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ORIGINAL ARTICLE

Amino acids regulate salinity-induced potassium efflux in barley root epidermis

Tracey Ann Cuin · Sergey Shabala

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Abstract The amino acid content increases substantially in salt-stressed plants. The physiological relevance of this phenomenon remains largely unknown. Using the MIFE ion flux measuring technique, we studied the effects of physiologically relevant concentrations of 26 amino acids on NaCl-induced K^+ flux from barley root epidermis. We show that 21 (of 26) amino acids caused a significant mitigation of the NaCl-induced K^+ efflux, while valine and ornithine substantially enhanced the detrimental effects of salinity on K^+ homeostasis. Our results suggest that physiologically relevant concentrations of free amino acids might contribute to plant adaptive responses to salinity by regulating K^+ transport across the plasma membrane, thus enabling maintenance of an optimal K^+/Na^+ ratio as opposed to being merely a symptom of plant damage by stress. Investigating the specific mechanisms of such amelioration remains a key issue for future studies.

Keywords Amino acids · Barley · Membrane · Potassium homeostasis · Stress · Adaptation

Abbreviations
BS Bath solution
MIFE Microelectrode ion flux

T. A. Cuin (✉) · S. Shabala
School of Agricultural Science,
University of Tasmania, Private Bag 54,
Hobart, TAS 7001, Australia
e-mail: Tracey.Cuin@utas.edu.au

Introduction

One of the earliest detectible responses to salt-stress is a massive K^+ efflux from cells (Shabala et al. 2003, 2006; Chen et al. 2005; Cuin and Shabala 2005), reducing the intracellular K^+ pool (Fricke et al. 1996; Carden et al. 2003; Cuin et al. 2003). Consistent with the key role of K^+ homeostasis in salt-tolerance mechanisms (Maathuis and Amtmann 1999), a reduction of this efflux correlates with increased salt-tolerance (Flowers and Hajibagheri 2001; Carden et al. 2003; Chen et al. 2005).

Substantial increases in the amount of proline and other amino acids in salinised plants has been widely reported (Fougère et al. 1991; Di Martino et al. 2003; Kavi Kishor et al. 2005). It has been proposed that an increase in free amino acids in stressed plants is merely a symptom of damage (Dubey and Rani 1990; Silveira et al. 2001; Di Martino et al. 2003). An alternative suggestion is that free amino acids contribute to osmotic adjustment by acting as osmolytes (Ford 1984). Accordingly, amino acids have been classified as one of the four major groups of compatible solutes, alongside sugars, polyoles and quaternary amines (Yancey et al. 1982; Hasegawa et al. 2000). However, similar to other compatible solutes, the overall concentration of amino acids is often far too low for a conventional osmotic effect (Chen and Murata 2002; Kavi Kishor et al. 2005). Furthermore, some reports show that while the actual composition of the amino acid pool may change, the total amount does not (Wang et al. 2003) ruling out a direct involvement of amino acids in cell osmoregulation. This suggests an indirect role in cytosolic osmotic adjustment, through regulatory or osmoprotective functions (Bohnert and Sheveleva 1998; Hasegawa et al. 2000).

In a previous study, we showed that exogenously supplied physiologically relevant concentrations of proline and glycine betaine rapidly ameliorate NaCl-induced K^+ efflux from barley roots (Cuin and Shabala 2005). In this work, we used the non-invasive microelectrode ion flux (MIFE) measuring technique to determine whether other amino acids have a similar mitigating effect. Altogether, 26 protein- and non-protein-amino acids at physiologically relevant exogenous concentrations (Curl and Truelove 1986) were studied. We show that 21 (of 26) amino acids caused significant ($P < 0.05$) mitigation of NaCl-induced K^+ efflux. Two more amino acids (valine and ornithine) substantially enhanced the detrimental effects of salinity on K^+ homeostasis. Taken together, our results suggest that physiologically relevant concentrations of free amino acids might contribute to plant adaptive responses to salinity by regulating K^+ transport across the plasma membrane, enabling maintenance of an optimal K^+/Na^+ ratio.

Material 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 an aerated hydroponic solution (0.1 mM $CaCl_2$ and 0.5 mM KCl) as described elsewhere (Chen et al. 2005).

