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# An in Vivo Nuclear Magnetic Resonance Investigation of Ion Transport in Maize (*Zea mays*) and *Spartina anglica* Roots during Exposure to High Salt Concentrations[1]

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**The response of maize (*Zea mays* L.) and *Spartina anglica* root tips to exposure to sodium chloride concentrations in the range 0 to 500 mM was investigated using $^{23}Na$ and $^{31}P$ nuclear magnetic resonance spectroscopy (NMR). Changes in the chemical shift of the pH-dependent $^{31}P$-NMR signals from the cytoplasmic and vacuolar orthophosphate pools were correlated with the uptake of sodium, and after allowing for a number of complicating factors we concluded that these chemical shift changes indicated the occurrence of a small cytoplasmic alkalinization (0.1–0.2 pH units) and a larger vacuolar alkalinization (0.6 pH units) in maize root tips exposed to salt concentrations greater than 200 mM. The data were interpreted in terms of the ion transport processes that may be important during salt stress, and we concluded that the vacuolar alkalinization provided evidence for the operation of a tonoplast $Na^+/H^+$-antiport with an activity that exceeded the activity of the tonoplast $H^+$ pumps. The intracellular pH values stabilized during prolonged treatment with high salt concentrations, and this observation was linked to the recent demonstration (Y. Nakamura, K. Kasamo, N. Shimosato, M. Sakata, E. Ohta [1992] Plant Cell Physiol 33: 139–149) of the salt-induced activation of the tonoplast $H^+$-ATPase. Sodium vanadate, an inhibitor of the plasmalemma $H^+$-ATPase, stimulated the net uptake of sodium by maize root tips, and this was interpreted in terms of a reduction in active sodium efflux from the tissue. *S. anglica* root tips accumulated sodium more slowly than did maize, with no change in cytoplasmic pH and a relatively small change (0.3 pH units) in vacuolar pH, and it appears that salt tolerance in *Spartina* is based in part on its ability to prevent the net influx of sodium chloride.**

High external salt (NaCl) concentration is detrimental to many plants both as a result of the decreased external water potential, which inhibits cell expansion, and the build-up of sodium and chloride in the cytoplasm, which results in the inhibition of metabolic processes. Because salinity is often a problem in irrigated soils and can lead to severe decreases in crop yield, an understanding of salt tolerance and the basis of salt-induced damage is likely to be valuable. Early work mainly involved characterization of the physiological response of plants to salt, but it is also necessary to understand the mechanisms involved at the subcellular level, and recent studies have identified a number of different ion transport systems of possible relevance to NaCl uptake, exclusion, and compartmentation.

One transport system that is likely to be important in the response of plant cells to high external salt concentrations is the $Na^+/H^+$-antiport, a secondary ion pump that utilizes the proton gradient generated by primary pumps, such as the $H^+$-ATPase or the $H^+$-PPase, to drive sodium transport. $Na^+/H^+$-antiport activity has been shown to exist in a number of halophytes such as *Atriplex* (Braun et al., 1988; Matoh et al., 1989), sugar beet (Blumwald and Poole, 1987), *Dunaliella* (Katz et al., 1989, 1991), and *Plantago maritima* (Staal et al., 1991), as well as in the glycophyte barley (Gabarino and Dupont, 1988; Fan et al., 1989). However, other investigators have been unable to find antiport activity in another glycophyte, maize (Cheeseman, 1982; Jacoby and Rudich, 1985), or *Plantago media*, which is less salt tolerant than *P. maritima* (Staal et al., 1991). In some studies, for example, in *Atriplex nummularia* (Braun et al., 1988) and *Dunaliella salina* (Katz et al., 1991), the $Na^+/H^+$-antiport appears to be associated with the plasmalemma, but in the majority of studies the antiport activity has been found in the tonoplast, where it appears to be responsible for the uptake and sequestration of sodium into the vacuole, thus preventing the accumulation of toxic ions in the cytosol. Evidence for the importance of the tonoplast $Na^+/H^+$-antiport in salt tolerance is provided by the discovery that the antiport can be activated by salt treatment in barley (Gabarino and Dupont, 1989) and in *P. maritima* (Staal et al., 1991), although it is not yet clear how activation is achieved.

The activity of the $H^+$-ATPases may also be involved in determining the level of salt tolerance because these pumps are considered to be the main source of the proton gradient

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Abbreviations: NTP, phosphate of nucleoside triphosphate; PPase, pyrophosphatase; ppm, parts per million; PPP, tripolyphosphate; TTHA, triethylenetetraminehexacetic acid; UDPG, UDP-Glc.

that drives the $Na^+/H^+$-antiports. Braun et al. (1986) found an increased ATPase capacity in plasmalemma vesicles of *A. nummularia* after salt adaptation, and similar results have also been obtained for the tonoplast $H^+$-ATPase of *Nicotiana tabacum* (Reuveni et al., 1990). These results are in accord with the idea of an increased energy requirement for efflux of sodium from the cytosol during salt treatment. However, the importance of increased proton pumping to salt tolerance at the plasmalemma is unclear because it has also been well documented that nonionic osmotica such as sorbitol or PEG result in increased rates of proton extrusion from plant cells (Reinhold et al., 1984; Reuveni et al., 1987; Spickett et al., 1992).

Although a number of ion pumps and channels have been characterized in isolated membrane preparations, such studies do not give an unequivocal indication as to whether these mechanisms are active in vivo and the direction in which they usually operate under conditions such as ionic osmotic shock. One possible solution to this problem is to use in vivo NMR spectroscopy, because this technique allows the metabolic responses of plant tissues to be investigated noninvasively (Ratcliffe, 1987; Ratcliffe and Roberts, 1990). This approach has been used by a number of investigators studying the effects of exposure to salt, and evidence for the involvement of ion transport in the response to salt has come from the observation of apparent pH changes using in vivo $^{31}$P-NMR (Packer et al., 1987; Katsuhara et al., 1989; Kuchitsu et al., 1989). In particular, vacuolar alkalinization and the influx of sodium have been linked with activation of the tonoplast $Na^+/H^+$-antiport (Fan et al., 1989; Guern et al., 1989). On the other hand, some NMR studies have been unable to show effects on pH either in one or both of the intracellular compartments (Ben-Hayyim and Navon, 1985; Gerasimowicz et al., 1986; Fan et al., 1989).

