Plant Cell Physiol. 46(12): 1924–1933 (2005)
doi:10.1093/pcp/pci205, available online at www.pcp.oupjournals.org

Exogenously Supplied Compatible Solutes Rapidly Ameliorate NaCl-induced Potassium Efflux from Barley Roots

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It has been suggested that the role of compatible solutes in plant stress responses is not limited to conventional osmotic adjustment, but also includes some other regulatory or osmoprotective functions. In this study, we hypothesized that one such function is in maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cell, a feature that may confer salt tolerance in many species, particularly in barley. This hypothesis was investigated using the non-invasive microelectrode ion flux (MIFE) measuring technique. We show that low (0.5–5 mM) concentrations of exogenously supplied proline or betaine significantly reduced NaCl-induced K^+ efflux from barley roots in a dose–response manner. This effect was instantaneous, implying that large intracellular concentrations of compatible solutes are not required for an amelioratory role. Exogenously supplied betaine also significantly enhanced NaCl-induced H^+ efflux, but only in pre-incubated roots, implying some alternative mechanism of regulation. Sap K^+ and Na^+ analysis and membrane potential measurements are also consistent with the model that one function of compatible solutes is in maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cell, possibly through the enhanced activity of H^+-ATPase, controlling voltage-dependent outward-rectifying K^+ channels and creating the electrochemical gradient necessary for secondary ion transport processes. These data provide the first direct evidence for regulation of ion fluxes across the plasma membrane by physiologically relevant low concentrations of compatible solutes.

Keywords: H^+ extrusion — *Hordeum vulgare* — K^+ homeostasis — Salinity — Stress adaptation.

Abbreviations: AFS, apparent free space; DAPC, depolarization-activated outward-rectifying potassium channel; LIX, liquid ion exchanger; MIFE, microelectrode ion flux.

Introduction

When confronted with a saline environment, plants respond with a significant elevation in the level of compatible solutes in the cytosol which ameliorates the detrimental effects of salinity (Bray 1997, Bohnert and Shen 1999, Hasegawa et al. 2000). The mechanisms of such amelioration are not fully understood. Compatible solutes were originally thought to function as cytosolic osmoticum involved in cellular osmoregulation (Bray 1997, Bohnert and Shen 1999, Hasegawa et al. 2000). Consistent with this view, the increase in cellular osmolality which results from the accumulation of non-toxic (thus 'compatible') osmotically active solutes is accompanied by the influx of water into, or at least reduced efflux from cells, thus providing the turgor necessary for cell expansion (Storey and Wyn Jones 1977, Wyn Jones and Storey 1978). However, the measured levels of many compatible solutes often appear to be too low to act as osmolytes (Holmström et al. 2000, Chen and Murata 2002, Sakamoto and Murata 2002), unless they are restricted to some intracellular compartment. It is now apparent that the functions of compatible solutes are not as straightforward as initially believed (Bohnert and Sheveleva 1998, Hare et al. 1998, Hasegawa et al. 2000). It has been proposed that the role of compatible solutes in cytosolic osmotic adjustment is indirect, through the plethora of regulatory or osmoprotective functions. The latter may include a possible role for compatible solutes in stabilizing the structure and activities of enzymes and protein complexes, scavenging radical oxygen species and maintaining the integrity of membranes under dehydration stress conditions (Shen et al. 1997, Bohnert and Sheveleva 1998, Bohnert and Shen 1999, Hasegawa et al. 2000). However, most of this evidence was obtained from in vitro experiments and, to our knowledge, no in planta studies reporting the possibility of compatible solutes directly controlling the activity of plasma membrane transporters are available in the literature.

Ion transport processes are central to the understanding of the complex and multigenic nature of salt tolerance in crop plants (Flowers and Yeo 1986, Yeo 1998, Tyerman and Skerret 1999). In particular, the crucial role of K^+ homeostasis in salt tolerance mechanisms of salinized plants has been placed centre stage (Maathuis and Amtmann 1999, Tester and Davenport 2003). Imposition of salt stress results in a massive efflux of K^+ from plant cells (Shabala 2000, Shabala et al. 2003, Chen et al. 2005) and significantly reduces the intracellular pools of K^+ (Carden et al. 2003, Cuin et al. 2003). Mitigation of this loss strongly correlates with the level of salt tolerance in barley cultivars (Flowers and Hajibagheri 2001, Carden et al. 2003, Chen et al. 2005). The regulatory and signalling pathways involved in the physiological functioning of K^+ transport sys-

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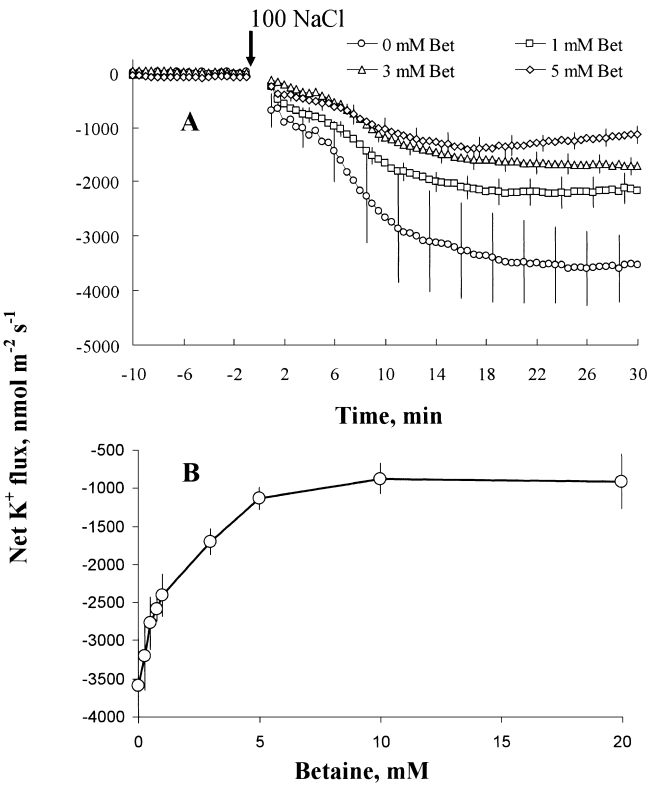

Fig. 1 (A) Salt-induced K^+ efflux from the elongation zone of barley roots after pre-incubation at various levels of exogenous betaine (0, 3 and 5 mM) for 1 h. Means ± SE (n = 6). (B) Dose dependence of betaine effects on K^+ flux from barley roots. Means ± SE (n = 6). Note that for all ion flux data the sign convention is influx positive.

