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# Specificity of Polyamine Effects on NaCl-induced Ion Flux Kinetics and Salt Stress Amelioration in Plants

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Polyamine (PA) levels in plants increase considerably under saline conditions. Because such an increase is believed to be beneficial for stress resistance, exogenous application of PAs has often been advocated as a means of ameliorating the detrimental effects of salinity. Results, however, are rather controversial, ranging from a significant amelioration to being ineffective or even toxic. The reasons for this controversy remain elusive. The ability of a root to retain K<sup>+</sup> in the presence of NaCl was used as a physiological indicator to evaluate the ameliorative effects of PA. Pre-treatment with 1 mM Spm<sup>4+</sup> (spermine), Spd<sup>3+</sup> (spermidine) or Put<sup>2+</sup> (putrescine) prevented salt-induced K<sup>+</sup> leak only in the mature root zone of hydroponically grown maize and *Arabidopsis*. In contrast, in the distal elongation root zone, PA pre-treatment resulted in an even larger NaCl-induced K<sup>+</sup> efflux, with the effect ranging from Spm<sup>4+</sup> > Spd<sup>3+</sup> = Put<sup>2+</sup>. A similar sequence has been also reported for H<sup>+</sup> pump inhibition, measured for both root zones. It appears that PAs affect cell membrane transporters in a highly specific way, with a relatively narrow 'window' in which amelioration is observed. We suggest that the ameliorative affect of PAs is the result of a complex combination of factors which might potentially include PA transport and accumulation in the cell cytosol, their metabolization and the functional expression of the specific target proteins or signaling elements.

**Keywords:** *Arabidopsis* • Ion transport • Maize • Potassium • Sodium • Tissue specificity.

**Abbreviations:** ADC, arginine decarboxylase; BSM, basal salt medium; CCCP, carbonyl cyanide *m*-chloro phenyl hydrazone; DAO, diamine oxidase; DEZ, distal elongation zone; HACC, hyperpolarization-activated cation channels; MIFE, microelectrode ion flux estimation; NSCC, non-selective cation channel; ODC, ornithine decarboxylase; PA, polyamine; PAO, polyamine oxidase; Put, putrescine; ROS, reactive oxygen species; SAMDC, S-adenosylmethionine decarboxylase; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine.

## Introduction

Polyamines [PAs: putrescine (Put), spermidine (Spd) and spermine (Spm)] are small aliphatic polycations that are classified as growth regulators, although their specific mechanism of action is not well understood (Tabor and Tabor 1984, Galston and Sawhney 1990). A variety of roles have been proposed for PAs, including cell division, root growth, flower and fruit development, and apoptosis (Evans and Malmberg 1989, Paschalidis and Roubelakis-Angelakis 2005a, Paschalidis and Roubelakis-Angelakis 2005b). In addition to their role in plant development, PAs may also play an important role in plant stress responses. It is widely reported that the PA concentration inside the cell is very responsive to external conditions, and a rapid change in light, temperature and various environmental stress agents may increase PA levels many fold (Flores 1990, Galston and Kaur-Sawhney 1995, Liu et al. 2000). One such environmental stress is salinity.

It has been demonstrated that salt-resistant plant varieties contain higher PA levels under stress conditions (Erdei et al. 1990, Basu and Ghosh 1991). The physiological rationale behind this elevation is still disputed. Recent experiments with gain- or loss-of-function mutants have also suggested a close association between plant stress tolerance and the level of endogenous PAs in plants (Alcázar et al. 2006). In addition, overexpression of heterologous genes for arginine decarboxylase (ADC) or ornithine decarboxylase (ODC), two key enzymes involved in Put biosynthesis, improves plant salt tolerance in tobacco (Kumira and Rajam 2002) and oat (Roy and Wu 2001). Increased salt tolerance is also reported in plants overexpressing SAMDC (S-adenosylmethionine decarboxylase; Wi et al. 2006) and SPDS (spermidine synthase; Kasukabe et al. 2004). Thus, a possible causal link between stress-induced elevation in the PA level and plant adaptive responses to salinity was postulated. However, the specific mechanisms behind this phenomenon remain largely unexplored.

Our early studies show that PAs are potent blockers of both slow (SV) and fast (FV) vacuolar channels (Brüggemann et al. 1998, Dobrovinskaya et al. 1999a, Dobrovinskaya et al. 1999b),

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so may be involved in the regulation of vacuolar ion compartmentation and cytosolic  $K^+/Na^+$  homeostasis. Also, we have recently shown (Shabala et al. 2007) that exogenously applied PAs ameliorate the detrimental effects of salinity by reducing NaCl-induced  $K^+$  efflux through non-selective cation channels (NSCCs), a major route of  $Na^+$  uptake into the plant cell (Demidchik and Tester 2002). These results are consistent with reports from the animal literature suggesting a specific interaction of PAs with almost every type of cation channel (Lopatin et al. 1994, Bowie et al. 1998, Gomez and Hellstrand 1999, Lu and Ding 1999, Huang and Moczydlowski 2001). In plant systems, PA inhibition of inward  $K^+$  currents across the plasma membrane of guard cells and in roots is also reported (Liu et al. 2000, Zhao et al. 2007). Furthermore, it is suggested that PAs may regulate the activity of numerous ion channels indirectly by affecting plasma membrane potential via the activation of  $H^+$ -ATPases through the enhancement of their interaction with 14-3-3 proteins (Garufi et al. 2007).

Plant membranes host hundreds of transport proteins. For cations alone, 46 unique families are known, containing approximately 880 members in Arabidopsis (Maser et al. 2001). This comprises about 5% of the entire Arabidopsis genome. For  $K^+$  transport alone, 75 genes from seven different families are known (Very and Sentenac 2002, Shabala 2003). These are differentially expressed in various cellular membranes and tissue types (Roberts and Tester 1995, Berthomieu et al. 2003, Shabala 2003, Cuin and Shabala 2006). Keeping in mind the large number of different types of membrane transporters controlled by PAs (see above), one should expect a high tissue specificity of the effect of PAs on cell ionic relations and, ultimately, salt tolerance. Surprisingly, to the best of our knowledge, no such information is available in the literature.

The main aim of the present work was to explore the tissue specificity of ameliorative effects of PAs on salinity-induced changes to ion fluxes in plant roots and provide some insights into the underlying mechanisms conferring such specificity. Given the reported strong correlation between a plant's ability to maintain cytosolic  $K^+$  homeostasis and its salt tolerance (Chen et al. 2005, Chen et al. 2007a, Chen et al. 2007b, Shabala and Cuin 2008, Smethurst et al. 2008), and the high inheritance of this trait (Chen et al. 2005, Chen et al. 2008),  $K^+$  efflux was used as a 'physiological marker' to estimate the ameliorative effects of PAs on cell membrane transporters in plant roots.