In this investigation $^{23}$Na-NMR and $^{31}$P-NMR were used to characterize the response of the glycophyte *Zea mays* to different levels of NaCl. The effect of external NaCl on the concentrations of bioenergetic metabolites in this plant has been discussed elsewhere (Spickett et al., 1992), so in this paper we focus on the ion transport processes that may operate under these conditions, with the object of determining their relationship to the ability of the plant to withstand high salt concentrations. Similar experiments were also carried out on the halophyte *Spartina anglica* to compare the responses of salt-sensitive and salt-tolerant plants.

## MATERIALS AND METHODS

### Sample Preparation for NMR Experiments

Maize seeds (*Zea mays* L. cv LG11) were germinated in the dark for 3 d at 25°C between sheets of absorbent paper soaked in 0.1 mM $CaSO_4$. *Spartina anglica* C.E. Hubbard was obtained from a salt marsh on the Exe Estuary, Devon, UK, and was maintained in a glasshouse in vermiculite moistened with one-fifth strength Long Ashton nitrate medium (Hewitt and Smith, 1975). Root tips (5 mm) were excised and maintained in oxygenated standard buffer (1 mM KCl, 0.5 mM $CaSO_4$, 10 mM Mes [pH 6.0]). *Spartina* root tips were vacuum-infiltrated to improve the quality of the spectra by removing air bubbles from the tissue. Approximately 160 maize root tips or 80 to 100 *Spartina* root tips were placed in a 10-mm diameter NMR tube, and oxygenated standard buffer was circulated through the sample at a rate of 8 mL $min^{-1}$ (Lee and Ratcliffe, 1983). The tissue was allowed to equilibrate for 30 min before the NMR experiment. For control treatments, the tissue was incubated for 6 h on standard buffer; for salt treatments, samples were maintained on standard buffer for 90 min before changing to a solution consisting of standard buffer plus NaCl at concentrations from 0 to 500 mM for an additional 4- to 5-h incubation. For $^{23}$Na uptake experiments, the shift reagent $Dy(PPP)_2^{7-}$ was included in the circulating buffer at the following concentrations: 5 mM $Dy(PPP)_2^{7-}$ for 100 mM NaCl, 10 mM $Dy(PPP)_2^{7-}$ for 200 mM NaCl, and 12.5 mM $Dy(PPP)_2^{7-}$ for 300 mM NaCl. The inhibitors sodium vanadate and amiloride were used at concentrations of 5 and 0.1 mM, respectively, and were included in the standard buffer as well as the salt treatment buffer to allow 90 min for the inhibitors to take effect.

### Preparation of Shift Reagents

The shift reagent $Dy(PPP)_2^{7-}$ was produced by a method similar to that of Ramasamy et al. (1989). Pentasodium PPP was passed through a column of Dowex-50W (protonated form) into a solution of tetramethylammonium hydroxide. The resulting tetramethylammonium PPP was freeze-dried and redissolved before combining with $DyCl_3$ to give $Dy(PPP)_2^{7-}$ in Mes buffer. $Dy(TTHA)^{3-}$ was produced directly by the reaction of $DyCl_3$ with the fully protonated ligand, $H_6TTHA$.

### NMR Methods

$^{31}$P-NMR spectra were recorded with a double-tuned $^{13}$C/$^{31}$P probe on a Bruker CXP300 spectrometer operating at a phosphorus-31 frequency of 121.49 MHz. All spectra were acquired with a spectral window of 8064 Hz. Spectra were recorded from tissue in vivo using a 90° pulse angle and a 0.8-s recycle time because fully relaxed spectra were not required. These spectra were acquired sequentially during the time course in files of 1125 scans (equivalent to 15 min), and each free induction decay contained 1K data points. Chemical shifts were measured relative to the signal from a capillary containing a 2% (v/v) aqueous solution of the tetraethyl ester of methylenediphosphonate. This signal occurs at 22.49 ppm relative to the signal from 85% $H_3PO_4$, and its line shape was improved by $^1$H-decoupling.

The pH values of the intracellular compartments were calculated from the chemical shifts of the Pi resonances after construction of appropriate calibration curves. The calibration solution for the cytoplasmic calibration contained 100 mM KCl, 5 mM $MgSO_4$, 2 mM $NaH_2PO_4$, and 3 mM $Na_2HPO_4$, and the vacuolar calibration solution contained 20 mM KCl, 50 mM $MgCl_2$, 10 mM citric acid, 5 mM malic acid, and 5 mM $NaH_2PO_4$ (pH 5.25). The effect of NaCl on the chemical shift of Pi was determined using the same cytoplasmic and vacuolar calibration solutions at pH 7.35 and 5.25, respectively, with NaCl concentrations of 0 to 2 M included in the solutions.

$^{23}$Na spectra were acquired using a broad-band probe tuned

to 79.38 MHz with a spectral window of 4032 Hz, a pulse angle of 60°, and a recycle time of 0.2 s. The spectra were acquired sequentially during the time course in files of 6000 scans (corresponding to 20 min), and each free induction decay contained 4K data points. Chemical shifts were referenced to the unshifted intracellular resonance at 0 ppm, and the extracellular resonance was shifted upfield using the shift reagent $Dy(PPP)_2^{7-}$ (Chu et al., 1984). The concentration of sodium in the tissue was calculated by comparing the intensity of the intracellular sodium resonance with that of a previously calibrated capillary containing 2 M NaCl, 40 mM dysprosium TTHA, which gives a resonance downfield of the intracellular sodium (Chu et al., 1984). The sample volume was measured as described by Spickett et al. (1992), and this was used to calculate the intracellular sodium concentration; the change in tissue volume during treatment was taken into account in the calculations.

### Determination of Sodium Visibility

The visibility of sodium by NMR in vivo was determined by extracting the tissue at the end of the experiment. The root tips were washed for 5 min in 10 mM $CaSO_4$ to remove apoplastic sodium and then dried in an oven at 80°C. The dried tissue was extracted by addition of a few drops of concentrated nitric acid, and the cell debris was removed by centrifugation at 12,000*g*. The sample was then neutralized and made up to 5 mL. The sodium concentration in vitro was measured by $^{23}$Na-NMR.

