shoots in the presence of salt so that it maintained a considerably high K/Na ratio in the shoot. This was the result of greater exclusion of Na^+ and higher uptake of K^+. These results reconfirm the findings of an earlier study in which these two lines were compared with some other lines of known degree of salt tolerance (Ashraf & O'Leary 1996). By contrast, these results slightly deviate from those of a recent study (Ashraf & O'Leary 1997), in which ion distribution in leaves of varying age in these two lines was examined, and it was found that both the lines did not differ in leaf K/Na ratios under saline conditions, although they showed differential pattern of Na^+ accumulation in their shoots as in the other two studies (Ashraf & O'Leary 1996, 1997). This variation in the results of the two studies can be explained on the basis of two reasons. First, in the present study ion analysis of a bulk sample of leaves was carried out, but in the latter study each individual leaf was separated into lamina and sheath and analysed separately. Secondly, in the present study plants of both lines were subjected to saline conditions for only 8 days, whereas in the other study a long-term treatment of salt was employed, i.e. from the seedling stage to to booting stage. Under such contrasting conditions it is naive to expect some variation in the results. In the present study the K/Na ratio in the shoot of Selection line was almost double that of the salt-sensitive line in the salt treatment. These results agree with the earlier findings of Wyn Jones *et al.* (1984), Schachtman *et al.* (1989) and Gorham (1990a, 1990b), in which they showed that

salt tolerance in hexaploid wheat was correlated with a high K/Na ratio in the shoots, and this high K/Na ratio was found to be maintained due to exclusion of Na^+ from the shoots.

Examination of data for ion distribution and PM-ATPase activity shows that high PM-ATPase activity in the leaves of the salt-tolerant Selection line may have been responsible for providing energy to pump out Na^+ from the cytoplasm through a secondary active transport (Na^+/H^+ exchanger). There is now strong evidence that the H^+-ATPase on the plasma membrane pumps out H^+ from the cytoplasm to the apoplast which results in establishing a charge and pH gradient, that can be harnessed to the transport of other ions through secondary transport (Sze 1985; Serrano 1985; Briskin 1990; Dupont 1992). Na^+ extrusion from cytoplasm to apoplast in the shoot of Selection line may have been responsible for maintaining normal functioning of metabolic processes in the cytoplasm. The low discrimination between Na^+ and K^+ in ion transport to the shoots of Yecora Rojo under salt stress is similar to what was found in barley (Gorham 1990b).

In our study, V-ATPase activity in spring wheat was not affected by the salt treatment, agreeing with results found in other glycophytes (Staal *et al.* 1991; Nakamura *et al.* 1992; Colombo & Cerana 1993). However, our results are in contrast to those of other researchers who found that V-ATPase activity increased with salinity in barley (Matsumoto & Chung 1988; Dupont & Morrisey 1991) and *Nicotiana* cultured cells (Reuveni *et al.* 1990). However, they do not contradict the hypothesis that lack of salt effect in the V-ATPase activity corresponds to the inability of the cell to store Na^+ in the vacuole. It is unknown if wheat cells are able to sequester Na^+ in the vacuole, as suggested for barley (Gorham 1990b) and demonstrated for *Nicotiana* cells (Hasegawa *et al.* 1990). In *Phaseolus vulgaris* L., one of the most salt-sensitive plant species known, an effective intracellular Na^+ compartmentation is lacking (Seemann & Critchley 1985). In addition, in halophytes, Na^+ is accumulated in the vacuole (Harvey *et al.* 1981; Matoh *et al.* 1987), and we have found an increased V-ATPase activity in the halophyte *Salicornia bigelovii* Torr grown in 200 mmol/L NaCl (Ayala *et al.* 1996).

The high activity of plasma membrane H^+-ATPase of the roots of Yecora Rojo is consistent with the observation that pumping out Na^+ from cytoplasm to apoplast leads to translocation of this ion to the shoot through the xylem stream (Pitman & Cram 1973; Flowers 1985), and could contribute to the high concentration of Na^+ in the shoots of Yecora Rojo. In contrast, the lack of effect of salt on the activity of root PM-ATPase in Selection line might have accounted for restricting the excessive transport of Na^+ to the shoots. A parallel situation has been observed in halophytes under saline conditions, in which a low level of salt concentration in the xylem compared to the growth medium prevents the halophytic species from excessive salt build-up in the shoot (Flowers 1985). This indicates that the major site of ion flux control is in the root of wheat rather than in the shoot, possibly due to the active role of xylem parenchyma cells (B. DeBoer 1985). Similar results were found in a relatively salt-tolerant crop, cotton, in which the degree of salinity during growth did not influence activity of H^+-ATPase in plasma membrane vesicles of roots (Hassidim *et al.* 1986).

The results presented here clearly support our hypothesis that plasma membrane H^+-ATPase activity differs in lines of wheat differing in salt tolerance, and suggest that salt-stimulation of plasma membrane H^+-ATPase activity in the shoots coupled with salt-insensitive activity in the roots might be important salt-tolerant traits in spring wheat.

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