## Results

### Effect of plant species

Maize roots grown under hydroponics condition showed some modest net  $K^+$  uptake in the mature root zone (20 mm from the tip) (Fig. 1). Pre-treatment with 1 mM Spm<sup>4+</sup> significantly ( $P < 0.05$ ) increased this uptake (from  $33 \pm 15$  to  $127 \pm 32$  nmol m<sup>-2</sup> s<sup>-1</sup>; Fig. 1). Two other PAs (Put<sup>2+</sup> and Spd<sup>3+</sup>) had no significant effect on net  $K^+$  uptake under these conditions (Fig. 1B). The onset of salt stress caused a significant

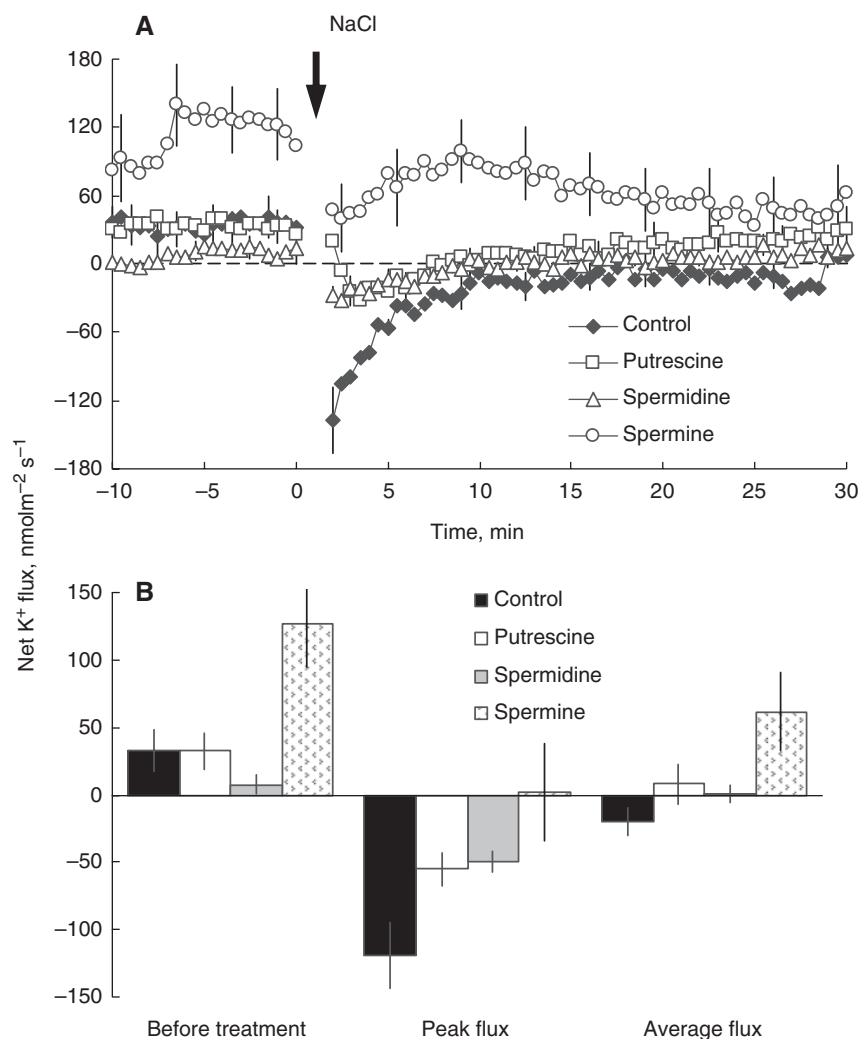
( $P < 0.05$ ) shift in  $K^+$  flux towards net efflux (Fig. 1). All PAs were efficient in preventing such NaCl-induced  $K^+$  efflux from the mature zone compared with the control (Fig. 1). Peak efflux was much higher in the control than in the three PA treatments, and the average efflux also showed the efficiency of PAs in reducing the overall  $K^+$  leakage from salt-treated roots (Fig. 1B). The most efficient was Spm<sup>4+</sup>, with no net NaCl-induced  $K^+$  efflux measured at any time in Spm<sup>4+</sup>-pre-treated maize roots.

To investigate this issue further, similar experiments were performed on the mature zone of Arabidopsis roots. Here, no significant ( $P < 0.05$ ) effect of PA pre-treatment at the initial (prior to stress) rate of  $K^+$  transport across the root surface was found (Fig. 2). All roots showed some small net  $K^+$  efflux of 20–30 nmol m<sup>-2</sup> s<sup>-1</sup>, most probably the result of transferring the plant from nutrient-rich Murashige–Skooog medium (containing ~20 mM  $K^+$ ) to poorer (0.2 mM  $K^+$ ) BSM (basic salt medium) solution. However, a significant amelioration of NaCl-induced  $K^+$  efflux was observed for all PAs, with their efficiency as follows: Put<sup>2+</sup> > Spd<sup>3+</sup> > Spm<sup>4+</sup>. This is consistent with our previously reported pea mesophyll data (Shabala et al. 2007), and is in contrast to the maize data shown in Fig. 1. Thus, it appears that the ameliorative effects of PAs on NaCl-induced  $K^+$  efflux are highly tissue and/or species specific.

### Effect of the root zone

When the same measurements (as in Fig. 1) were performed at the distal elongation zone (DEZ; 2 mm from the tip), no amelioration was found (Fig. 3). For Put<sup>2+</sup>- and Spd<sup>3+</sup>-pre-treated roots, NaCl-induced  $K^+$  fluxes were not significantly ( $P < 0.05$ ) different from the control. At the same time, Spm<sup>4+</sup>-treated roots showed a much higher  $K^+$  efflux in both steady-state (before NaCl treatment) conditions and after salt stress (Fig. 3). This implies a detrimental effect of 1 mM Spm<sup>4+</sup> pre-treatment on the DEZ. This is in stark contrast to the results from the mature zone (Fig. 1) where 1 mM Spm<sup>4+</sup> was the most efficient in terms of a decrease in the NaCl-induced  $K^+$  efflux. Thus, in addition to tissue or species specificity (as shown in the previous section), PA efficiency in regulating the activity of plasma membrane transporters appears to differ dramatically between functionally different root zones, ranging from amelioration to neutral and then to toxic for the same concentration of a particular PA (Table 1).

The specificity of the PA action on the root zone was also obvious when net NaCl-induced  $H^+$  fluxes were compared between PA-treated roots (Fig. 4). The onset of NaCl stress caused a transient increase in net  $H^+$  efflux in both the mature zone (Fig. 4A) and the DEZ (Fig. 4B) of maize roots, with a much stronger response from the latter. This stimulation was strongly (~80%) suppressed by 0.5 mM vanadate, a known inhibitor of P-type  $H^+$ -ATPase, and completely abolished by 50  $\mu$ M of the protonophore CCCP (carbonyl cyanide *m*-chloro phenyl hydrazone; Fig. 4B, insert). Root pre-treatment with PAs substantially reduced the ability of roots to respond to



**Fig. 1** NaCl-induced K<sup>+</sup> flux kinetics measured from the mature root zone (20 mm from the tip) of 5-day-old maize seedlings grown in hydroponics (true water culture). (A) Transient K<sup>+</sup> flux kinetics. Maize roots were pre-treated in 1 mM of the appropriate PA and then 100 mM NaCl was added at time zero (indicated by an arrow). (B) Mean pre-treatment flux (after 1 h of PA exposure under control conditions), peak K<sup>+</sup> efflux and average K<sup>+</sup> efflux (measured over the 20 min after NaCl application) for PA-pre-treated maize roots shown in A. Mean  $\pm$  SE ( $n = 5-9$ ). For all MIFE data, the sign convention is 'influx positive'.