### Estimation of Relative Cytoplasmic and Vacuolar Volumes

Patel et al. (1990) showed that the cytoplasmic and vacuolar compartments each occupies about 45% of the total tissue volume in 5-mm pea root tips, with the remaining 10% of the volume being occupied by the cell wall and intercellular spaces. In this work the volume corresponding to the apoplast and intercellular spaces was ignored because only the intracellular volume was of interest. The relative cytoplasmic volumes in maize and *Spartina* were estimated by comparing the protein content of the tissues with that of 5-mm pea root tips.

### Determination of Protein Concentration

Root tips (5 mm) from maize, pea (*Pisum sativum* L. cv Meteor), or *Spartina* were extracted by homogenization in 100 mM potassium phosphate (pH 7.5), 0.5 mM EDTA, and 2% PVP-40, and the homogenate was clarified by centrifugation. The protein concentration was determined using the method of Bradford (1976), and the assay was calibrated with γ-globulin.

## RESULTS

In vivo $^{31}$P-NMR spectra of maize root tips were acquired during time courses of up to 4 h during treatment with NaCl at concentrations from 0 to 500 mM. The effect of 500 mM NaCl over time on the phosphorus spectra of maize is shown in Figure 1. It can be seen that the levels of Glc-6-P and UDPG decreased considerably during the treatment and that

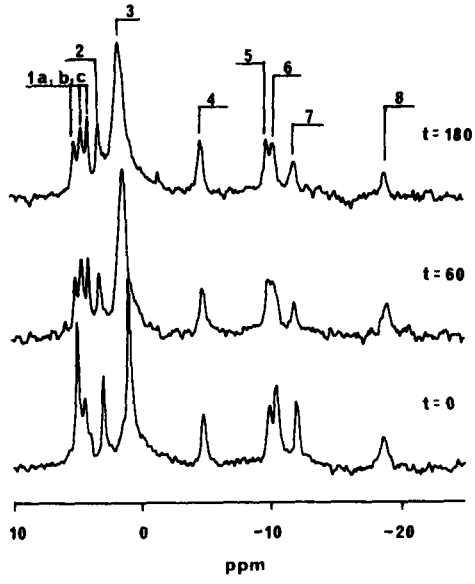


**Figure 1.** $^{31}$P-NMR spectra of excised maize root tips after treatment with 500 mM NaCl for different lengths of time. The spectra were acquired in 15 min with a 0.8-s recycle time and are the sum of 1125 transients. The resonance assignments are as follows: 1a, Glc-6-P; 1b, Fru-6-P and other phosphomonoesters; 1c, phosphocholine; 2, cytoplasmic phosphate; 3, vacuolar phosphate; 4, γ-phosphate of NTP and β-phosphate of nucleoside diphosphate; 5, α-phosphates of NTP and nucleoside diphosphate; 6, UDPG and NAD(P)(H); 7, UDPG; and 8, β-phosphate of NTP.

there was a substantial increase in the concentration of phosphocholine. These changes are larger than those observed in control-treated tissue (data not shown), and they indicate that high salt concentrations adversely affect the energy metabolism in the tissue. These metabolic changes, which could be due to either an increased energy requirement for ion transport or the inhibition of bioenergetic pathways, such as oxidative phosphorylation and glycolysis, have been discussed elsewhere (Spickett et al., 1992) and are in agreement with responses observed in other excised root tissues (Gerasimowicz et al., 1986; Fan et al., 1989). Although the changes are similar to those observed in starved tissue (Roby et al., 1987), it is unlikely that starvation contributes significantly to the response, because the changes in metabolite levels are reversible (data not shown). It is more probable that the effects are due to a limiting turnover of bioenergetic metabolites compared to the rate of energy utilization. It can also be seen that the vacuolar phosphate resonance broadened and shifted downfield during the period of treatment and that there was also a less noticeable but nevertheless significant shift of the cytoplasmic phosphate resonance in the same direction. Such changes in the chemical shifts of the intracellular phosphate resonances and the broadening of the vacuolar phosphate signal have been noted previously and have been attributed to changes in intracellular pH (Packer et al., 1987; Fan et al., 1989; Katsuhara et al., 1989; Takagishi et al., 1991).

Figure 2 shows the concentration-dependent response to salinity of the chemical shift of the cytoplasmic and vacuolar

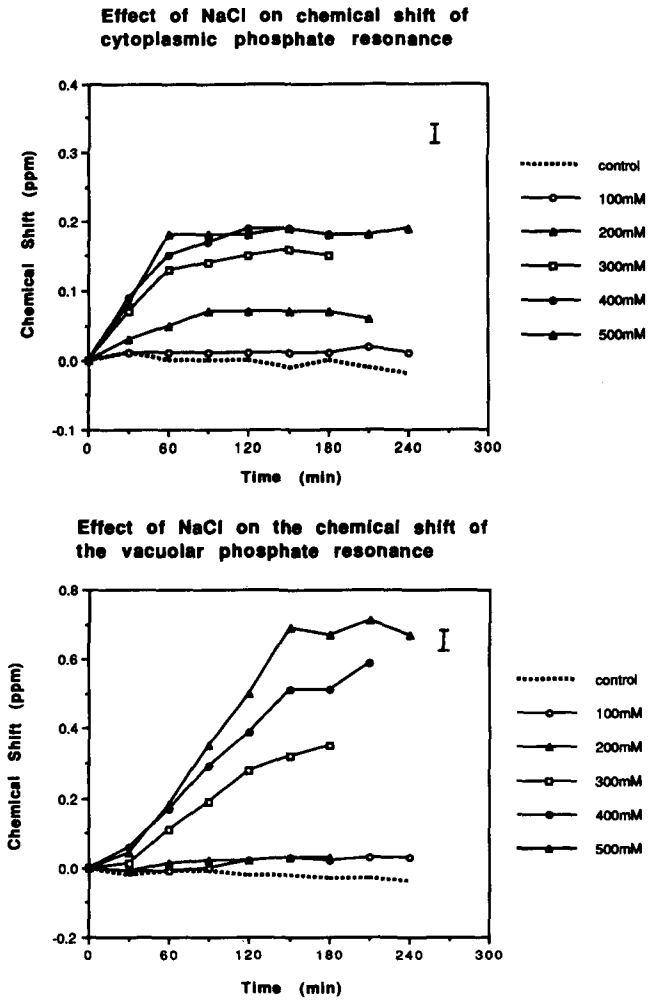


**Figure 2.** The effect of increasing salt concentration on the chemical shifts of the cytoplasmic and vacuolar phosphate resonances of maize root tips. The changes in chemical shift for each resonance are shown during the time course of the treatments. The error bars correspond to the averaged SD (control, $n = 9$; 100 and 200 mM salt, $n = 3$; 300 and 400 mM salt, $n = 4$; 500 mM salt, $n = 7$).