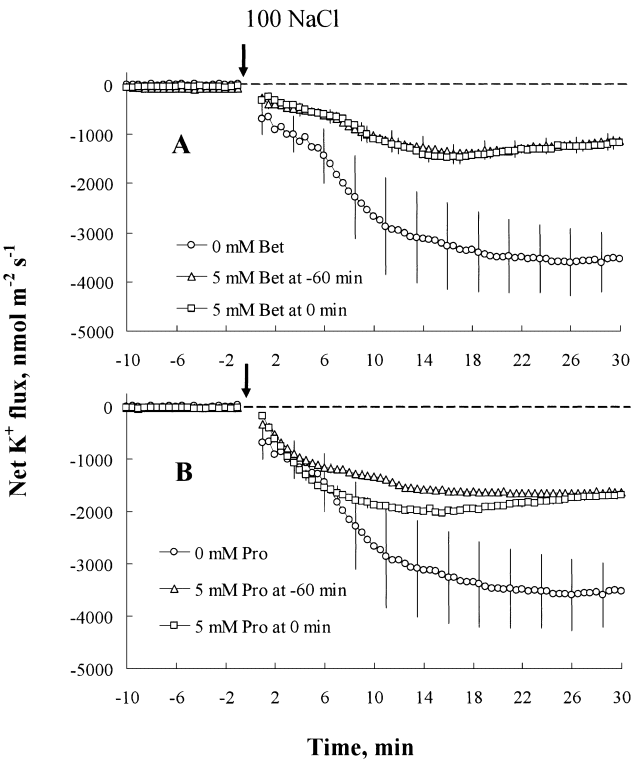

Fig. 2 (A) Evidence for the extracellular mode of betaine action. K^+ fluxes were compared between roots pre-incubated in 5 mM betaine for 1 h prior to the addition of 100 mM NaCl, and those where 5 mM betaine was supplied simultaneously with the salt stress. Means ± SE (n = 6). (B) Effect of exogenous proline on K^+ flux from barley roots. Fluxes were compared between roots pre-incubated in 5 mM proline for 1 h prior to the addition of 100 mM NaCl, and those where 5 mM proline was supplied simultaneously with the salt stress. Means ± SE (n = 6).

tems under salinized conditions are, however, poorly understood (Hasegawa et al. 2000). Are compatible solutes somehow implicated in this process?

The other important issue in salt tolerance mechanisms concerns the role of the plasma membrane ATP-dependent electrogenic H^+ pump. There is evidence to suggest that the stimulation of H^+-ATPases by salt stress may provide a driving force for a plasma membrane Na^+/H^+ exchanger to move Na^+ from the cytoplasm to the apoplast, thereby providing a significant contribution to the salt adaptation of plant cells (Nakamura et al. 1992, Ayala et al. 1996). The role that compatible solutes may have in this regulation is currently unknown.

This study addressed the above issues by directly measuring the effects of compatible solutes on the activity of plasma membrane transporters in epidermal cells in the elongation zone of barley roots. Using the non-invasive microelectrode ion flux (MIFE) measuring technique (Shabala et al. 1997, Newman 2001), net fluxes of K^+, H^+, Na^+ and Ca^{2+} were measured from NaCl-stressed roots pre-incubated or exposed to various concentrations of proline or betaine. Our results show that one function of compatible solutes may be in maintaining cytosolic K^+ homeostasis by preventing NaCl-induced K^+ leakage from the cell. This may be achieved through the enhanced activity of the H^+-ATPase, controlling voltage-dependent outward-rectifying K^+ channels and creating the electrochemical gradient necessary for secondary ion transport processes.

Results

Effect of compatible solutes on K^+ fluxes from NaCl-stressed roots

Consistent with previous reports (Babourina et al. 2000, Shabala 2000, Shabala et al. 2003, Chen et al. 2005), the imposition of NaCl resulted in a large efflux of K^+ from the elongation zone of barley roots (Fig. 1). A 1 h pre-incubation of the root in low concentrations of betaine substantially reduced the NaCl-induced K^+ efflux (Fig. 1A) in a dose-dependent manner (Fig. 1B). Most of these concentrations (those in the mM range) significantly ($P < 0.05$) reduced the magnitude of NaCl-induced net K^+ efflux, with K_m ~1.3 mM (Fig. 1B).

No statistically significant ($P > 0.05$) difference was found between NaCl-induced K^+ flux responses from roots pre-incubated in betaine for 1 h compared with those where 5 mM