Experimental solutions and protocols for MIFE measurements

Net fluxes of K^+ were measured non-invasively using the MIFE technique (University of Tasmania, Hobart, Australia) as described previously (Shabala et al. 1997).

For steady-state measurements of NaCl-induced K^+ fluxes, 3–4 days old excised barley roots were immobilised in a horizontal position in a 4 ml Perspex measuring chamber as described elsewhere (Cuin and Shabala 2005). The bath solution (BS) contained 0.5 mM KCl and 0.1 mM $CaCl_2$ plus the required amino acid. Solution pH was adjusted to 5.7 with HCl/KOH. No pH buffer was used in order to enable H^+ flux measurements. After 1 h incubation, a double stock of NaCl (final concentration: 80 mM) was applied. Steady-state K^+ fluxes were measured for 5 min, 60 min after the imposition of salt. All measurements were made in the mature zone, ~10 mm from the root tip.

For transient measurements of K^+ and H^+ fluxes, steady-state ion fluxes were recorded for 10 min from the root mature zone following pre-incubation in BS plus amino acid for pre-incubation experiments and minus the amino acid for simultaneous addition with NaCl. The double stock of NaCl solution plus the required amino acid was then applied and transient ion flux responses recorded for 1 h. In all experiments, pH values of the bath solution were continuously monitored with a pH microelectrode.

Membrane potential measurements

Conventional KCl-filled Ag/AgCl microelectrodes (Shabala and Lew 2002) with tip diameter ~0.5 μm were used to measure the membrane potential of epidermal cells from the mature zone. Barley roots were excised and mounted in the holder as described above. Steady-state membrane potential values were measured in roots exposed for 1 h to 0 or 80 mM NaCl, added after a 1 h pre-incubation in 1 mM of selected amino acids. Measurements were taken from at least five individual plants for each treatment, with no more than three measurements taken from any one root. Membrane potentials were recorded for 1.5–2 min after the potential stabilised following cell penetration.

Statistical analysis

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

Results

A potential mitigating effect of amino acids on salt-induced K^+ efflux is dependent on the amino acid supplied

Pre-incubation of barley roots for 1 h in a range of amino acids at a physiologically relevant (Curl and Truelove 1986) concentration of 1 mM prior to the addition of NaCl substantially modified the steady-state NaCl-induced K^+ efflux from the mature zone (Fig. 1). Twenty-one (of 26) amino acids tested, significantly ($P < 0.05$ or better) decreased the magnitude of net K^+ efflux, while valine and ornithine substantially enhanced K^+ efflux from barley roots. The presence of amino acids in the bathing medium affected neither the Na^+ concentration in the bathing medium (as evident from flame photometry measurements; data not shown), nor the response of the ion-selective electrodes (verified by calibrating microelectrodes in the presence of the relevant amino acid).

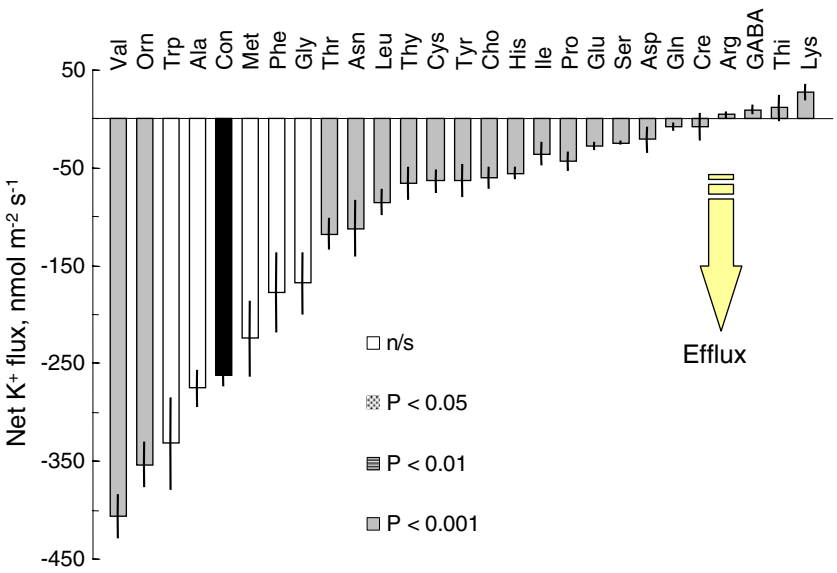

Fig. 1 Net K^+ fluxes measured from mature epidermis of barley root 1 h after 80 mM NaCl treatment. Roots were pre-incubated in a range of amino acids at a concentration of 1 mM, pH 5.7, unbuffered, for 1 h prior to salt stress. The average flux was calculated from recordings over 5 min. Different shading on a graph indicates significance level (Student's *t* test). All amino acids are ranked according to their mitigating effect. Data are mean ± SE ($n = 8$)