phosphate peaks determined from the $^{31}$P-NMR spectra. For the cytoplasmic phosphate resonance, NaCl concentrations of 200 mM and greater caused immediate changes in chemical shift compared to control treatment (no NaCl), whereas NaCl concentrations of at least 300 mM were required to cause a clear shift in the vacuolar phosphate resonance, and there was a lag period of approximately 30 min before the change in chemical shift of the vacuolar phosphate resonance became apparent. Figure 2 also shows that the changes in chemical shift reached a maximum after approximately 60 min for cytoplasmic phosphate and 150 min for vacuolar phosphate. It can be seen that the cytoplasmic response appears to show a saturation effect at high external salt concentrations, because the curves for the chemical shift changes at 300 to 500 mM NaCl are much closer together than those for the lower salt treatments. No such effect was observed for the vacuolar response.

Although the chemical shifts of phosphate resonances are often taken to be directly related to the intracellular pH in this type of experiment (Packer et al., 1987; Fan et al., 1989; Katsuhara et al., 1989), changes in ionic composition can also affect their chemical shifts (Roberts et al., 1981). The effect of NaCl concentration on the chemical shift of phosphate under conditions mimicking those in the cytoplasm and vacuole was determined in vitro, and the results are shown in Figure 3. Increasing the NaCl concentration caused the phosphate signals to shift downfield, i.e. the same effect as increasing the pH of the solution. It can be seen that the phosphate chemical shift was more sensitive to the NaCl concentration under cytoplasmic conditions than under vacuolar conditions, but that there was a significant shift in both cases over the concentration range 0 to 500 mM. Thus, if sodium and chloride accumulate in the tissue during treatment with salt it may not be possible to relate changes in chemical shift directly to changes in intracellular pH.

The uptake of sodium at various external concentrations of NaCl was monitored in vivo by $^{23}$Na-NMR. Figure 4 shows the changes in the sodium spectra of maize during several hours of treatment with 200 mM NaCl. Comparison of the sodium content in salt-treated maize root tips before and after extraction with nitric acid showed that the visibility of intracellular sodium was approximately 100% (data not shown). Figure 5 shows the calculated net uptake during treatment with external NaCl concentrations of 100 to 300 mM, and it can be seen that there is a lag of approximately 30 min before the maximum rate of uptake is reached. This is similar to the lag observed in the vacuolar phosphate chemical shift

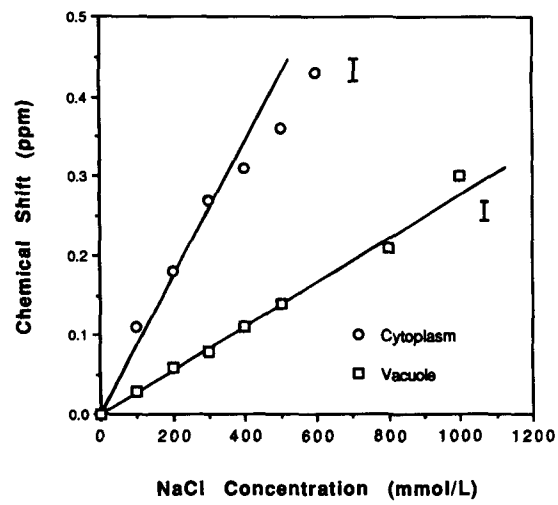


**Figure 3.** The effect of NaCl concentration on the chemical shift of the phosphate resonance in vitro under conditions that mimic the cytoplasmic and vacuolar environment. The cytoplasmic calibration was carried out in 5 mM sodium phosphate (pH 7.35), 100 mM KCl, and 5 mM $MgSO_4$ plus varying concentrations of NaCl, and the vacuolar calibration was carried out in 5 mM sodium phosphate (pH 5.25), 20 mM KCl, 50 mM $MgCl_2$, 10 mM citric acid, and 5 mM malic acid plus varying concentrations of NaCl. The error bars correspond to the averaged SD ($n = 3$ for cytoplasmic conditions and $n = 4$ for vacuolar conditions).

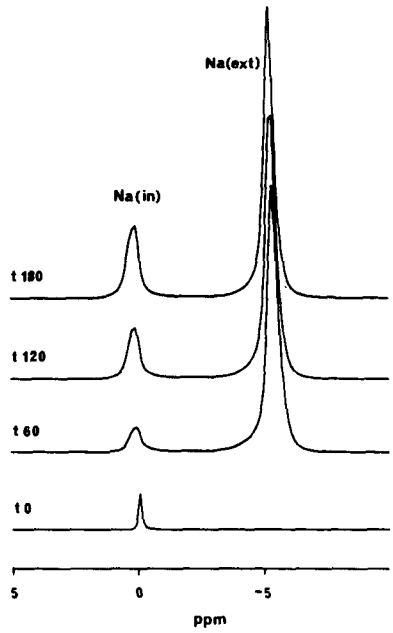


**Figure 4.** $^{23}$Na-NMR spectra of maize root tips treated with 200 mM NaCl at different times after the start of treatment. The spectra are the sum of 6000 scans, acquired over 20 min with a recycle time of 0.2 s. The external (ext) sodium resonance was shifted upfield using the shift reagent $Dy(PPP)_2^{7-}$, and chemical shifts are given relative to the internal (in) sodium resonance before salt treatment was initiated. The resonance corresponding to the intensity standard (2 M NaCl, 40 mM $Dy[TTHA]^{3-}$) occurred downfield of the internal resonance and is not shown in these spectra.