NaCl by increasing the rate of H<sup>+</sup> efflux. Consistent with the K<sup>+</sup> data (Fig. 1), the strongest effect on net H<sup>+</sup> flux was observed in Spm<sup>4+</sup>-treated roots (Fig. 4A); the effects of Spd<sup>3+</sup> and Put<sup>2+</sup> were much weaker and similar to each other.

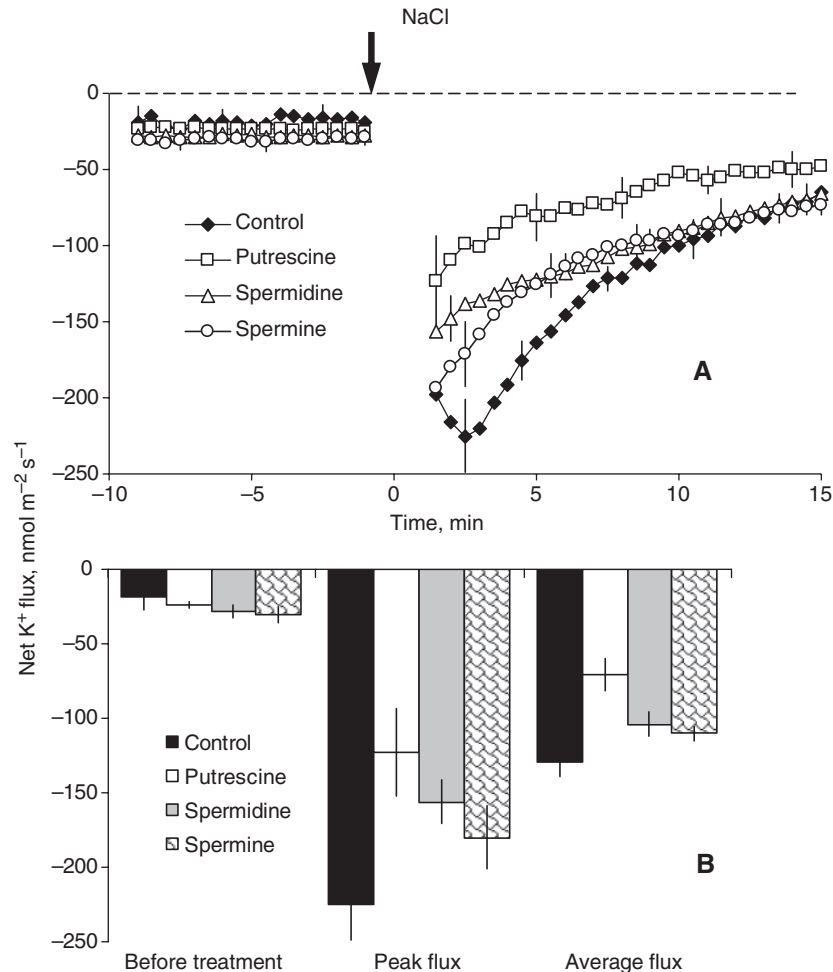
### Effect of growth conditions

The effects of PAs also depend strongly on plant growth conditions. When plants were grown in a paper roll (see Materials and Methods for details), the net K<sup>+</sup> uptake in the mature zone of the control plant was about 5-fold higher (Fig. 5) when compared with hydroponically grown plants (Fig. 1) ( $179 \pm 23$  vs.  $33 \pm 15$  nmol m<sup>-2</sup> s<sup>-1</sup>;  $P < 0.05$ ). Root pre-treatment with PAs reduced this initial K<sup>+</sup> uptake by 30–70% (Fig. 5). Salinity stress (80 mM NaCl) reduced the net K<sup>+</sup> uptake in all treatments. However, at no time did it reach negative values (net efflux). Most efficient were Spd<sup>3+</sup> and Spm<sup>4+</sup>, with less if any reduction

by Put<sup>2+</sup>, when judged by the relative changes in the shift in net K<sup>+</sup> flux before and after stress (Fig. 5B).

When ion flux kinetics were measured from the DEZ of paper roll-grown roots, all PA treatments exhibited detrimental effects on the root K<sup>+</sup> retention ability (Fig. 6). Not only were the initial flux levels of PA-treated roots significantly ( $P < 0.05$ ) lower than those of the control, but root pre-treatment with PAs appears to exacerbate the detrimental effects of salinity on K<sup>+</sup> flux. The worst-case scenario was observed for Spm<sup>4+</sup> (net peak efflux  $-435 \pm 70$  nmol m<sup>-2</sup> s<sup>-1</sup>) followed by Spd<sup>3+</sup> ( $-281 \pm 35$  nmol m<sup>-2</sup> s<sup>-1</sup>). Salinity-induced K<sup>+</sup> fluxes in Put<sup>2+</sup>-treated roots were not significantly different from those of the control (Fig. 6).

The overall effects of various growth conditions and root zone specificity of PA effects on net K<sup>+</sup> transport in maize roots are summarized in Table 1.



**Fig. 2** NaCl-induced K<sup>+</sup> flux kinetics measured from the mature root zone (10 mm from the tip) of 8-day-old Arabidopsis (WT Columbia) roots. (A) Transient K<sup>+</sup> flux in response to 50 mM NaCl treatment (added at time zero) to roots pre-treated in 1 mM of the appropriate PA. (B) Mean pre-treatment flux (after 1 h of PA exposure under control conditions), peak K<sup>+</sup> efflux and average K<sup>+</sup> efflux (measured over the 15 min after NaCl application) for PA-pre-treated Arabidopsis roots shown in A. Mean  $\pm$  SE ( $n = 6$ ).

Growth conditions also affected the ability of roots to generate H<sub>2</sub>O<sub>2</sub> under both control and NaCl conditions. Hydroponically grown plants showed intrinsically higher levels of H<sub>2</sub>O<sub>2</sub> near the root apex (Fig. 7A, B) and responded much more strongly to NaCl treatment (Fig. 7A, C).

One of the likely reasons potentially explaining the difference in ion fluxes patterns between hydroponically grown vs. paper roll-grown roots (e.g. Fig. 5) is a mechanical disturbance caused by aerators. Accordingly, some additional experimentation was conducted looking at effects of mechanical stimulation on root growth and ion transport and accumulation. Plants were grown in paper rolls in the presence of 80 mM NaCl in the growth solution. Half of the plants were used as control (no shaking), and the other half were constantly shaken at a high frequency (see Materials and Methods for details). The root oxygen availability was identical in both treatments. As shown in Fig. 8, mechanical disturbance (shaking) not only slightly reduced the root length, but also resulted in much