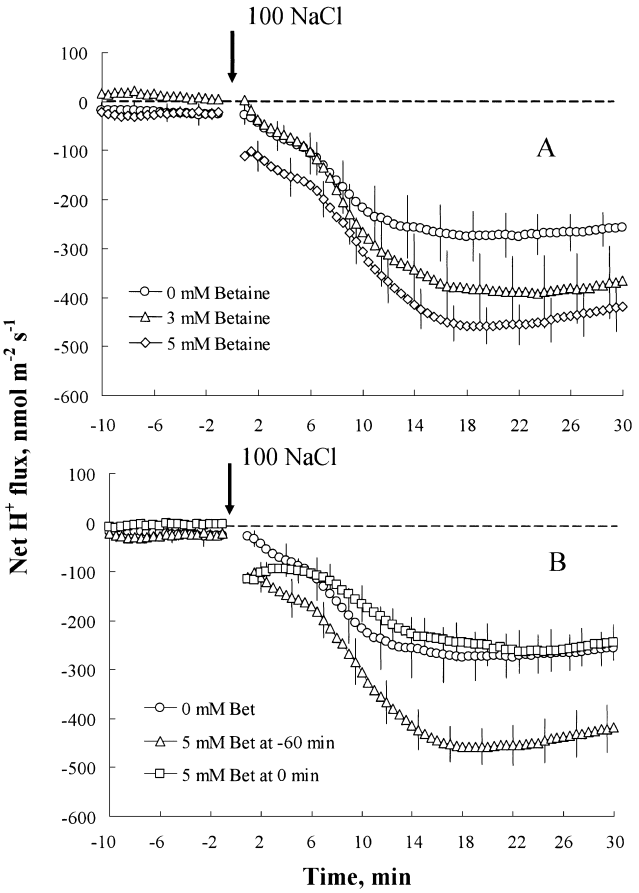

Fig. 3 Salt-induced H^+ efflux from the elongation zone of barley roots. Means ± SE (n = 6). (A) The elongation zone after pre-incubation at various levels of exogenous betaine (0, 1, 3 and 5 mM) for 1 h. (B) The elongation zone either pre-incubated in 5 mM betaine for 1 h prior to the addition of 100 mM NaCl, or with 5 mM betaine supplied simultaneously with the salt stress.

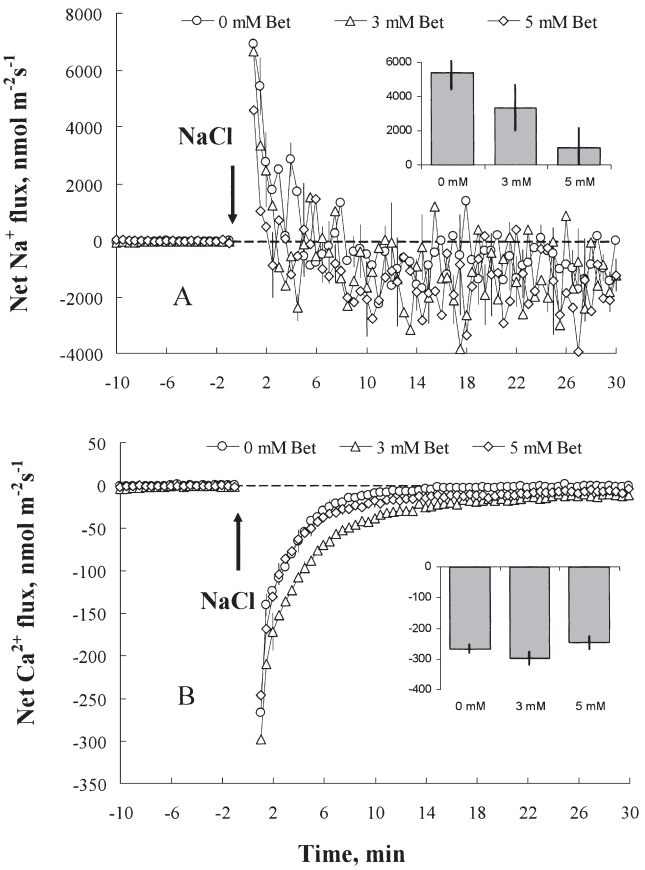

Fig. 4 Salt-induced Na^+ (A) and Ca^+ (B) efflux from the elongation zone of barley roots after pre-incubation at various levels of exogenous betaine (0, 3 and 5 mM) for 1 h in response to 100 mM NaCl treatment. Means ± SE (n = 6). The inset in (A) shows the peak Na^+ influx (nmol m^{-2} s^{-1}) measured over the first 3 min after NaCl treatment.

betaine was supplied simultaneously with 100 mM NaCl (Fig. 2A). Indeed, the effect on the NaCl-induced K^+ efflux in the elongation zone was instantaneous (within the time resolution of the MIFE system; Fig. 2A), ruling out the possibility of betaine accumulation in the cytosol being responsible for the observed amelioration of detrimental salinity effects on K^+ flux.

We also wanted to ascertain whether this dramatic ameliorative response on NaCl-induced K^+ fluxes was specific to the protective mechanism of betaine alone, or whether other compatible solutes have a similar mitigating affect. Simultaneously supplying 5 mM proline with 100 mM NaCl again resulted in a significant ($P < 0.05$) decrease in the magnitude of the NaCl-induced K^+ efflux from the root elongation zone (Fig. 2B), although, in contrast to betaine, the ameliorative effects of proline were not apparent until about 10 min after the imposition of NaCl.

Effect of compatible solutes on H^+ fluxes from NaCl-stressed roots

Also consistent with our previous reports on other tissues (Babourina et al. 2000, Shabala 2000, Shabala and Newman 2000, Shabala et al. 2003), salt stress substantially increased net H^+ extrusion from salinized barley roots (Fig. 3). Root pre-incubation in 3 or 5 mM betaine resulted in a further increase in the salt-induced H^+ efflux (Fig. 3A). The effect is statistically significant at $P < 0.05$ (t-test). When 5 mM betaine was supplied simultaneously with 100 mM NaCl, H^+ flux responses were virtually similar to those in the control (no betaine; Fig. 3B). This is in contrast to K^+ data (Fig. 2B) and may indicate multiple pathways of regulation of activity of plasma membrane transporters by compatible solutes.