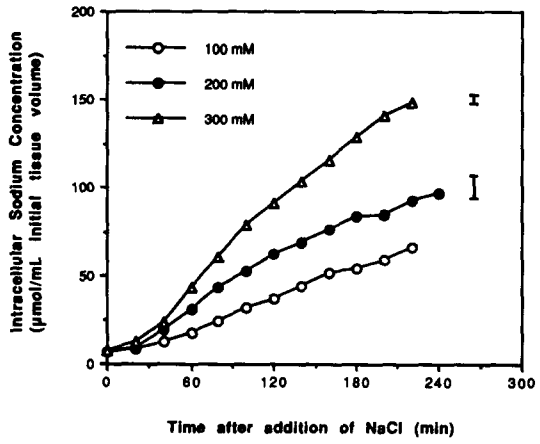


**Figure 5.** The profile of sodium uptake by maize root tips as measured by $^{23}$Na-NMR, at external salt concentrations of 100, 200, and 300 mM. The intracellular sodium concentration was calculated by comparison of the resonance intensity with that of a previously calibrated standard containing 2 M NaCl, 40 mM dysprosium TTHA, taking into account the volume occupied by the tissue in the NMR tube. The error bars show the averaged SD (100 mM salt, $n = 2$; 200 mM salt, $n = 4$; 300 mM salt, $n = 3$).

changes and would be consistent with the majority of the sodium being taken up into the vacuole. The rates of net sodium influx, calculated over the linear part of the slopes, were found to be 19.0 $\mu$mol mL$^{-1}$ h$^{-1}$ for 100 mM NaCl, 32.5 $\mu$mol mL$^{-1}$ h$^{-1}$ for 200 mM NaCl, and 49.0 $\mu$mol mL$^{-1}$ h$^{-1}$ for 300 mM NaCl. These influx rates appear to be approximately linearly dependent on concentration over the range investigated, and there was no indication of saturation of the uptake mechanism at the higher salt concentrations. After the lag period, uptake was reasonably constant over the observed time course, with possibly a slight decrease in rate after the first 2 h of treatment at the higher concentrations. It was found that for an external NaCl concentration of 200 mM the rate of uptake decreased after 5 to 6 h, although influx continued to approximately 10 h before reaching a steady state with an intracellular concentration approaching the external concentration (data not shown).

Figure 6 shows the effect of amiloride, an inhibitor of the $Na^+/H^+$-antiport (Brumwald et al., 1987), and sodium vanadate, an inhibitor of the plasmalemma $H^+$-ATPase (Brummell et al., 1986), on the rate of sodium uptake at an external concentration of 300 mM. It can be seen that while amiloride had little effect on the uptake profile (net uptake rate = 45.5 $\mu$mol mL$^{-1}$ h$^{-1}$), treatment with vanadate resulted in a substantial increase in the rate of sodium influx (net uptake rate = 84.0 $\mu$mol mL$^{-1}$ h$^{-1}$). Because both of these compounds are thought to be incomplete inhibitors in vivo (Brummell et al., 1986; Blumwald et al., 1987), the lack of inhibition observed with amiloride does not necessarily indicate that an $Na^+/H^+$-antiport is not operating under these conditions.

Although $^{23}$Na-NMR cannot distinguish between sodium in the cytoplasm and in the vacuole, the maximum possible concentration in these compartments, corresponding to a situation in which all of the sodium is sequestered in one compartment, can be calculated from the NMR data using estimates of the cytoplasmic and vacuolar volumes. The protein content of 5-mm maize root tips (55 ± 2 mg g$^{-1}$ fresh weight) was very similar to the value for pea root tips (57 ±

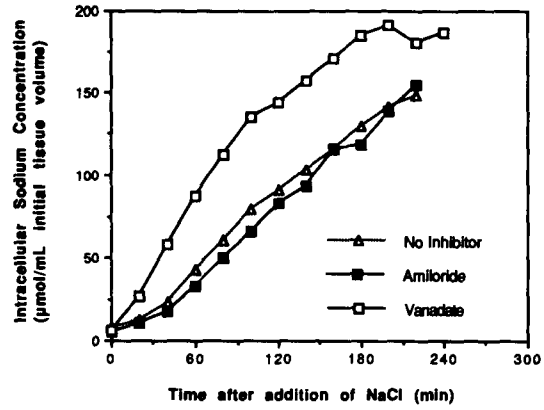


**Figure 6.** The effect of the inhibitors vanadate and amiloride on uptake of sodium by maize root tips treated with 300 mM NaCl. The intracellular sodium concentration was calculated as described for Figure 5. $n = 3$ for standard treatment and treatment with amiloride; $n = 2$ for treatment with sodium vanadate.

2 mg $g^{-1}$ fresh weight), indicating that in 5-mm maize root tips the relative cytoplasmic volume is similar to the 50% of intracellular volume found in pea (Patel et al., 1990). Thus, in 5-mm root tips, the maximum possible sodium concentration in either the cytoplasm or the vacuole is approximately twice the intracellular sodium concentration.

The changes in chemical shift of the cytoplasmic and vacuolar phosphate resonances that could be produced by this maximum possible sodium concentration, assuming that the chemical shift change is due entirely to the ionic strength effect, can be calculated using the data in Figure 3. The calculated values for treatment with 300 mM salt are compared with the changes in chemical shift measured by $^{31}$P-NMR for the same treatment (Fig. 7). It can be seen that the calculated change in vacuolar chemical shift is much smaller than the measured change, indicating that the change in chemical shift must be largely due to an alkalinization of the vacuole. For the cytoplasmic compartment, the data between 0 and 60 min indicate a small increase in pH, and because it is unlikely that all of the intracellular sodium is confined to the cytoplasm, it can be concluded that a cytoplasmic alkalinization occurred for at least the first 2 h of salt treatment.

Experiments similar to those described above were carried out on root tips of the halophyte *S. anglica*. Although $^{31}$P-NMR experiments showed that *Spartina* had similar phosphorus spectra to maize, the quality of the spectra was not as good because of the smaller amount of tissue available and the lower percentage of cytoplasm in the root tips. Comparison of the protein content of 5-mm *Spartina* root tips (44 ± 8 mg $g^{-1}$ fresh weight) with the protein concentration of pea root tips indicated a cytoplasmic volume of only 38% in *Spartina*. Figure 8 shows that upon treatment with 500 mM NaCl the vacuolar phosphate resonance broadened and was shifted slightly downfield, whereas no change in the position of the cytoplasmic phosphate resonance was observed. This can be seen more clearly in Figure 9, which gives the change in the chemical shifts of the phosphate resonances during the time course of the experiment. The most obvious difference between *Spartina* and maize was the absence of a change in the chemical shift of the cytoplasmic phosphate. The vacuolar response was similar to that of maize in that there was a lag of 30 min before any change occurred, and the