Nine amino acids were selected for more detailed studies in kinetics experiments (Fig. 2): one having detrimental effect (Val), one "neutral" (showing no significant effect; Ala), and the others (Arg, GABA, Glu, Gln, Lys, Pro and Ser), mitigating NaCl-induced K^+ efflux in static experiments (Fig. 1). Except for glutamate, steady-state K^+ fluxes were essentially unaffected by the presence of amino acids, indicating little effect on fluxes in the absence of salt (Fig. 2a). Five minutes after the imposition of salt-stress, a mitigating effect was already obvious for six (of nine) amino acids tested (Fig. 2b). After a temporary recovery, the K^+ efflux increased steadily over the next 60 min in control, alanine- and valine-treated roots (Fig. 2c); the latter resulting in significantly ($P < 0.05$) higher K^+ efflux compared with the control. At the same time, plants subjected to the other six treatments maintained relatively constant with a significantly ($P < 0.001$) smaller net K^+ efflux (Fig. 2c). Of these, lysine was the most efficient, not only preventing NaCl-induced K^+ efflux but also causing net K^+ uptake in salt-treated roots.

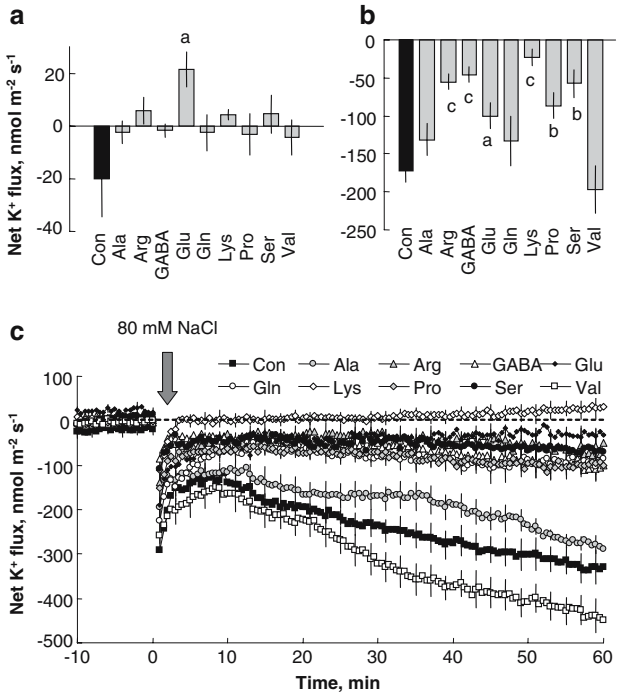

Fig. 2 **a** Effects of selected amino acids (1 h pre-incubation) on the steady-state K^+ flux before the imposition of the salt-stress. The average flux was calculated from recordings over 5 min (mean ± SE; $n = 6$). **b** Average K^+ efflux measured in response to acute (80 mM NaCl) salt-stress during the first 5 min after stress imposition (mean ± SE; $n = 6$). **c** Transient K^+ flux kinetics from amino acid-pre-treated roots during the first hour after salt-treatment (mean ± SE; $n = 6$). Significance levels are: $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$

Effects of amino acids on H^+ efflux after the imposition of salt

Root pre-incubation in amino acid solution significantly shifted steady-state net H^+ fluxes towards bigger efflux for all amino acids tested except proline, valine, and glutamine (Fig. 3a). The imposition of 80 mM NaCl further enhanced the net efflux of H^+ (Fig. 3b), similar to previous results for the mature zone of barley (Shabala et al. 2003). The most efficient were valine and glycine (activation up to 70 nmol m^{-2} s^{-1}; Fig. 3b). No substantial changes in H^+ flux activity in response to NaCl treatment was found in roots pre-treated with GABA and lysine (Fig. 3b).