Effect of compatible solutes on Na^+ and Ca^{2+} fluxes

Massive Na^+ influx was measured from barley roots immediately upon NaCl application (Fig. 4A). Root pre-treatment with betaine appears to reduce the overall Na^+ uptake over the first several minutes, with some indication of the

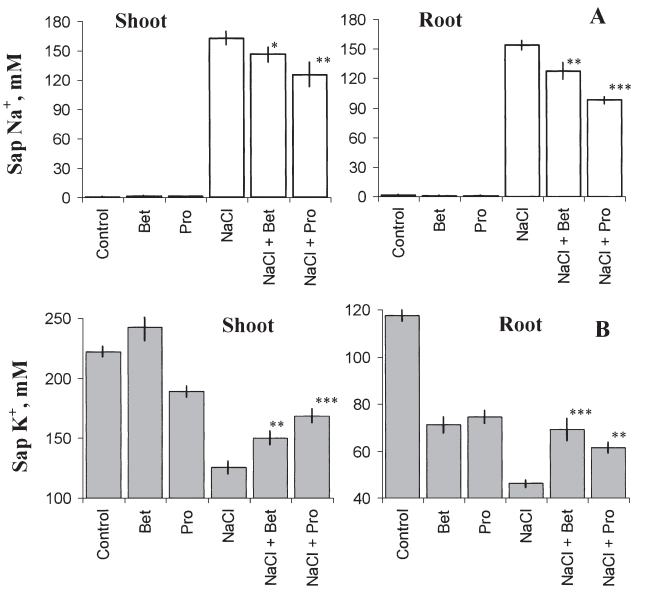

Fig. 5 Sap Na^+ (A) and K^+ (B) concentrations measured from leaf and root tissues of barley plants grown for 21 d at various concentrations of NaCl and compatible solutes (5 mM proline or betaine added to the growth solution). Means ± SE (n = 6). Asterisks near the chart bars indicate the level of significance compared with NaCl treatment at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t-test).

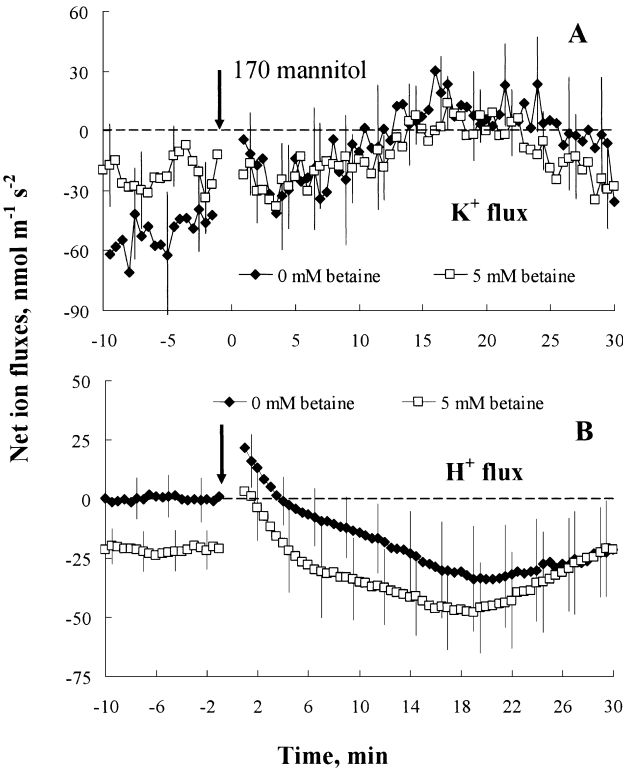

Fig. 6 Effect of isotonic mannitol solution on net K^+ (A) and H^+ (B) fluxes from barley roots pre-incubated in 0 or 5 mM betaine for 1 h. Means ± SE (n = 6).

effect being dose dependent (Fig. 4A inset). However, a low signal to noise ratio made these results statistically insignificant ($P > 0.05$). Furthermore, analysis of Na^+ flux data was complicated by the poor discrimination of the Na^+ LIX (liquid ion exchanger) between Na^+, K^+ and Ca^{2+} (Chen et al. 2005). This results in a substantial underestimation of the magnitude of Na^+ influx. Thus, the issue of the effect of compatible solutes on Na^+ uptake should be revisited using better (more selective) Na^+ LIX (once it becomes commercially available).

No clear effect of betaine of NaCl-induced Ca^{2+} flux was found (Fig. 4B). Massive Ca^{2+} efflux was measured immediately after NaCl application, being the result of the Donnan exchange in the cell wall (Shabala and Newman 2000). This massive Ca^{2+} efflux masked the possible ameliorating effect (if any) of betaine on the activity of plasma membrane Ca^{2+} transporters. As a result, no significant difference between Ca^{2+} flux responses from control and betaine-pre-incubated roots was found (Fig. 4B inset).

Sap K^+ and Na^+ concentrations

Another way to provide an insight into the effect of compatible solutes on Na^+ uptake in plants was via sap analysis. Barley plants were grown hydroponically at various ratios of NaCl and compatible solutes. After 3 weeks, leaf and root samples were collected, and sap extract analysed for Na^+ and K^+ content (Fig. 5).

Adding low concentrations of compatible solute to the growth media (5 mM of proline or betaine) had some positive effect on Na^+ accumulation in both leaf and root tissues (Fig. 5A). For most treatments, the effect was significant at $P < 0.05$ (labelled as asterisks in Fig. 5). Even more dramatic was the effect of compatible solutes on sap K^+ content (Fig. 5B). Plants grown in the presence of a low concentration of proline or betaine were able to retain much higher amounts of K^+ in both roots and shoots under saline conditions (significant at $P < 0.05$; Fig. 5B). Interestingly, application of compatible solutes in the absence of salt stress caused a significant ($P < 0.05$) reduction of sap K^+ in root tissues compared with control.

Effect of compatible solutes on ion fluxes is NaCl specific

When roots were treated with 170 mM mannitol (isotonic to 100 NaCl), no significant ($P > 0.05$) effect of betaine on the net K^+ flux from roots was found (Fig. 6A). Consistent with our previous reports (Shabala 2000, Shabala and Lew 2002), hyperosmotic stress increased K^+ uptake in both control and betaine-treated roots (Fig. 6A). Also not significant was the effect of betaine on mannitol-induced H^+ fluxes (Fig. 6B). Taken together, these results suggest that the regulatory effects of betaine on K^+ and H^+ transport across the plasma membrane of barley roots are NaCl specific and are not related to the osmotic component of salt stress.