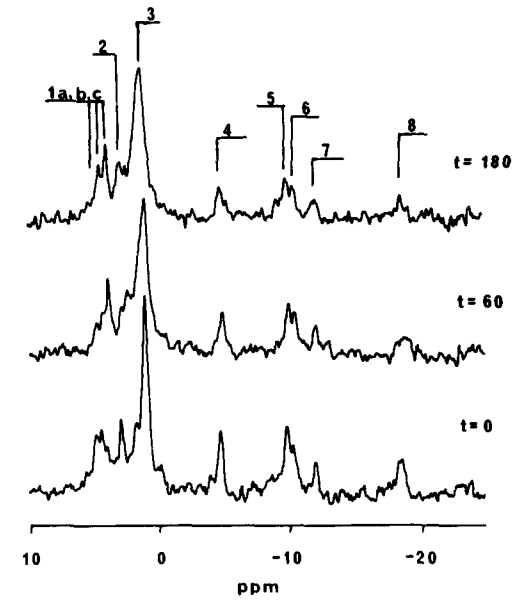


**Figure 8.** $^{31}$P-NMR spectra of *S. anglica* root tips at different times after the start of treatment with 500 mM NaCl. The acquisition parameters are the same as those described in Figure 1, except that the spectra are the sum of 2250 scans collected over 30 min.

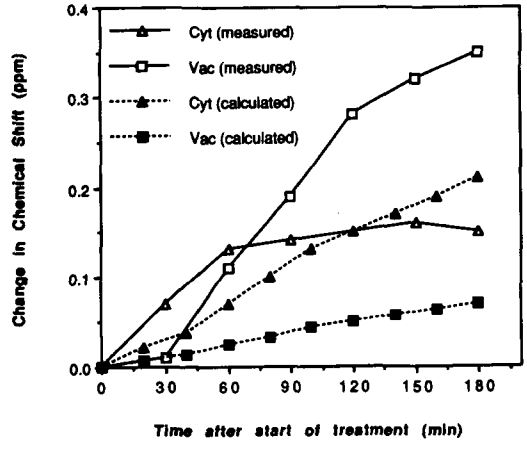


**Figure 7.** Comparison of the changes in chemical shift of the cytoplasmic and vacuolar phosphate resonances measured by $^{31}$P-NMR during 300 mM salt treatment with the changes calculated from the maximum possible sodium concentration, assuming that the changes were due solely to ionic strength and using the information in Figure 3. The maximum sodium concentration was taken to be twice the average intracellular concentration measured by $^{23}$Na-NMR during exposure to 300 mM salt (Fig. 5), because the volumes of the cytoplasm and the vacuole were each estimated to be approximately 50% of the intracellular volume.

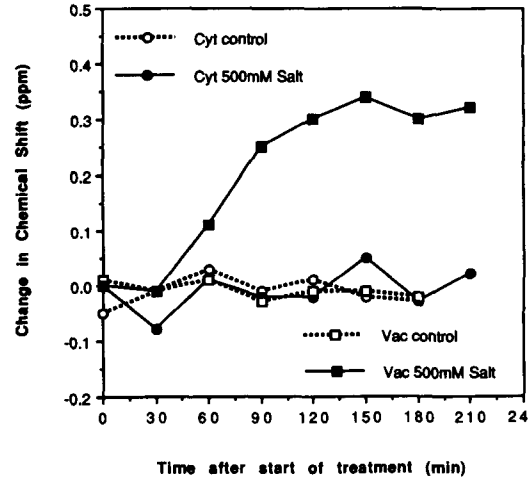


**Figure 9.** The changes in chemical shift of cytoplasmic and vacuolar phosphate resonances of *S. anglica* root tips during control treatment and treatment with 500 mM salt. $n = 3$ for treatment with salt; $n = 2$ for control treatment.

maximum change was reached after about 150 min, but the downfield shift of the vacuolar peak was considerably smaller than that observed in maize (0.3 ppm as opposed to 0.7 ppm). This suggests either that less salt is taken up into *Spartina* or that the tonoplast proton pump(s) are able to cope better with the dissipation of the proton gradient by the $Na^+/H^+$-antiport. The rate of sodium uptake by *Spartina* at an external NaCl concentration of 200 mM was measured using $^{23}$Na-NMR. Comparison of the uptake profiles of maize and *Spartina* at this concentration showed that, although the rate was initially comparable to that of maize, it decreased after approximately 60 min to give a much slower rate during the following 4 to 6 h (Fig. 10). After 4 h, the concentration of sodium in *Spartina* was approximately 40% of that observed in maize under the same conditions.

## DISCUSSION

The aim of this work was to investigate the response of some of the ion transport processes in maize root tips to salt treatment, using the noninvasive techniques of in vivo $^{23}$Na- and $^{31}$P-NMR. This experimental approach enabled the uptake of sodium to be correlated with changes in the chemical shifts of the pH-dependent $^{31}$P-NMR signals from the cytoplasmic and vacuolar phosphate pools. However, two related factors complicated the subsequent interpretation of the data in terms of the operation of proton-linked transport systems. First, there is as yet no technique for distinguishing the contributions of the cytoplasmic and vacuolar sodium pools to the in vivo $^{23}$Na-NMR signals. Although the observed 100% visibility of the tissue sodium provided weak support for the vacuolar location of the bulk of the sodium (because the molecular interactions that lead to the quadrupolar broadening of the in vivo $^{23}$Na-NMR signal are more likely to occur in the protein- and nucleic acid-rich cytoplasm), it was necessary to make assumptions about the subcellular distribution of the sodium on the basis of earlier investigations. Second,

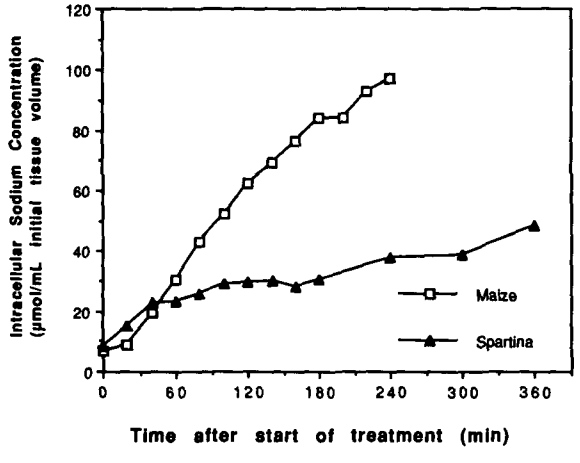


**Figure 10.** Comparison of the rates of sodium uptake by *Spartina* and maize root tips during treatment with 200 mM salt. The intracellular sodium concentration of *Spartina* was calculated as described for maize in Figure 5 ($n$ = 2 for *Spartina* and $n$ = 4 for maize).

it was found that the concentration of NaCl had a direct effect on the chemical shift of the $^{31}$P-NMR phosphate signal under both cytoplasmic and vacuolar conditions (Fig. 3), implying that changes in chemical shift observed in vivo could be due, at least in part, to changes in the ionic composition of these compartments during sodium uptake. This effect was not considered fully in earlier NMR investigations (Packer et al., 1987; Fan et al., 1989; Katsuhara et al., 1989; Kuchitsu et al., 1989), and it limits the extent to which changes in chemical shift can be interpreted in terms of changes in pH.