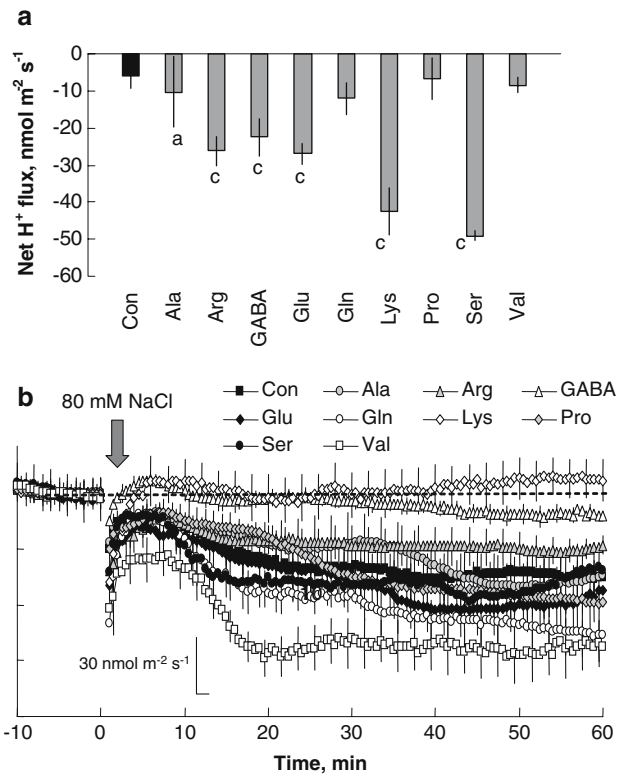

Fig. 3 **a** Effects of selected amino acids (1 h pre-incubation) on the steady-state H^+ flux before the imposition of the salt-stress. The average flux was calculated from recordings over 5 min (mean ± SE; $n = 6$). **b** Transient H^+ flux kinetics from amino acid-pre-treated roots during the first hour after salt-treatment (mean ± SE; $n = 6$). All solutions were unbuffered and adjusted to pH 5.7

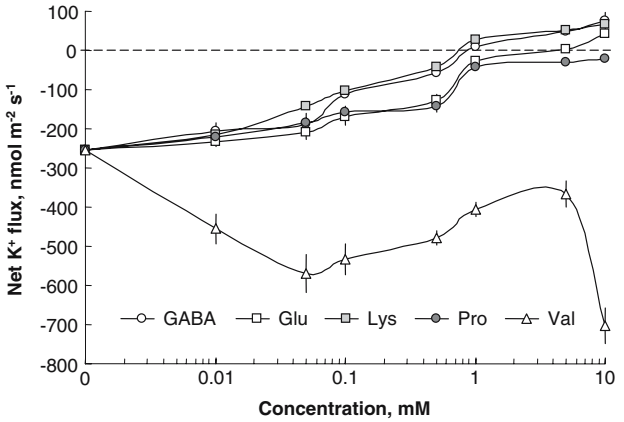

Fig. 4 Dose-dependency of the effect of selected amino acids on NaCl-induced K^+ efflux. Roots were pre-treated in a range of amino acid concentrations for 1 h before 80 mM NaCl was added 1 h prior to measurement. Recordings were made over 5 min and the average flux calculated. Note the absence of any clear dose dependency for valine (an amino acid lacking a mitigating effect). Data are mean ± SE ($n = 6$). All solutions were unbuffered and adjusted to pH 5.7

The ameliorative effect of amino acids on salt-induced K^+ efflux is dose-dependent

Five amino acids were selected for dose-response studies. These were chosen based on their extreme effect on NaCl-induced K^+ efflux (Val, Lys; Fig. 1), known mitigating effects on salinity (Pro) (Hasegawa et al. 2000; Kavi Kishor et al. 2005), or due to other putative roles in plants such as signalling or control over cation fluxes (GABA, Glu) (Kinnersley and Turano 2000; Demidchik et al. 2002, 2004; Essah et al. 2003; Bouché and Fromm 2004). Experiments showed that the mitigating effect saturated in a concentration range; 1–5 mM, with K_m values being in the range 0.1–0.5 mM (Fig. 4). Importantly, net K^+ uptake was measured from salt-treated roots not only for lysine, but also for GABA- and glutamate-treated roots, when high concentrations of amino acids were used. Interestingly, valine, which appears to have an exacerbating effect on the NaCl-induced K^+ efflux showed no such dose-dependency.