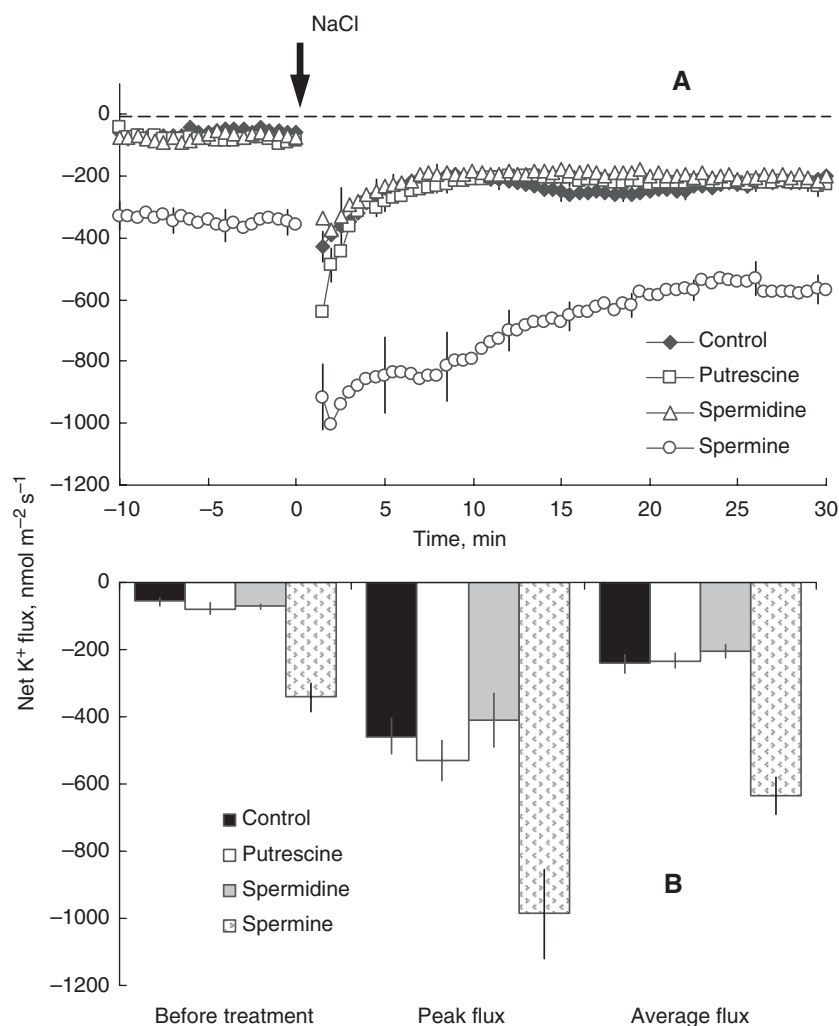
higher Na<sup>+</sup> accumulation in roots and especially in shoots of mechanically stimulated seedlings compared with unshaken controls (all the results are significant at  $P < 0.05$ ).

## Discussion

### Exogenous application of PAs is not practically viable

The use of PA sprays has been widely advocated in the literature as an efficient way of improving crop performance under saline conditions (Mishra and Sharma 1994, Chattopadhyay et al. 2002). Here we question the feasibility of this approach, given the high specificity of PA effects on membrane transport activity. It appears that a relatively narrow 'window' exists in which beneficial effects of PAs are observed. Most probably, this 'window' is not constant but is determined by many factors, including internal (tissue and age specificity) and external





**Fig. 3** NaCl-induced K<sup>+</sup> flux kinetics measured from the distal elongation zone (2 mm from tip) of 5-day-old maize seedlings grown in hydroponics (true water culture). (A) Transient K<sup>+</sup> flux kinetics. Maize roots were pre-treated in 1 mM of the appropriate PA and then 100 mM NaCl was added at time zero (indicated by an arrow). (B) Mean pre-treatment flux (after 1 h of PA exposure under control conditions), peak K<sup>+</sup> efflux and average K<sup>+</sup> efflux (measured over the 20 min after NaCl application) for PA-pre-treated maize roots shown in A. Mean  $\pm$  SE ( $n = 5-9$ ).

(e.g. light intensity, ambient temperature and humidity) factors. Given such complexity, exogenous application of PAs is unlikely to be practically viable.

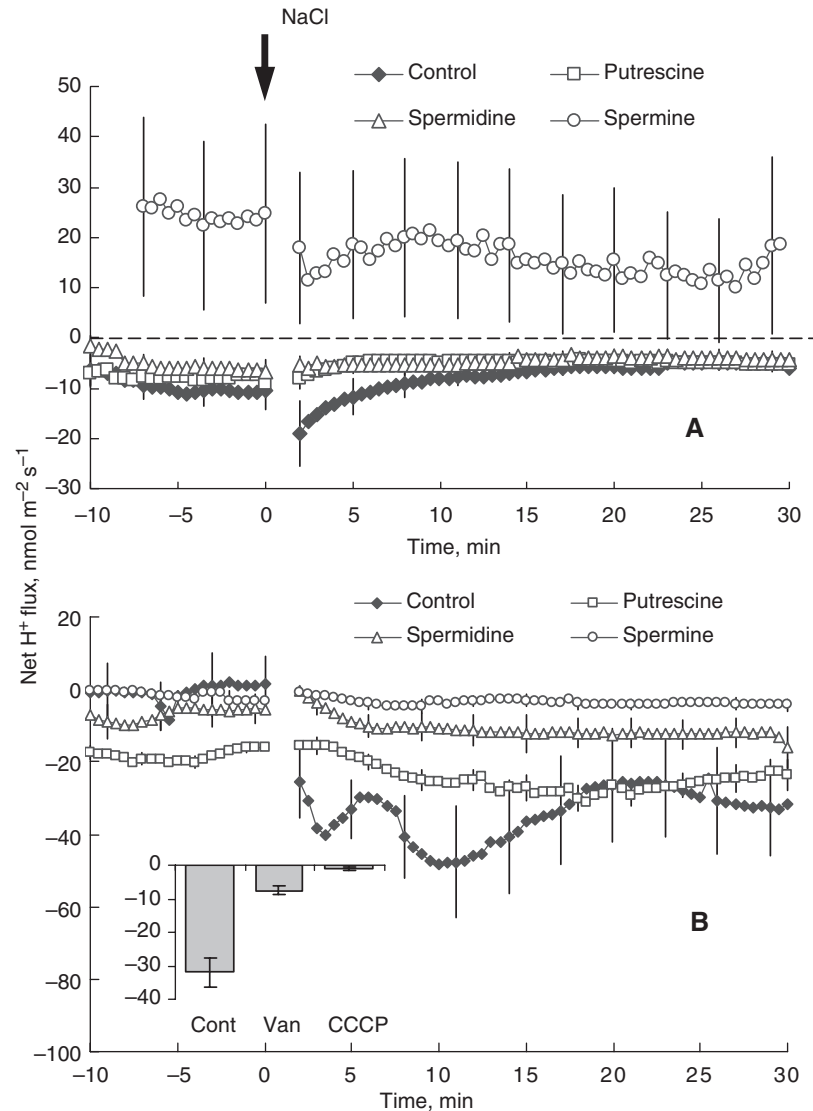
### PA effects are specific to species, tissue and growth condition

The effects of PAs were found to be specific to species, tissue and growth conditions for both K<sup>+</sup> and H<sup>+</sup> transport activities in the plasma membrane of maize and Arabidopsis roots (Figs. 1–6). The detailed explanation of the observed phenomena requires a separate investigation and is beyond the scope of this study. At this stage, several possible explanations may be put forward, as discussed further below. First, one can assume some substantial differences in the functional expression of plasma membrane ion transporters between different tissues and functional root zones. These, in turn, may be specifically affected by PAs, either directly or via some second messengers

**Table 1** Qualitative effects of PA pre-treatment on the NaCl-induced K<sup>+</sup> efflux from maize roots

Growth conditions	Hydroponics	Paper roll
Mature zone	Ameliorative effects	No amelioration
Distal elongation zone	No amelioration (Spe <sup>4+</sup> toxicity)	Detrimental effects

elicited by PAs. Secondly, differential expression of PA transporters and PA metabolism in the cytosol could affect their actual cytosolic concentrations and, consequently, the apparent efficiency sequence for Put<sup>2+</sup>, Spd<sup>3+</sup> and Spm<sup>4+</sup> effects. Thirdly, the observed effects may be explained by the variable activity of the apoplast diamine oxidase (DAO) and polyamine oxidase (PAO), as well as reductases and antioxidant enzymes affecting reactive oxygen species (ROS) production, scavenging



**Fig. 4** Effects of PA pre-treatment on NaCl-induced  $H^+$  flux kinetics measured from (A) the mature zone and (B) the DEZ of hydroponically grown 5-day-old maize seedlings. Roots were pre-treated in 1 mM of the appropriate PA and then 100 mM NaCl was added at time zero (indicated by an arrow). Mean  $\pm$  SE ( $n = 5-11$ ). The inset in B shows the effect of 1 h root pre-treatment in 0.5 mM vanadate, a known inhibitor of P-type  $H^+$ -ATPase, and 50  $\mu$ M CCCP (a protonophore) on the magnitude of NaCl-induced  $H^+$ -flux measured in the DEZ.