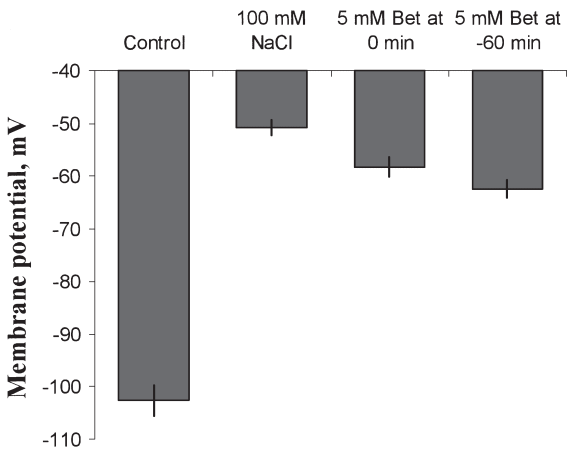

Fig. 7 Steady-state values of membrane potential of barley root epidermal cells in control and after 15 min of 100 mM NaCl treatment. Means ± SE (n =8).

NaCl-induced membrane potential depolarization is decreased by betaine

Keeping in mind the strong voltage dependence of many plasma membrane ion transporters, we tested the effects of compatible solutes on membrane potential in NaCl-stressed roots. Adding 100 mM NaCl to the bathing medium resulted in a substantial and highly significant ($P < 0.001$) depolarization (from –103 ± 2.9 to – 51 ± 1.5 mV; Fig. 7) of epidermal root cells. Adding 5 mM betaine simultaneously with 100 mM NaCl led to a less severe depolarization (–58 ± 1.8 mV; significant at $P < 0.05$). Pre-incubation of roots in 5 mM betaine for 1 h prior to NaCl addition had an even greater ameliorative effect (–62 ± 1.7 mV; significant at $P < 0.05$).

Measuring betaine efflux from barley roots

To support the 'apoplastic mode of betaine action' model, a series of experiments was conducted to determine whether the concentrations of betaine leaked from roots might be sufficient to account for the observed regulation of K^+ fluxes from plant roots in natural conditions. Accordingly, the amount of betaine leaked from roots into the external media was measured using an HPLC technique. A typical experimental trace from one experiment is shown in Fig. 8. Our results showed that on average, over the 70 h period, 1 g of roots was able to leak betaine to account for a concentration of 0.84 ± 0.23 mM (n = 3) in a 1 ml volume. Unfortunately, direct measurements of betaine released from roots grown in high salt (100 mM NaCl) solution were hindered by a methodological problem of interference with high Na^+ in the HPLC column.

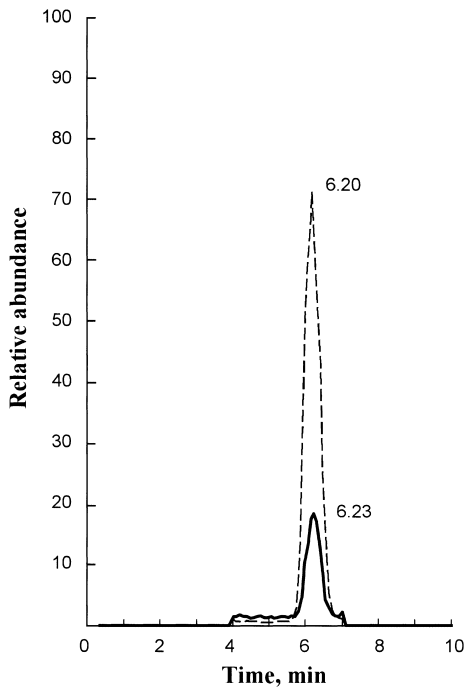

Fig. 8 HPLC chromatograms for betaine measurements in root growth solution. All traces were scaled up to a 1 mM betaine sample. Solid line, a typical trace for one experimental sample (measurements of the betaine in 10 ml of growth solution after roots were incubated for 70 h); dashed line, 0.75 mM betaine standard. Betaine retention time was about 6.2 min.

Discussion

Compatible solutes rapidly regulate membrane transport under salinized conditions

The results reported here demonstrate a substantial and rapid effect of compatible solutes on salt-induced H^+ and K^+ fluxes from barley roots (Fig. 1–3). To our knowledge, this is the first report of this sort in the literature. Both reduced K^+ efflux (e.g. retained K^+) and enhanced H^+ extrusion (leading to membrane hyperpolarization) are beneficial to plants, contributing to better performance under saline conditions. This is evident from both sap analysis (Fig. 5) and plant growth responses (data not shown). Previously, an immediate effect of proline and betaine on plasma membrane permeability to urea and methylurea has been recorded, but the levels of compatible solutes supplied were non-physiologically high (50–250 mM; Mansour 1998). Therefore, in addition to the conventional osmoprotective role of compatible solutes in transgenic plants reported elsewhere (Hayashi et al. 1997, Sakamoto and Murata 2002), we suggest that the beneficial effects of overexpressed levels of compatible solutes in salinized plants may be a result of a protective effect on plasma membrane integrity and its associated transporter proteins, particularly those responsible for K^+ transport across the plasma membrane.

Extracellular mode of betaine control over K^+ transport across the plasma membrane

The observed effect of exogenously applied betaine on NaCl-induced K^+ fluxes was almost instantaneous (within the time resolution of the MIFE system; Fig. 2A). The resulting efflux was identical to the one measured from roots pre-incubated with 5 mM betaine (Fig. 1A). This strongly refutes the need for substantial intracellular betaine concentrations in this salt tolerance mechanism and points to an extracellular mode of betaine control over K^+ transport across the plasma membrane.