The mitigating effect of amino acids on salt-induced K^+ efflux correlates with depolarisation of the membrane potential

Due to the strong voltage-dependence of K^+ efflux channels, we tested the effects of the above five amino acids on the membrane potential before and after the application of NaCl (Table 1). Prior to the application of NaCl, significant differences were found between the membrane potential in mature zone epidermal cells in both lysine- (-142.8 ± 4.7 mV) and valine- (-99.7 ± 1.9) treated roots, compared to the control (-125.2 ± 2.6). The membrane potential in GABA-, glutamate- and proline-treated roots were not significantly different (at $P < 0.05$) from the control. After 1 h NaCl treatment, a significant depolarisation of the membrane potential was observed for all treatments, but the extent of this depended on the specific type of amino acid supplied. Valine-treated roots were even more depolarised than control plants (-48.1 ± 1.6 and -68.2 ± 2.2 mV, respectively). All other amino acids tested significantly reduced the extent of the NaCl-induced depolarisation after 1 h of 80 mM NaCl (Table 1). Lysine, by far had the largest ameliorative effect on the NaCl-induced depolarisation in the root epidermal cells; after 1 h salt-treatment the membrane potential was -110.8 ± 1.6 mV. Furthermore, the extent of the membrane depolarisation showed a strong positive correlation with the extent of the NaCl-induced efflux of both K^+ ($r^2 = 0.85$) and H^+ ($r^2 = 0.69$) fluxes (Fig. 5).

Table 1 Steady-state value of membrane potential of barley root epidermal cells in control and after 1 h pre-treatment of 1 mM of selected amino acids before 1 h after 80 mM NaCl application

Treatment	0 mM NaCl	80 mM NaCl
Control	−125.2 ± 2.6	−68.2 ± 2.2
GABA	−126.9 ± 3.1	−93.4 ± 2.5[c]
Glu	−119.9 ± 1.9	−81.2 ± 1.6[c]
Lys	−142.8 ± 4.7[b]	−110.8 ± 1.1[c]
Pro	−125.0 ± 2.2	−93.0 ± 2.0[c]
Val	−99.7 ± 1.9[c]	−48.1 ± 1.6[c]

Significance levels are [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ for ±NaCl compared to the control. Means ± SE ($n = 15$)

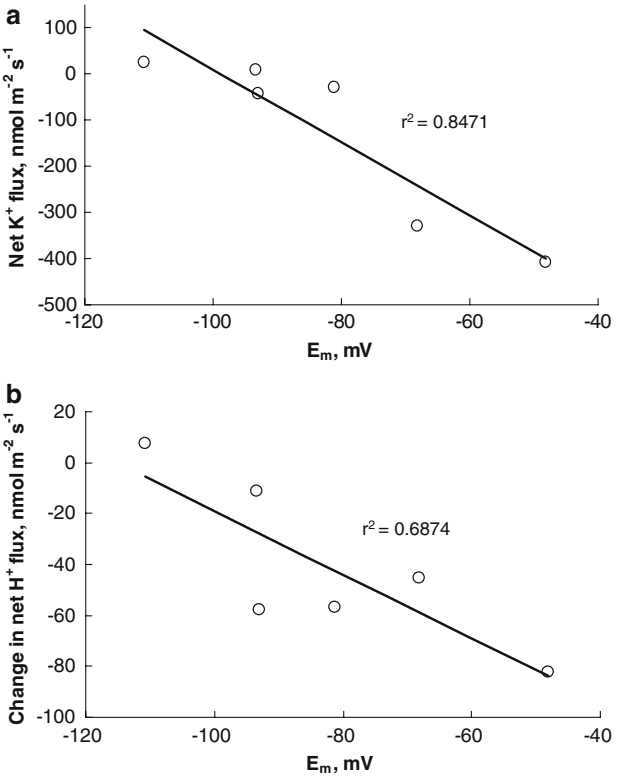

Fig. 5 Linear correlation between **a** net K^+ and **b** net H^+ fluxes and membrane potential of epidermal cells in barley roots pre-incubated in selected amino acids at a concentration of 1 mM. Recordings were made 1 h after the imposition of 80 mM NaCl