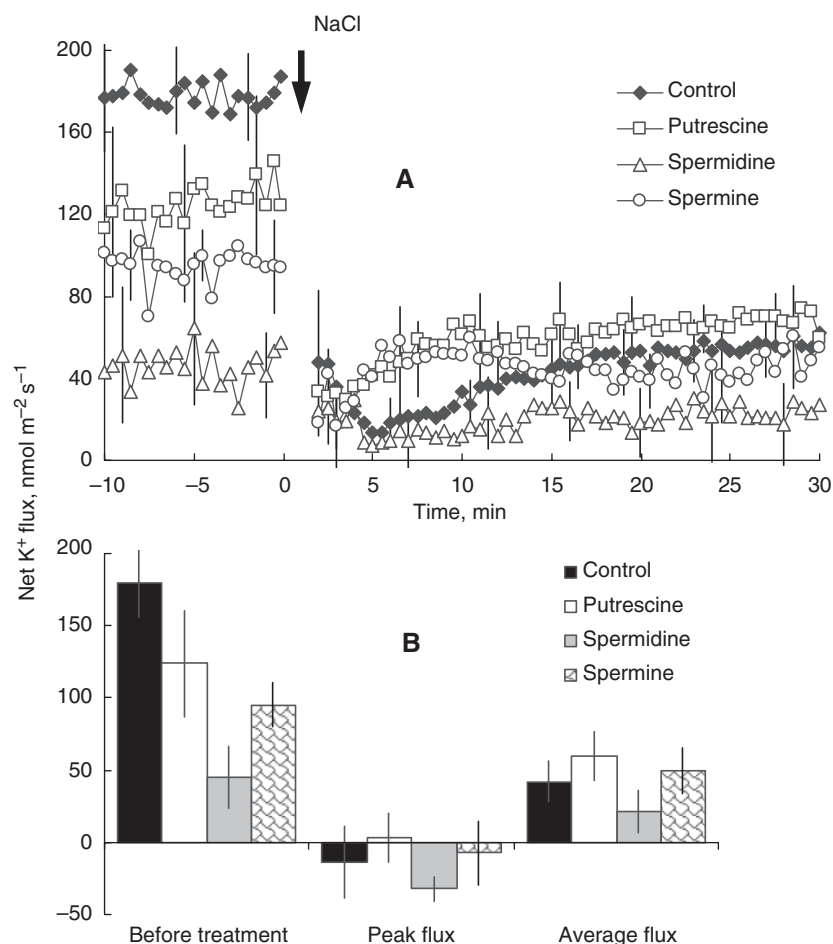
and ROS-dependent signaling. Fourthly, the existence of additional signaling cascades affected by PAs, e.g. phosphoinositide metabolism, and possible alterations of the intracellular  $Ca^{2+}$  homeostasis, cannot be excluded. As a result, the PA effect on membrane transport activity may range from beneficial through neutral to detrimental (Table 1).

### PA effects on $K^+$ transport

In the mature zone of barley roots, NaCl-induced  $K^+$  efflux is mediated mainly by outward rectifying  $K^+$ -selective channels (Chen et al. 2007a), with the activity of these channels being determined by the degree of the salt-induced membrane depolarization. Increased  $H^+$ -ATPase activity may serve as a negative feedback mechanism, partially restoring an otherwise

depolarized membrane potential (Chen et al. 2007a). Salt stress is known to induce plasma membrane  $H^+$ -ATPase gene expression (Niu et al. 1993) and stimulate vanadate-sensitive  $H^+$ -pumping (Shabala 2000; Fig. 4B insert). This could be responsible for the salt-induced increase of  $H^+$  efflux observed in this study (Fig. 6), probably as a result of increased 14-3-3 protein binding (Babakov et al. 2000). Importantly,  $Spm^{4+}$  but not  $Spd^{3+}$  or  $Put^{2+}$  stimulated a direct interaction of 14-3-3 proteins with the C-terminal autoinhibitory domain of the  $H^+$ -ATPase, increasing its hydrolytic activity (Garufi et al. 2007).

Earlier we speculated that PAs would decrease NaCl-induced  $K^+$  efflux either directly, by blocking available cation or  $K^+$  channels, or indirectly via better control of membrane potential



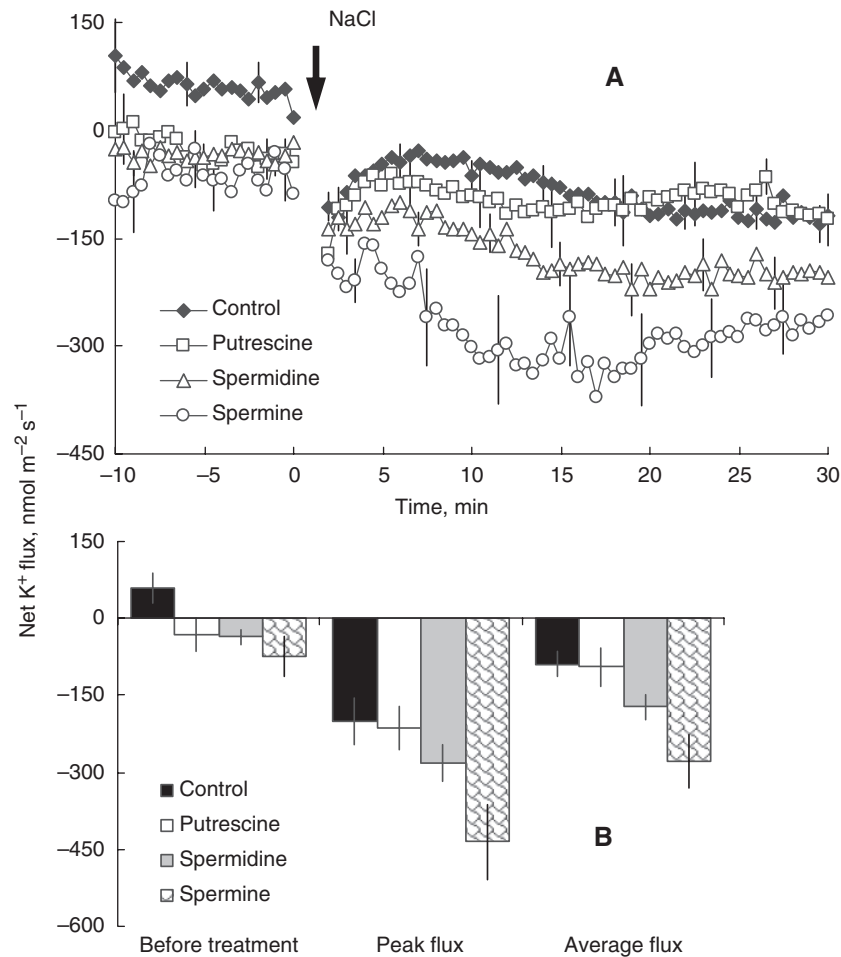
**Fig. 5** NaCl-induced K<sup>+</sup> flux kinetics measured from the mature root zone (20 mm from tip) of 5-day-old maize seedlings grown in paper rolls. (A) Transient K<sup>+</sup> flux kinetics. Maize roots were pre-treated in 1 mM of the appropriate PA and then 100 mM NaCl was added at time zero (indicated by an arrow). (B) Mean pre-treatment flux (after 1 h of PA exposure under control conditions), peak K<sup>+</sup> efflux and average K<sup>+</sup> efflux (measured over the 20 min after NaCl application) for PA-pre-treated maize roots shown in A. Mean  $\pm$  SE ( $n = 6-8$ ).