Earlier, Chen et al. (2000) reported that maize accumulated exogenously supplied betaine at 5 mM at a rate of 8.2 μmol h^{-1} g^{-1} DW. For barley, the betaine content of roots exposed to 1 mol m^{-3} betaine for 24 h was 2.77 μmol g^{-1} FW (Ahmad et al. 1987) which would correspond to an accumulation in the cytosol of about 2.1 mol m^{-3} h^{-1}. Assuming a similar rate of accumulation in this study, it is unlikely that large intracellular concentrations of betaine would have accumulated in the cytosol during the 1 h incubation period.

Possible sources of the external betaine in nature

In this study, acute salt stress was used. In nature, soil salinization is a more gradual process. As a result, significant amounts of betaine can be synthesized and/or accumulated. Between 40 and 400 μmol g^{-1} DW has been reported in a number of accumulators under natural or experimental salinized conditions (Rhodes and Hanson 1993). Much of this is likely to be located in the cytosol (Hall et al. 1978, Leigh et al. 1981, Matoh et al. 1987), with a large proportion [e.g. 30–40% of the leaf total (Robinson and Jones 1986)] in the chloroplasts (Robinson and Jones 1986, Schröppel-Meier and Kaiser 1988, Murata et al. 1992, Papageorgiou and Murata 1995). If transported across the plasma membrane into the apoplast, betaine may regulate the magnitude of K^+ efflux from the cell. This was supported further by our HPLC measurements of the amount of betaine leaked into growth solution (Fig. 8). As mentioned above, over the 70 h period, 1 g of root was able to leak betaine to account for a concentration of 0.84 ± 0.23 mM (n = 3) in a 1 ml volume. Assuming an apparent free space (AFS) volume in barley roots of approximately 10% (Marschner 1995), this would be equivalent to an increase in the apoplastic betaine concentration of 8.4 mM over a 3 d period. From our results, 1 mM of externally applied betaine was sufficient to reduce NaCl-induced K^+ efflux by about 50% (Fig. 1). Neglecting some potential diffusion of betaine from the AFS into the soil solution, and assuming it is not metabolized by soil microorganisms, such a concentration (e.g. 1 mM) may be achieved within 8 h. Under saline conditions, production of betaine is known to increase several-fold (Rhodes and Hanson 1993). Accordingly, it might be assumed that the amount of betaine leaked from the root should be at least the same as from control roots, more probably much higher, making betaine control over K^+ efflux from salinized roots physiologically relevant.

A further external source of betaine for accumulation could be available from the soil. Betaine and its precursor choline (Rhodes and Hanson 1993) are often present within the rooting medium due to release into the soil by microbial producers, root exudation, from decaying plants and animals and/or excretion from mammals (Goldmann et al. 1991, Boncompagni et al. 1999, de Rudder et al. 1999, Morbach and Krämer 2002, Räsänen et al. 2004). The extent to which such sources of betaine are available for mitigation of salt stress by plants is unknown, although they are available for osmoregulation by rhizospheric bacteria (Miller and Wood 1996).

Specificity of regulation of K^+ transport by compatible solutes

The ameliorative effect on NaCl-induced K^+ fluxes was also observed when exogenously supplied proline was used (Fig. 2B). However, in contrast to betaine, the mitigating effects of proline were not apparent until about 10 min after the imposition of NaCl. Pre-incubation in 5 mM proline further decreased K^+ efflux. This requirement for pre-incubation may indicate that some level of cytosolic proline is required for a mitigating effect to be seen and suggests that the modes of action for proline and betaine are slightly different. Induction of transcript levels of the high affinity proline transporter *HvProT* has been reported within 30 min after the imposition of 200 mM NaCl for barley roots (Ueda et al. 2001), thus indicating a rapid response to enable uptake of proline. Furthermore, *HvProT* is strongly expressed in the root tip region under salt stress (Ueda et al. 2001). However, the extent to which such transporters operate in unsalinized conditions in roots exposed to proline is unreported for barley, although *AtProT1* and *AtProT2* from *Arabidopsis* are expressed at low levels in roots as well as other parts of the plant (Rentsch et al. 1996).

Possible involvement of the H^+ pump

Pre-incubation of barley roots in betaine increased H^+ efflux from the roots (Fig. 3). This could represent either an increase of the plasma membrane H^+ pump or, alternatively, a decreased activity of secondary inward H^+ transporters at the plasma membrane. Previous reports on NaCl-induced increases in H^+ efflux and the effects of metabolic inhibitors such as vanadate, supports the notion that an ATP-driven H^+ pump is involved (Shabala 2000, Shabala and Newman 2000). Salt stress has been shown to stimulate H^+-ATPase activity at the plasma membrane of plant cells (Nakamura et al. 1992) and NaCl-induced activation of the H^+-ATPase pump has been reported for many halophytic species (Braun et al. 1986, Ayala et al. 1996, Vera-Estrella et al. 1999). The resulting acidification of the external solution is proposed to be important in providing the driving force for a plasma membrane Na^+/H^+ exchanger to move Na^+ from the cytoplasm into the apoplast (Ayala et al. 1996).

The betaine-enhanced NaCl-induced H^+ efflux (Fig. 3) could therefore increase the driving force for Na^+/H^+ exchange. In addition, increased H^+ efflux could be important in the restoration of an otherwise depolarized membrane potential and prevent K^+ losses from the cell through depolarization-activated outward-rectifying K^+ channels (DAPCs; Maathuis and Sanders 1995, Maathuis et al. 1998). Indeed, a decrease in the extent of the NaCl-induced membrane potential depolarization was found in roots to which 5 mM betaine was supplied (Fig. 7). In addition, the activation of the proton pump and resulting extrusion of H^+ ions may enhance K^+ uptake via a high affinity H^+/K^+ symporter (Maathuis and Sanders 1994), improving the K^+/Na^+ ratio in the cytosol. This is supported further by results of the sap analysis (Fig. 5) reporting both an increased ability of plants to retain K^+ and a small but significant effect on reducing the amount of Na^+ in both roots and shoots (Fig. 5) in plants supplied with external proline or betaine. Some indication of the rapid betaine control over Na^+ uptake may be seen in Fig. 4A. Unfortunately, unambiguous interpretation of Na^+ flux data is tempered by the poor selectivity of commercially available Na^+ LIX (Carden et al. 2001, Chen et al. 2005). Once an Na^+ LIX with improved selectivity becomes available, the issue of betaine control over the activity of the plasma membrane Na^+/H^+ exchanger may be resolved.