Despite these complications, Figure 7 shows that the change in chemical shift of the vacuolar phosphate signal cannot be explained simply by the accumulation of sodium and a counterion in the vacuole. The same is true for the cytoplasmic phosphate signal for the first 2 to 3 h of salt treatment, and although the subsequent values of the chemical shift could be explained on the assumption that all of the sodium remains in the cytoplasm, this seems unlikely because it is thought that salt is usually sequestered in the vacuole (Flowers et al., 1977; Greenway and Munns, 1980). It can be calculated from the data in Figures 2, 3, and 5 that exposure to 300 mM NaCl for 3 h caused a vacuolar alkalinization of at least 0.6 pH units. Similar calculations for the cytoplasm indicate a pH change of between −0.05 unit (i.e. an acidification), if all the sodium and a counterion accumulated in this compartment, and +0.2 unit, if the sodium was sequestered in the vacuole. It seems reasonable to conclude that there was a small alkalinization of the cytoplasm, corresponding to at least 0.1 pH unit, but as with the vacuolar pH, it is not possible to measure the pH change with great accuracy because of the uncertainty in the ionic composition of the individual compartments during sodium uptake. This uncertainty is reflected in both the compartmental location of the sodium and the extent to which compensating movements of other ions (e.g. potassium) influence the net effect on the subcellular ionic strength. As a result, the pH changes reported here are effectively lower limits to the actual pH changes that occur in response to salt uptake. These pH changes are comparable with many of those inferred from other in vivo $^{31}$P-NMR studies, although comparisons are difficult because of the wide range of salt concentrations that have been used. Gerasimowicz et al. (1986) observed no pH changes in excised maize root tips, but their experiments were conducted at a relatively low salt concentration (160 mM), and Figure 2 confirms that this salt concentration has no effect in maize under these conditions. Increases in the cytoplasmic and/or vacuolar pH have been observed in several other in vivo NMR studies (Packer et al., 1987; Fan et al., 1989; Kuchitsu et al., 1989). The only exception appears in the data of Katsuhara et al. (1989), who reported a cytoplasmic acidification in *Nitellopsis obtusa* in response to 100 mM salt, but this could have resulted from lack of oxygenation of the tissue, and quantitation would not have been facilitated by the poor quality of the spectra.

Intracellular pH changes have also been found to occur in plant tissue treated with nonionic osmotica, and these effects have been attributed to an activation of the plasmalemma $H^+$-ATPase as part of the detection and signaling process of osmoregulation (Reinhold et al., 1984; Reuveni et al., 1987;

Spickett et al., 1992). In maize, sorbitol, mannitol, and PEG produced a cytoplasmic alkalinization of similar magnitude (0.1 unit) to that observed with salt treatment, whereas the vacuolar alkalinization was smaller, approximately 0.3 unit (Spickett et al., 1992). The time course of the pH changes was different, because the effects of nonionic osmotica became apparent after about 90 min of treatment, in contrast to the salt-induced changes, which occurred immediately in the cytoplasm and after 30 min in the vacuole. Because salt treatment causes osmotic shock as well as ion overload, the observed pH changes may be due in part to an osmotic effect, but it is clear that other effects resulting from the ionic component of the treatment occur simultaneously.

The alkalinization of the vacuole during salt treatment is most probably caused by tonoplast ion transport, because there are relatively few metabolic processes occurring in the vacuole, and it is consistent with tonoplast $Na^+/H^+$-antiport activity. However, it also implies that the antiport activity exceeds the activity of the primary tonoplast pumps ($H^+$-ATPase and $H^+$-PPase) that normally maintain $\Delta pH$ across the tonoplast. At external salt concentrations of 200 mM and below, there was no vacuolar alkalinization, indicating that the primary proton pumps were able to match the rate of proton movement by the antiport, but at higher salt concentrations, the rate of the primary pumps was insufficient to prevent dissipation of the proton gradient. This suggests that the antiport has a greater capacity than the $H^+$ pumps. However, the external salt concentration at which the changes in pH became apparent corresponded to the concentration at which effects on the levels of bioenergetic metabolites such as ATP, Glc-6-P, and UDPG were first observed (Spickett et al., 1992), and so it is also possible that the activity of the $H^+$ pumps was limited by substrate supply rather than by their intrinsic capacity. An alternative explanation for the salt-induced alkalinization of the vacuole is that it reflects an inhibition of the tonoplast $H^+$-PPase by cytoplasmic sodium (Katsuhara et al., 1989; Nakamura et al., 1992b). It is difficult to assess the relative merits of these two explanations, but in the absence of any evidence for a high cytoplasmic sodium concentration in the root tissue, it seems more likely that it is the $Na^+/H^+$-antiport that is responsible for the alkalinization.

It was also found that the changes in chemical shift of the phosphate resonances reached a maximum after approximately 60 min for the cytoplasmic peak and 150 min for the vacuolar one. It has been shown that in the vacuole the majority of the chemical shift change was due to a pH effect, and, therefore, it can be concluded that the pH stabilized after this time. This suggests that there was either a decrease in the activity of the $Na^+/H^+$-antiport or an activation of the primary $H^+$ pumps that prevented further dissipation of the proton gradient. In this study, the rate of sodium uptake was found to be maintained for considerably longer than 150 min, although it is not known in which compartment it is located, so a decrease in the antiport activity (possibly resulting from the decrease in proton gradient) would imply that sodium accumulates in the cytoplasm. It seems more probable that the stabilization is due to increased activity of either the tonoplast $H^+$-ATPase or $H^+$-PPase. Nakamura et al. (1992a) observed salt-induced activation of the tonoplast $H^+$-ATPase, whereas the $H^+$-PPase was found to be inhibited at high salt concentrations, suggesting that the ATPase is most likely to be responsible for the pH stabilization. In the cytoplasm where the salt concentration has a substantial direct effect on chemical shift, stabilization may be due to the achievement of a steady-state level of cytoplasmic sodium as sodium entering the tissue is sequestered in the vacuole.