due to the stimulation of H<sup>+</sup>-ATPase pumping activity and a decrease of Na<sup>+</sup> influx through non-selective channels (Shabala et al. 2007, Zepeda-Jazo et al. 2008). The results of the present study demonstrate that this prediction is qualitatively fulfilled only in the case of the mature zone of the hydroponically grown maize (Fig. 1) and Arabidopsis (Fig. 2) roots. Based on the available data on PA binding sites in enzymes and ion channels, the blocking effect of PAs is expected to increase with PA valency (Kusano et al. 2007). In the case of Arabidopsis (Fig. 2), the apparent effective sequence was in the reverse order, i.e. Put<sup>2+</sup> > Spd<sup>3+</sup> > Spm<sup>4+</sup>. These results may be explained by assuming that PAs exert their effect on ion channels from the cytosolic side (e.g. Liu et al. 2000) and that the amount of PAs accumulated in the cytosol differs between various PAs as a result of the difference in their transport rate across the plasma membrane, as PAs and the diamine Put<sup>2+</sup> are believed to be transported via separate routes (Kusano et al. 2007). This hypothesis has to be validated in direct experiments.

### Polyamine effects on H<sup>+</sup> transport

PA treatment prevented the stimulation of H<sup>+</sup> extrusion by salt both in the mature zone and the DEZ (Fig. 4). Given the fact that the above stimulation was also strongly suppressed by vanadate and CCCP, two agents affecting H<sup>+</sup>-ATPase activity, a possible mediation of the observed H<sup>+</sup> efflux by the plasma membrane H<sup>+</sup>-ATPase pump is likely. If so, the observed results were unexpected and are in disagreement with previously published data (Garufi et al. 2007). Several possible explanations may be given. First, to the best of our knowledge, no information on the modulation of a coupling factor H<sup>+</sup>/ATP by Spm<sup>4+</sup> is available in the literature, as only the ATPase activity but not H<sup>+</sup> pumping was assayed in previous studies. At the same time, an increase in the ATPase activity per se does not imply an increased rate of H<sup>+</sup> pumping. Thus, a possible uncoupling action of Spm<sup>4+</sup> on the plasma membrane H<sup>+</sup>-ATPase may be suggested and should be verified in future experiments. Secondly, the comparison of H<sup>+</sup> and K<sup>+</sup> flux traces obtained in the





**Fig. 6** NaCl-induced K<sup>+</sup> flux kinetics measured at the DEZ of 5-day-old maize seedlings grown in paper rolls. (A) Transient K<sup>+</sup> flux kinetics. Maize roots were pre-treated in 1 mM of the appropriate PA and then 100 mM NaCl was added at time zero (indicated by an arrow). (B) Mean pre-treatment flux (after 1 h of PA exposure under control conditions), peak K<sup>+</sup> efflux and average K<sup>+</sup> efflux (measured over the 20 min after NaCl application) for PA-pre-treated maize roots shown in A. Mean  $\pm$  SE ( $n = 5-10$ ).

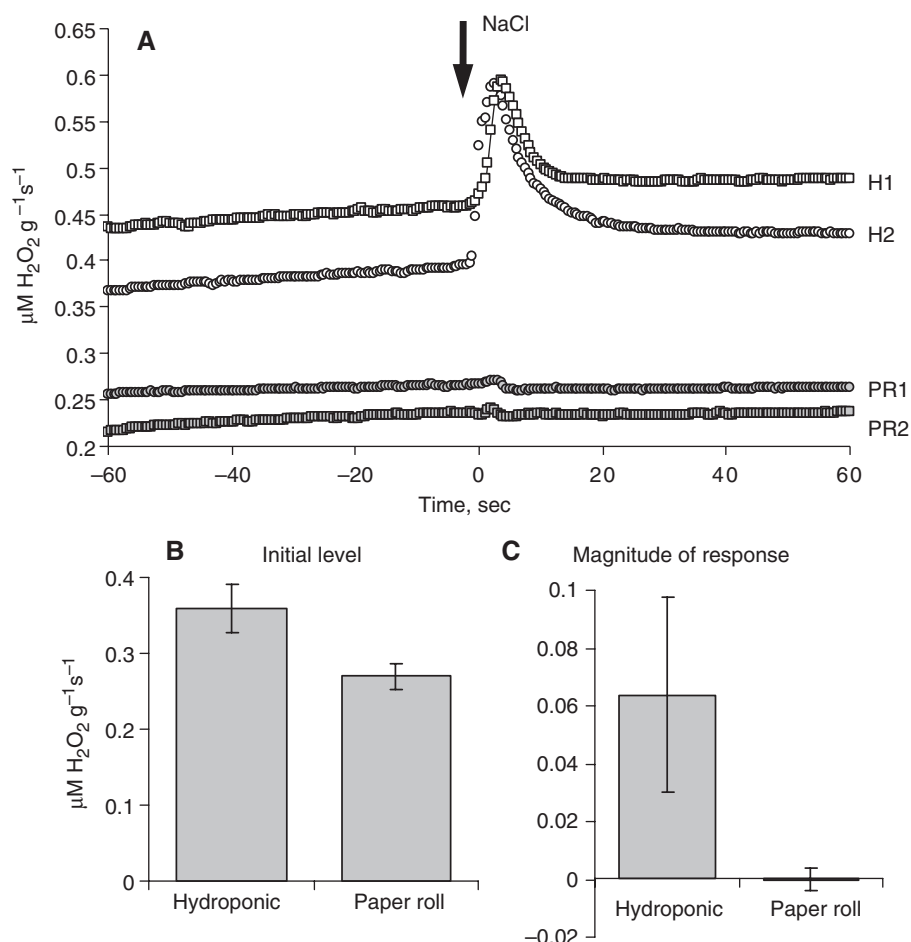
presence of Spm<sup>4+</sup> (Figs. 1A, 4A) may imply that in PA-treated roots, a high-affinity K<sup>+</sup>/H<sup>+</sup> symport starts to dominate over other K<sup>+</sup> and H<sup>+</sup> transport mechanisms, including H<sup>+</sup> pumping and K<sup>+</sup> uptake via inward rectifying potassium channels. Finally, there are additional phosphorylation sites in the plasma membrane H<sup>+</sup>-ATPase that regulate the activity of the enzyme. Phosphorylation of some of these sites depends on the increase in the intracellular Ca<sup>2+</sup> and provokes an inhibition of the H<sup>+</sup> pumping by plasma membrane ATPases in several plant species (Dubey and Boutru 2009). PAs could also alter the intracellular Ca<sup>2+</sup> balance, leading to indirect inhibition of the plasma membrane H<sup>+</sup>-ATPase activity.