Betaine apparently controls H^+ from the cytosolic side

In stark contrast to its effect on K^+ flux, betaine control over H^+ flux was only apparent in pre-incubated roots (Fig. 3). Indeed, when 5 mM betaine was supplied simultaneously with 100 mM NaCl, no response was seen in the H^+ flux compared with the 0 mM compatible solute treatments (Fig. 3B). This indicates a need for accumulation of cytosolic compatible solutes for mitigation of the NaCl-induced H^+ flux responses. At the same time, the restricted period allowing for the accumulation of cytosolic compatible solutes before the imposition of 100 mM NaCl (<1 h), and the small amount supplied (3 or 5 mM) imply that only small quantities are required within the cytosol for an effect to be seen. Therefore, an osmosensing mechanism is not likely to be involved in mediating betaine effects on H^+ transport across the plasma membrane.

Specific molecular mechanisms for betaine transport into root cells remain unknown. Although betaine was found not to be an efficient substrate for heterologously expressed HvProT (Ueda et al. 2001), both *Arabidopsis* and tomato ProT transporters are reported to have a higher affinity for betaine than proline (Schwacke et al. 1999, Grallath et al. 2005) and are the most likely candidates. This is supported by the finding of at least three betaine/proline transporters in mangrove (Waditee et al. 2002), which, like barley, is a betaine accumulator under salinized conditions. It is likely, therefore, that as yet undetermined mechanisms operate to accumulate betaine within these barley roots to a level at which modifications to the H^+-pumping ATPase can take place.

Effect of proline and betaine is not related to the osmotic component of salt stress

When an isotonic mannitol concentration was used instead of 100 mM NaCl, no significant increase in efflux of either K^+ or H^+ was observed (Fig. 6). This is consistent with both literature reports (Vera-Estrella et al. 1999) and our previous observations on other species (Shabala 2000). Also not significant was the difference in either the K^+ or H^+ flux responses to 170 mM mannitol between control and betaine-pre-treated roots (Fig. 6). This suggests that the observed modification of ion transport activity in NaCl-stressed roots by betaine is not related to the osmotic component of salt stress. The most likely explanation is that NaCl and osmotic stress target different K^+ transporters at the plasma membrane. Experiments on *Arabidopsis* mutants with altered K^+ transport activity may provide more specific details on this matter. Such experiments are currently in progress in our laboratory. Both MIFE and patch–clamp experiments suggested that DAPC is the major contributor to NaCl-induced K^+ efflux from *Arabidopsis* root epidermal cells (S. Shabala, V. Demidchik, J. Davies, unpublished). Therefore, it is logical to suggest that DAPCs may be regulated by betaine, either directly or indirectly (via altered membrane potential). A reduction in the extent of the membrane depolarization (Fig. 7) would certainly result in a lower K^+ efflux, an effect which could be almost immediate upon the application of the osmolytes.

Physiological implications

Due to the importance of K^+ retention by the cell in salinity tolerance (Maathuis and Amtmann 1999), the ameliorating affect of proline and betaine on the NaCl-induced K^+ efflux would have significant consequences on the salt tolerance of the plant. In barley, the extent of the NaCl-induced K^+ efflux is strongly correlated with the degree of salt sensitivity in different cultivars (Chen et al. 2005), and retention of intracellular K^+ is greater in salt-tolerant barley cultivars (Flowers and Hajibagheri 2001, Carden et al. 2003). Thus, by decreasing the NaCl-induced K^+ efflux, the tolerance of barley to salinized conditions could be improved by the supply of very low levels of exogenous betaine or proline.

In the majority of transgenic plants, the levels of betaine accumulated are rarely more than 1 μmol g^{-1} FW (Lilius et al. 1996, Hayashi et al. 1997, Nuccio et al. 1998, Holmström et al. 2000, Huang et al. 2000). Similarly small amounts of proline accumulation have also been reported (Kavi Kishor et al. 1995, Nanjo et al. 1999, Hong et al. 2000). This is far too low for conventional osmoregulation, even assuming all these solutes are located in the cytosol. In all the cases listed above, however, growth of the transgenic plants was increased under saline conditions. Since the maintenance of a high cytosol K^+ concentration is considered to be one of the most fundamental salt tolerance mechanisms in plants (Maathuis and Amtmann 1999), our data reported here indicate that this acquired tolerance may be a result of mitigation of NaCl-induced K^+ efflux (Fig. 1, 2).

In addition, the increase in proton pumping as seen by the increase in H^+ efflux in roots pre-treated with betaine may enhance K^+ uptake (Shabala and Lew 2002) providing at the same time the driving force for a plasma membrane Na^+/H^+ exchanger to move Na^+ from the cytoplasm into the apoplast (Ayala et al. 1996) thus contributing to improved K^+/Na^+ ratios in plant tissues (Fig. 5). Understanding the specific ionic and molecular mechanisms underlying the regulation by compatible solutes on K^+ and H^+ transport across the plasma membrane may be critical for increasing the efficiency of the mode of action of compatible solutes, thus contributing to the genetic engineering of salt-tolerant crops.