Cytosolic accumulation of amino acids is required for an effect on NaCl-induced K^+ efflux

In order to ascertain whether the effect of amino acids on salt-induced K^+ efflux was due to an external effect on K^+ transporter systems, or whether accumulation within the cytosol is necessary for an effect to be seen, 1 mM of either glutamate, proline, GABA, lysine or valine was supplied to roots simultaneously with 80 mM NaCl. Under such conditions, none of the supplied amino acid had any significant effects on the net K^+ efflux (Fig. 6). This strongly implies that cytosolic accumulation is required for an effect on K^+ transport under conditions of salt-stress.

Amino acids act additively on NaCl-induced K^+ efflux

To check whether the effects of amino acids on salt-induced K^+ influx follow an additive pattern, roots were pre-treated in solution containing both 1 mM lysine (completely preventing NaCl-induced K^+ efflux) and 1 mM valine (exacerbating the detrimental effects of NaCl on K^+ flux). After a 1 h incubation, the resultant NaCl-induced K^+ efflux was between the responses from roots treated with valine and lysine separately (Fig. 7). Thus, both amino acids appear to have a comparable effect to that observed when individually supplied; when supplied together, valine and lysine appear to act additively.

Discussion

The results clearly show that the supply of exogenous amino acids significantly modifies (mitigating in most cases), the extent of the NaCl-induced K^+ efflux. This may be crucial for maintaining optimal K^+/Na^+ homeostasis in the cell cytosol, thus enhancing salt-tolerance (Maathuis and Amtmann 1999; Flowers and Hajibagheri 2001; Volkov et al. 2003; Chen et al. 2005). Therefore, our results rule out earlier suggestions in the literature that increases in the level of free amino

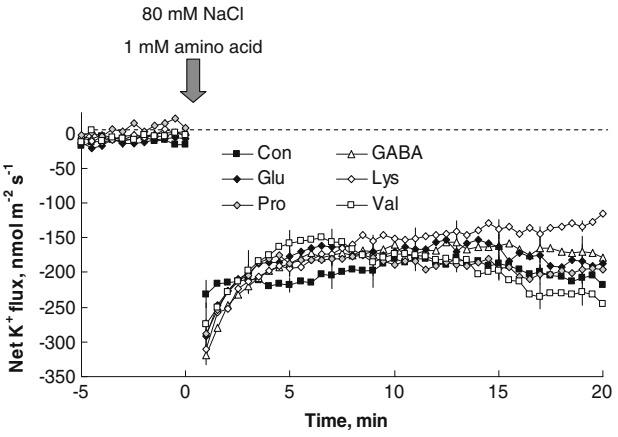

Fig. 6 Evidence for the internal mode of action of amino acids on NaCl-induced K^+ efflux. Transient K^+ flux kinetics were measured from barley root mature zone to which 1 mM of selected amino acids were added simultaneously with 80 mM NaCl. No significant difference was found in the net K^+ flux responses between the roots supplied with amino acids and the control for the first 20 min after the imposition of salt-stress (mean ± SE; $n = 6$)

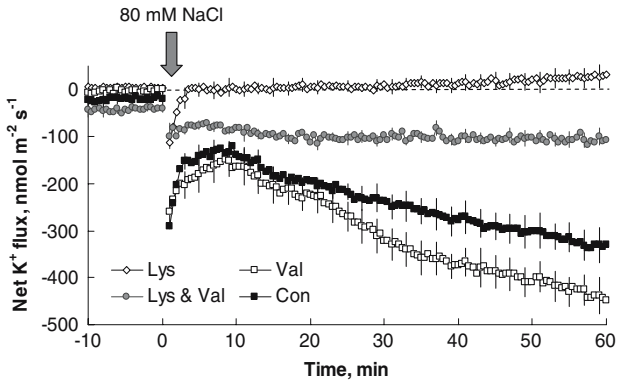

Fig. 7 Additivity of the effects of exogenously supplied amino acids on salt-induced K^+ efflux. Roots were pre-incubated in solution containing both 1 mM Lys and 1 mM Val for 1 h prior to the imposition of 80 mM NaCl. The result for Lys and Val alone and the control are taken from Fig. 2c, and are presented on this graph for comparison (mean ± SE; $n = 6$)