For sodium to be sequestered in the vacuole by the $Na^+/H^+$-antiport, it must first enter the cell across the plasmalemma, and this could occur via an $Na^+/H^+$-antiport or a cation channel. Reuveni et al. (1987) suggested that in cultured carrot cells an antiport is responsible for sodium influx to explain the large external acidification observed during salt treatment. However, the plasmalemma antiport is usually conceived as a mechanism for maintaining a low intracellular sodium concentration by using the plasmalemma proton gradient to export sodium from the cell, although it is likely to be reversible and the direction of the fluxes should be dependent on the relative magnitudes of $\Delta\tilde{\mu}_{H+}$ and $\Delta\tilde{\mu}_{Na+}$ (Reinhold et al., 1989). In the present experiments, it can be calculated that for a $\Delta pH$ of 1.5 units external sodium chloride concentrations greater than 300 mM could be expected to cause initial reversal of the antiport (sodium influx and proton efflux) but that the direction of transport would be rectified as soon as the intracellular sodium concentration increased. Sodium vanadate, an inhibitor of the plasmalemma $H^+$-ATPase, was found to increase the rate of sodium influx throughout the time course of treatment with 300 mM salt. This suggests that an active efflux of sodium was occurring at the antiport, driven by the $\Delta\tilde{\mu}_{H+}$ produced by the plasmalemma $H^+$-ATPase, and that inhibition of the ATPase by vanadate reduced sodium efflux and, therefore, increased the net influx. This result contrasts with the previous findings of Cheeseman (1982) and Jacoby and Rudich (1985) that there is no evidence for a plasmalemma $Na^+/H^+$-antiport in maize.

From these results, it appears that sodium is exported from the cytoplasm both at the tonoplast and at the plasmalemma. Sodium is, therefore, more likely to enter the cell via a plasmalemma cation channel or uniport, a process that would be favorable because of the large negative transmembrane potential. Cheeseman (1982) suggested a sodium uniport to account for passive influx of sodium in maize roots. Specific sodium channels have not yet been identified in plants, but tonoplast channels with equal selectivity for $K^+$ and $Na^+$ have been reported in tomato and sugar beet (Pantoja et al., 1989) and *Plantago* (Maathius and Prins, 1990), whereas in *Chara*, plasmalemma $K^+$ channels with a lower selectivity for $Na^+$ have been identified (Tester, 1990). In roots of intact maize seedlings, Schubert and Lauchli (1988) observed both active and passive components of sodium efflux.

Although chloride influx was not measured directly in this study, it was probably comparable to the sodium uptake, because measurement of intracellular malate content did not show any significant increase during salt treatment (data not shown). Malate may be synthesized endogenously in plant tissues if the cation influx greatly exceeds the anion influx (Osmond, 1976). Although it appears that $Cl^-$ enters the tissue, it is not clear whether it is compartmentalized in the vacuole with the sodium.

It is important to remember that the response of excised

maize root tips to salinity is likely to differ from that of intact seedlings, where salt may be transported to the shoot and accumulated to a high concentration in the leaf tissue. It is known that excised nontranspiring tissue and cell suspensions are more salt tolerant than intact plants, which typically cannot survive 100 to 200 mM salt (Flowers et al., 1977). However, in this study, we investigated a short-term cellular response as opposed to a physiological response during a period of days, and it is probable that the salt concentrations used would also be toxic to the excised root tips during a longer period, because it was noted that elongation was inhibited during the time course of the experiments (data not shown). It appears to be reasonable that the initial response should involve redirection of resources from growth and differentiation to adaptive metabolism, and, hence, the effects observed here should also be relevant to the salinity response in intact plants.

Comparison of the behavior of maize with *S. anglica* during salt treatment showed that the rate of sodium uptake by *Spartina* was much slower than that of maize under the same conditions and that there was a comparably smaller change in the chemical shift of the vacuolar phosphate resonance. The fact that the decrease in net sodium influx and the decrease in the vacuolar phosphate chemical shift change were similar supports the hypothesis that sodium in the tissue is largely compartmentalized in the vacuole. It appears that tolerance to salt in *Spartina* is at least partly based on its ability to prevent large net influxes of NaCl, although it is not clear whether this is achieved by preventing influx or by more efficient efflux at the plasmalemma. The sodium that is taken up seems to be sequestered in the vacuole in the same way as in maize, but there is no evidence for a more active tonoplast $Na^+/H^+$-antiport or $H^+$-ATPase in *Spartina*, which implies that these systems are not determining salt tolerance under these conditions.

## CONCLUDING REMARKS

The observations reported here suggest the occurrence of several ion transport processes during treatment of the glycophyte *Z. mays* with high external salt concentrations, and from this, a possible sequence of the events involved in the response to salt shock can be surmised. Sodium enters the tissue, probably by a plasmalemma cation channel, and accumulates briefly in the cytoplasm until uptake into the vacuole and efflux at the plasmalemma are initiated. At both sites the transport appears to involve a $Na^+/H^+$-antiport driven by a $H^+$-ATPase (or additionally a $H^+$-PPase in the tonoplast). At higher salt loads, the tonoplast proton pumps are initially unable to prevent the dissipation of the $H^+$ gradient by the antiport, and the result is an alkalinization of the vacuole, although there is little effect on the pH of the cytoplasm. Subsequently, the vacuolar alkalinization levels off, suggesting an activation of the tonoplast $H^+$-ATPase, because uptake of sodium continues for several hours. The active sequestration of sodium in the vacuole appears to result in a depletion of the energy resources of the tissue under extreme salt treatment, which may be one of the responses detrimental to the tissue. Results obtained with the halophyte *S. anglica* suggest that salt tolerance in root tips lies in the control of the rate of net salt influx into the tissue rather than the differential activities of the tonoplast proton pumps and $Na^+/H^+$-antiport.

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