### Dependence of polyamine effects on growth conditions may be attributed to both ROS production and signaling and root mechanosensitivity

Growth conditions affect the physiological status of plants, and could particularly affect the expression and a post-translational

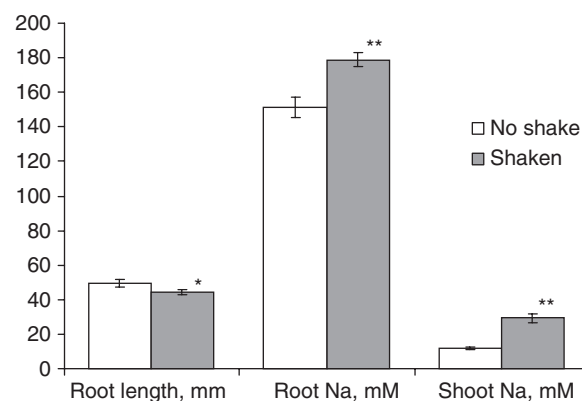
regulation of plasma membrane transporters. It is obvious from the data presented in Figs. 5 and 6 that in the maize roots grown in paper rolls, as opposed to hydroponically grown, K<sup>+</sup> uptake is greatly enhanced in control conditions. One explanation is the increase of a driving force for K<sup>+</sup> uptake due to the increased activity of the plasma membrane H<sup>+</sup>-ATPase. Alternatively/additionally the balance between K<sup>+</sup> uptake and efflux could be altered by the activation of additional non-selective channels, causing a membrane depolarization. Such non-selective channels could be ROS-activated channels (see below). Indeed, ROS production was higher by hydroponically grown roots (Fig. 7).

In PA-pre-treated roots, K<sup>+</sup> uptake was either completely and indiscriminately suppressed by all PAs (in the DEZ) or diminished, with a lesser effect of Put<sup>2+</sup> as compared with higher PAs (in the mature zone). A possible mechanism explaining the PA toxicity may be that suggested by Ditomaso et al. (1989) for Put<sup>2+</sup>. According to their model, part of the exogenously applied Put<sup>2+</sup> is catabolized by cell wall-located



**Fig. 7** Effect of growth conditions on  $\text{H}_2\text{O}_2$  production by maize roots. Plants were grown either hydroponically or in paper rolls, and the amount of  $\text{H}_2\text{O}_2$  produced by the root apex was quantified for control and saline conditions. (A) Two representative traces of  $\text{H}_2\text{O}_2$  recordings for each of the growth conditions studied (H, hydroponics; PR, paper roll). (B and C) The initial  $\text{H}_2\text{O}_2$  levels and the magnitude of the NaCl-induced  $\text{H}_2\text{O}_2$  response, respectively. Mean  $\pm$  SE ( $n = 5-7$ ).

DAO (Flores and Filner 1985). This catabolism produces  $\text{H}_2\text{O}_2$ , resulting in peroxidative damage of the plasma membrane and leading to an increased leakiness of cell membrane to  $\text{K}^+$  (McKersie et al. 1982). PAO, responsible for  $\text{Spd}^{3+}$  and  $\text{Spm}^{4+}$  catabolism, is also largely localized to the cell wall (Kaur-Sawhney et al. 1981, Flores and Filner 1985) and its increased activity has been shown within an hour of stress application (Politycka and Kubis 2000). In maize roots,  $\text{H}_2\text{O}_2$  is preferentially formed in the elongation zone; specifically in epidermal and vascular tissues (Liszkay et al. 2004). Cell wall peroxidase converts  $\cdot\text{O}_2^-$  into  $\cdot\text{OH}^-$  (Liszkay et al. 2003). At the same time, ROS ( $\cdot\text{OH}^-$  and  $\text{H}_2\text{O}_2$ ) are capable of activating both NSCCs and hyperpolarization-activated cation channels (HACCs) (Demidchik et al. 2003, Foreman et al. 2003, Cuin and Shabala 2007, Demidchik et al. 2007), the main routes for  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx in roots, respectively. Hence, it is reasonable to suggest that the observed dependence of PA effects on growth conditions may be attributed to the difference in ROS production between hydroponically and paper roll-grown



**Fig. 8** Effect of mechanical stimulation on root growth and  $\text{Na}^+$  accumulation in roots and shoots (see Materials and Methods for details). Mean  $\pm$  SE ( $n = 4-10$ ). The difference between control (no shaking) and mechanically stimulated (shaking) treatments is significant at the  $*P < 0.05$  or  $**P < 0.01$  level.

plants (Fig. 7). It is not clear whether the difference in the oxygen availability per se, or mechanical disturbance to roots caused by aeration, or some other factor, might cause this difference. This issue has to be addressed in future studies.

Due to the higher ROS production and expression of ROS-activated channels in the elongation zone (Demidchik et al. 2003), the impact would be higher in this tissue. In the mature zone, NaCl-induced increases in K<sup>+</sup> efflux were less in the PA-treated roots, so that the average K<sup>+</sup> flux under salt was comparable with that in control and PA-pre-treated roots. In contrast, in the elongation zone, NaCl induces a much higher K<sup>+</sup> efflux in PA-treated cells. The difference may be caused by the fact that NSCCs in the elongation, but not in the mature zone, are stimulated by external H<sub>2</sub>O<sub>2</sub> (Demidchik et al. 2007). Therefore, a larger membrane depolarization in PA-treated epidermal cells in the elongation zone may be the result of enhanced NSCC activity. For the sake of completeness, it should be mentioned that PAs are also capable of reducing stress-induced ROS production through the induction of antioxidant enzymes and increases in glutathione levels (Groppa and Benavides 2008, and references therein). It is conceivable that, depending on whether PAs stimulate ROS production or scavenging under salt stress, the overall effect may be detrimental or beneficial.

It also appears that mechanical disturbance to roots may have a substantial impact on the activity of root membrane transporters, causing an enhanced Na<sup>+</sup> influx via non-selective pathways (Fig. 8), and may also be responsible for their differential sensitivity to PAs. Indeed, mechanosensitive ion channels have been found in a variety of living organisms, including plants (Cosgrove and Hedrich 1991, Ding and Pickard 1993, Ramahaleo et al. 1996, Shepherd et al. 2002). At least 10 mechanosensitive ion channel-like proteins were found in the *Arabidopsis* genome and, recently, two of these channels (MSL9 and MSL10), which form relatively non-selective (Cl<sup>-</sup> > Ca<sup>2+</sup>) channels, were electrophysiologically characterized in root cells (Haswell et al. 2008). The activity of these mechanosensitive ion channels in maize roots, possibly provoked by aerator 'bubblers', would result in a more depolarized membrane potential. This in turn could give rise to a lower net K<sup>+</sup> uptake rate in aerated hydroponics as compared with paper roll-grown plants. The activity of mechanosensitive ion channels could also increase cytosolic Ca<sup>2+</sup> concentrations, thus affecting a plethora of membrane transport processes (Haswell et al. 2008).