acids in stressed plants is merely a symptom of damage, bearing no physiological significance (Dubey and Rani 1990; Silveira et al. 2001; Di Martino et al. 2003). It should be stressed again that these mitigating effects were obtained in situ at physiologically relevant (0.1–1 mM: Curl and Truelove 1986) concentrations. To the best of our knowledge, all previous reports of stabilising effects of amino acids on membrane permeability and enzymatic activity were obtained in vitro and for physiologically unrealistic (e.g. 100–500 mM; Heber et al. 1971; Nash et al. 1982) concentrations. Thus, our findings provide a significant conceptual advance in attributing a physiological role for the observed phenomenon.

It is known that amino acid uptake by plant roots occurs in co-transport with H^+ (Kinraide and Etherton 1980; Reinhold and Kaplan 1984; Li and Bush 1990, 1991, 1992; Bush 1993, 1999; Boorer and Fischer 1997; Boorer et al. 1996). Studies on *Arabidopsis* have confirmed that a range of amino acid transporters are present in roots (Fischer et al. 1998; Wipf et al. 2002). From this point of view, the observed increase in the rate of net H^+ efflux after root pre-treatment with some amino acids (Fig. 3a) may be considered as beneficial, increasing the steepness of $\Delta\mu_H$ gradient to facilitate amino acid uptake. Although specific mechanisms of such activation remains to be studied in the future, involvement of H^+-ATPase is likely (Kinraide and Etherton 1980). Interestingly, those amino acids which were efficient in activating H^+ efflux in steady-state conditions (e.g. GABA or lysine), prevented any further activation of H^+ efflux by NaCl treatment (Fig. 3b). On the contrary, root pre-treated with valine and glutamine (two amino acids that were not efficient in shifting steady-state H^+ fluxes; Fig. 3b) showed the greatest H^+ response to NaCl treatment (Fig. 3b). This phenomenon may be tentatively explained by additive effects of amino acids and NaCl on plasma membrane H^+-ATPase activity. A NaCl-induced increase in plasma membrane H^+-ATPase activity has been reported for both halophytic (Braun et al. 1986; Ayala et al. 1996; Vera-Estrella et al. 1999) and non-halophytic (Nakamura et al. 1992; Maeshima 2000; Gaxiola et al. 2001) species. It might be that in GABA- and lysine-pretreated roots, the activity of PM H^+-ATPase is already "saturated", with no further activation required in an attempt to restore an otherwise depolarised membrane potential under saline conditions (Table 1). Such "saturation" is not observed for valine- and glutamine-pretreated roots that responded to NaCl treatment by an increased rate of H^+ pumping. Validation of this hypothesis may come from assays of H^+-ATPase activity in amino acid- and NaCl-treated roots.

Although changes in the composition of amino acids are reported under conditions of salt-stress (Fougère et al. 1991; Di Martino et al. 2003; Wang et al. 2003), it is unclear whether such accumulation is functionally related to stress alleviation (Hoai et al. 2003), or is merely a "by-product" of salt-stress, such as a result of increased protein degradation (Dubey and Rani 1990), inhibition of protein synthesis (Silveira et al. 2001) or increased photorespiratory rate (Di Martino et al. 2003). Our results suggested that 21 (of 26) amino acids tested were efficient in preventing salt-induced K^+ leakage from the cell (Fig. 1). Of a special interest is lysine, whose efficiency in mitigating K^+ efflux was even higher than that for proline—the most commonly reported compatible solute (Kavi Kishor et al. 2005). Therefore, it appears that increased pools of amino acids may eventually contribute to cell osmotic adjustment, but not directly as suggested earlier (Ford 1984), but via regulating the cellular content of inorganic solutes (specifically, K^+) which would contribute to osmotolerance.

Interestingly however, we were unable to find any relationship between the extent of K^+ flux modifications and the grouping of amino acids according to their known stabilising effect on biological membranes (Heber et al. 1971), or to changes in membranes permeability to ions (Rai 2002). Thus, it appears that the amino acid effect bears no clear relationship to its structure or function.