Materials and Methods

Plant material and growth conditions

Seeds of the salt-sensitive barley variety (*Hordeum vulgare* cv. Franklin; Australian Winter Cereal Collection) were germinated and grown in the dark in aerated hydroponic solution (0.1 mM $CaCl_2$ and 0.5 mM KCl) as described elsewhere (Chen et al. 2005). Three- to four-day-old seedlings were used for ion flux and membrane potential measurements. All measurements were made on the elongation zone of the root, ~3 mm from the root tip.

Ion flux measurements

Net fluxes of Ca^{2+}, K^+, Na^+ and H^+ were measured non-invasively using the MIFE technique (University of Tasmania, Hobart, Australia) as described previously (Shabala et al. 1997, Shabala 2000). Briefly, microelectrodes were pulled and salinized with tributylchlorosilane. Electrode tips were filled with commercially available ion-selective cocktails (Ca^{2+}, 21,048; K^+, 60,031; Na^+, 71,178; H^+, 95,297; all from Fluka, Busch, Switzerland). Electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan), their tips put close together and positioned 40 μm above the root surface. During measurements, a computer-controlled stepper motor moved the electrode between two positions (40 and 80 μm, respectively) from the root surface in a 10 s square-wave manner. The CHART software (see Shabala et al. 1997 and Newman 2001 for details) recorded the potential difference between two positions and converted them into electrochemical potential differences using the calibrated Nernst slope of the electrode. Net ion fluxes were calculated by using the MIFEFLUX software for cylindrical diffusion geometry (Newman 2001).

Experimental solutions and protocols for MIFE measurements

Fifty minutes prior to measurement, a barley seedling was taken from the growth cabinet and its root immobilized in the horizontal position in a 4 ml Perspex measuring chamber as described elsewhere (Shabala et al. 1997). The bath solution was 0.5 mM KCl and 0.1 mM $CaCl_2$ (BSM) plus the required concentrations of compatible solute (proline or betaine in a concentration range 0–5 mM). Net ion fluxes from the root elongation zone were measured for 10 min to ensure steady-state initial flux values. A double stock of NaCl (100 mM final concentration) containing an appropriate amount of compatible solute (in both 0.5 mM KCl and 0.1 mM $CaCl_2$) was applied and transient ion flux responses were measured for another 30 min. The time required for the stock addition and the establishment of the diffusion gradients has been reported to be about 40 s (Shabala and Hariadi 2005). Accordingly, the first 60 s after the solution change were later discarded from the analysis and appears as a gap in all figures. In some experiments, isotonic mannitol solution (final concentration 170 mM) was used instead of 100 mM NaCl. After 50 min of pre-incubation in an appropriate solution, steady-state fluxes were recorded for 10 min. Then osmotic stress (mannitol double stock) was applied, and ion fluxes were recorded for a further 30 min.

Membrane potential measurements

Conventional KCl-filled Ag/AgCl microelectrodes (Shabala and Lew 2002) with tip diameter ~0.5 μm were used to measure membrane potential from epidermal cells in the root elongation zone (~3 mm from the root tip). Measurements were taken from at least five individual plants for each treatment, with not more than three measurements from each individual root. Membrane potentials were recorded for 1.5–2 min after the initial cell penetration. Measurements were carried out on barley root segments, excised and immobilized as described above. Measurements were made on roots either in the BSM alone, BSM additionally supplied with 100 mM NaCl, pre-incubated in 5 mM betaine prior to 100 mM NaCl exposure or, finally, in roots to which 100 mM NaCl was added simultaneously with betaine. Measurements were carried out 15–20 min after the addition of 100 mM NaCl.

Sap K^+ and Na^+ concentrations

Barley seeds were germinated on moist tissue paper in the dark before transfer to a modified Hoagland's solution (Walker et al. 1996) containing 5 mM K^+, when the primary root was approximately 1 cm in length. Plants were grown under greenhouse conditions in unamended nutrient solution for the first 7 d after sowing, then 100 mM NaCl ± 5 mM betaine or 5 mM proline was added to the growth medium.

Harvesting was carried out on 21-day-old plants. Roots and shoots were separated and the plant tissue placed into 1.5 ml microcentrifuge tubes in which there was a basal opening, allowing cell sap but not tissue fragments to pass through to a collection tube. The sample was frozen in liquid nitrogen and then thawed (two cycles) and spun for 3 min at 11,600×*g* in a microcentrifuge. The collected sample was measured for its K^+ and Na^+ concentration (in mM) using a flame photometer.

Betaine measurements

Barley seeds were germinated for 3 d in Petri dishes and then transferred into large-volume (500 ml) growth containers where they were grown for another 4 d in aerated basic (BSM) hydroponic solution (0.1 mM $CaCl_2$ + 0.5 mM KCl) as described above. Seven-day-old seedlings were then transferred into a small volume container (10 seedlings per 12 ml Falcon test tube filled with 10 ml of BSM solution). Constant aeration was provided. Plants were grown for another 70 h. Roots were then removed from the growth container, and the amount of betaine in solution (released by roots) was analysed using a Waters 2690 Alliance HPLC coupled to a Finnigan LCQ ion trap mass spectrometer. The column was a GROM-SIL 100 Amino-1 PR (3 μm), 126 mm×4 mm. The mobile phase was acetonitrile : water (75 : 25) at a flow rate of 0.5 ml min^{-1}. Detection of betaine was by electrospray mass spectrometry using a needle voltage of 4 kV and capillary temperature of 240°C. Under these conditions, betaine had a retention time of 6.3 min. The experiment was repeated in triplicate, each collective sample consisting of 10 roots (30 roots in total). To minimize potential contamination and confounding effects of microbial activity, all containers and solutions were thoroughly sterilized prior to use.

Statistical analysis

Results were analysed using the Student's *t*-test.

Acknowledgments

This work was supported by an ARC Discovery grant (DP0449856) to S.S. The technical assistance of Dr. Noel Davies with HPLC experiments is greatly appreciated.

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(Received April 7, 2005; Accepted September 19, 2005)