## Materials and Methods

### Plant material

Maize (*Zea mays* L. cv Sweetcorn) seeds were surface sterilized (3% H<sub>2</sub>O<sub>2</sub> for 10 min) and thoroughly rinsed with distilled water. Seeds were germinated in a dark growth cabinet at 24°C in Petri dishes for 2–3 d. Uniformly germinated seedlings were selected and grown in two different ways: (i) true hydroponics

(aerated water culture) or (ii) by using the paper roll method. Using the true hydroponics method, 15–20 germinated seedlings were transferred to a bubbled hydroponic culture unit comprising a 1.5 liter plastic container over which seedlings were suspended on a plastic grid so that their roots were almost completely immersed in the growth solution (BSM: 0.2 mM KCl and 0.1 mM CaCl<sub>2</sub>). Aeration was provided by two aquarium air pumps via flexible plastic tubing. When the paper roll method was used, germinated maize seedlings were placed between two layers of wet filter paper and oriented vertically. The size of the paper roll 'sandwich' was ~14×40 cm, and the seedlings were placed in the upper part of it, so that the distance between the root tip and the lower end of the 'sandwich' was at least 8 cm. About 200 ml of growth solution (as above) was poured into a 0.8 liter glass beaker of ~10 cm diameter. The double-layered paper roll 'sandwich' containing 10–12 vertically oriented seedlings was placed into the beaker in such a way that it adhered to the inner walls of the beaker and followed its circumference. The 'sandwich' was immersed into the growth solution to a depth of 2–3 cm, so the plant roots never reached the solution during the entire period of their growth (2–3 d). Capillary forces in the filter paper ensured that the plant roots remained wet, but well aerated. For both methods, seedlings were grown under constant (+24°C) conditions in the darkened growth cabinet until 5 d old. Roots of 8–9 cm long were used for measurements.

*Arabidopsis thaliana* (wild type Columbia) seeds were obtained from the NASC (Nottingham, UK). Plants were grown at 22°C with 24 h fluorescent lighting (100 μmol m<sup>-2</sup> s<sup>-1</sup> irradiance) in vertical Petri dishes containing 0.35% Phytigel (Sigma) with full-strength Murashige–Skoog medium (Duchefa, Haarlem, The Netherlands) and 1% (w/v) sucrose as previously described (Demidchik and Tester 2002, Cuin and Shabala 2007). Six- to eight-day-old plants were used for measurements, and all measurements were made at the mature zone.

### Ion flux measurements

Net K<sup>+</sup> and H<sup>+</sup> fluxes were measured using the non-invasive microelectrode ion flux estimation (MIFE) technique (UTas Innovation Ltd., Hobart, Tasmania). The principle of the MIFE measurement is that the concentrations of the ion of interest (e.g. H<sup>+</sup> or K<sup>+</sup>) are measured in the unstirred layer near the root surface at two well-defined distances (30 and 80 μm in our case) from the tissue. Based on the diffusion coefficient for protons and potassium ions, and the difference in the ion concentrations at the two measuring positions, net ion fluxes at the membrane surface are calculated. All details of microelectrode fabrication and calibration are available in our previous publications (Shabala et al. 1997, Cuin and Shabala 2005, Shabala et al. 2005), and the theory of the MIFE measurements is available elsewhere (Newman 2001, Shabala 2006).

During experiments, maize or *Arabidopsis* seedlings were placed in a 10 ml measuring chamber. Their roots were immobilized in a horizontal position as described elsewhere

(Cuin and Shabala 2005) and pre-incubated in a BSM solution containing 1 mM of the appropriate PA ( $\text{Spm}^{4+}$ ,  $\text{Spd}^{3+}$  or  $\text{Put}^2$ ) for 1 h. The choice of the above timing was determined by the fact that 1 h pre-treatment with PAs has caused approximately 50% reduction in NaCl-induced  $\text{K}^+$  efflux from cells (Shabala et al. 2007); the latter is often used as a 'physiological marker' and strongly correlates with plant salinity tolerance (Shabala and Cuin 2008). Also, numerous studies investigating ameliorative effects of exogenous PAs on plant performance under saline conditions used  $\text{Spm}^{4+}$ ,  $\text{Spd}^{3+}$  or  $\text{Put}^2$  at a concentration of 1 mM (e.g. Ndayiragije and Lutts 2006, on rice; Yamaguchi et al. 2006, on Arabidopsis; Liu et al. 2006, on barley). Hence, our choice of concentration was driven by an attempt to make our results comparable with published data.

The measuring chamber was transferred into the Faraday cage and immobilized on the computer-driven 3D hydraulic manipulator. Electrodes were positioned near the root surface, and net fluxes of  $\text{K}^+$  and  $\text{H}^+$  were measured for about 10 min. Then 80 mM NaCl treatment was given, followed by another 30 min of recording. In maize, for each of the growing conditions (i.e. hydroponics vs. paper roll), measurements were performed in the mature zone (~20 mm from the root apex) and at the DEZ (2 mm from the root cap junction). In Arabidopsis, all measurements were made at the mature zone, about 10 mm from the tip.

### $\text{H}_2\text{O}_2$ production measurements

Roots of both hydroponically and paper-roll-grown plants were used to study the effects of growth conditions and NaCl stress on  $\text{H}_2\text{O}_2$  production. A root segment of ~10 mm was cut from the apex, weighed and placed in the measuring chamber containing 1 ml of BSM measuring solution. Measurements were performed using the hydrogen peroxide selective micro-electrode ISO-HPO-2 sensor (WPI, Sarasota, FL, USA). The sensor consists of an internal working/counter electrode combination, which fits inside a disposable steel sleeve filled with electrolyte and separated from the external environment by a selective membrane. This electrode is then plugged into the Four-Channel Free Radical Analyzer TBR4100 (WPI) connected to a PC for data acquisition. The electrode was calibrated in a set of known  $\text{H}_2\text{O}_2$  standards (0.5–8  $\mu\text{M}$  range) before and after measurements.  $\text{H}_2\text{O}_2$  was measured for about 60 s. Salinity treatment was then given, followed by another 120 s of recording. Higher (500 mM NaCl) concentrations were used to increase the resolution of the method. The data obtained were then adjusted according the weight of each sample to give the final result in  $\mu\text{M g}^{-1}$ .

### Mechanical stimulation

Surface-sterilized maize seeds were germinated in Petri dishes for 3 d as described above. Uniformly grown plants were selected and transferred to the paper rolls (see above) and were grown for a further 4 d under room conditions in the presence of 80 mM NaCl. Half of them were used as control (no mechanical stimulation), while the other half were exposed to constant

mechanical disturbance. The latter was achieved by mounting the growth container on the top of two small size aquarium pumps (MJ-S250; 2.5 W; Minjiang Shuizu Co., Guangzhou, PR China) which provided a constant vibration at 50 Hz frequency. As the solution level in the container was well below the rot tip, root oxygen availability was identical in both treatments. At the end of the experiments, roots were thoroughly rinsed in 10 mM  $\text{CaSO}_4$  solution to remove all the apoplastic  $\text{Na}^+$  and the root length was measured. Root and shoot  $\text{Na}^+$  content was then analyzed from frozen–thawed samples using flame photometry (Corning 410C, Essex, UK) as described elsewhere (Cuin et al. 2009).

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