How this modification of NaCl-induced K^+ fluxes occurs is currently unknown. An internal mode of action is most likely (Fig. 6). A similar requirement for intracellular accumulation was also reported earlier for proline, but not glycine betaine, in barley elongation

zone Cuin and Shabala (2005). Also, as commented above, the modifications of the steady-state H^+ fluxes prior to the addition of NaCl (Fig. 3a) may be taken as further evidence for the uptake and consequent accumulation of exogenously supplied amino acids.

The correlation between the extent of the membrane depolarisation and the extent of the NaCl-induced K^+ efflux (Fig. 5a), strongly implicates depolarisation-activated K^+ channels (Maathuis and Sanders 1995; Maathuis et al. 1998). Support for such activation of outward-rectifying K^+ channels by NaCl-induced depolarisation has recently been presented in *Arabidopsis* (Shabala et al. 2006). Because membrane depolarisation is caused by a massive Na^+ influx into the cell (Shabala et al. 2003), salt-stress-mitigating amino acids could thus exert their mode of action by preventing Na^+ influx. Alternatively or additionally, their modifications of K^+ efflux could be due to a direct blockage of K^+ efflux channels, a role recently suggested for Ca^{2+} in salt-stressed *Arabidopsis* (Shabala et al. 2006). To address this issue in full, MIFE measurements will need to be complemented by measuring other key electrophysiological characteristics such as patch-clamp measurements of currents through specific ion channels, as well as by functional genomics studies. Such experiments are clearly outside the scope of this paper. Only a few speculative scenarios can be suggested at this time. For example, proline is known to minimise cellular damage by enhancing the stability of proteins and membranes (Hasegawa et al. 2000; Kavi Kishor et al. 2005) and several other amino acids also stabilise proteins against denaturation under various stress conditions (Heber et al. 1971; Paleg et al. 1981; Nash et al. 1982). A ROS-scavenging role for some amino acids has also been shown (Smirnoff and Cumbes 1989). Evidence for the existence of both GABA and glutamate receptors in plant systems has been reported (Kinnersley and Turano 2000; Bouché and Fromm 2004), and GABA and glutamate control over cation fluxes have been electrophysiologically characterised (Demidchik et al. 2002; Essah et al. 2003; Demidchik et al. 2004). Therefore, a plethora of mechanisms may operate in parallel, enabling plant adaptive responses to salinity. Elucidating the full extent of such regulatory networks remains a great challenge for the future.

The dose-dependency of the mitigation of the NaCl-induced K^+ response (K_m values within 0.1–0.5 mM range; Fig. 4) indicates that effects of amino acids on K^+ fluxes are seen at levels commonly found within salinised plants (Fougère et al. 1991; Di Martino et al. 2003; Wang et al. 2003), or soil (Curl and Truelove 1986). Plants can also take up amino acids from the rooting medium (Jones et al. 2005) and mechanisms exist for the uptake and internal transport of amino acids within the plant (Bush 1999). The question remains as to whether it is the actual amino acids supplied, or some products of their metabolism, that are having the effect on the NaCl-induced K^+ efflux. Amino acids are known to undergo rapid interconversions (Rai 2002), and the whole amino acid spectra changes upon the exogenous addition of proline (Carbonera et al. 1989) or other amino acids (Handa et al. 1986). Proline itself results from synthesis from glutamic acid, arginine, and ornithine (Verma and Zhang 1999). GABA is formed from glutamic acid (Bown and Shelp 1997) and methionine, aspartic acid, ornithine, and arginine are precursors in the synthesis of polyamines (Rhodes et al. 1999). Such interconversions in the longer term could increase the mitigation of the NaCl-induced K^+ efflux shown after pre-incubation in some amino acids. Indeed, a possible reason for the increased K^+ efflux in plants pre-incubated in exogenous valine (Figs. 1, 2c) could be due to its inhibition on the interconversion and synthesis of other amino acids as a result of its negative regulation of acetohydroxyacid synthase, an enzyme in a number of amino acid biosynthetic pathways (Bush 1999).

In conclusion, by decreasing the extent of the NaCl-induced K^+ efflux, most amino acids could substantially mitigate the effects of salt-stress on potassium homeostasis and, ultimately, enhance plant adaptation to salinity. This indicates that the common assumption that increases in free amino acid under abiotic stress is merely a symptom of damage (Dubey and Rani 1990; Silveira et al. 2001) may need reviewing. The mechanisms underlying this modification of the NaCl-induced K^+-efflux is unknown but is currently under investigation.

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