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Salinity-induced ion flux patterns from the excised roots of *Arabidopsis sos* mutants

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Abstract The SOS signal-transduction pathway is known to be important for ion homeostasis and salt tolerance in plants. However, there is a lack of *in planta* electrophysiological data about how the changes in signalling and ion transport activity are integrated at the cellular and tissue level. In this study, using the non-invasive ion flux MIFE technique, we compared net K^+, H^+ and Na^+ fluxes from elongation and mature root zones of *Arabidopsis* wild type Columbia and *sos* mutants. Our results can be summarised as follows: (1) SOS mutations affect the function of the entire root, not just the root apex; (2) SOS signalling pathway is highly branched; (3) Na^+ effects on SOS1 may by-pass the SOS2/SOS3 complex in the root apex; (4) SOS mutation affects H^+ transport even in the absence of salt stress; (5) SOS1 mutation affects intracellular K^+ homeostasis with a plasma membrane depolarisation-activated outward-rectifying K^+ channel being a likely target; (6) H^+ pump also may be a target of SOS signalling. We provide an improved model of SOS signalling and discuss physiological mechanisms underlying salt stress perception and signalling in plants. Our work shows that *in planta* studies are essential for understanding the functional genomics of plant salt tolerance.

Keywords Potassium · Sodium · Membrane · Stress · Ion channels · Signalling

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Introduction

Extensively studied over the last few years, the SOS signal-transduction pathway is important for ion homeostasis and salt tolerance in plants (Hasegawa et al. 2000; Sanders 2000; Zhu 2000, 2003). The current model for the SOS signal-transduction pathway is that high Na^+ initiates changes in cytosolic-free Ca^{2+} (Knight et al. 1997). A myristoylated Ca^{2+}-binding protein encoded by SOS3 senses this salt-elicited Ca^{2+} signal and translates it to downstream responses (Liu and Zhu 1998; Ishitani et al. 2000). SOS3 interacts with and activates SOS2, a serine/threonine protein kinase (Halfter et al. 2000; Liu et al. 2000). This SOS2/SOS3 complex regulates the expression level of a salt tolerance effector gene encoding SOS1, a plasma membrane Na^+/H^+ antiporter, which extrudes excess Na^+ from the cytosol (Shi et al. 2000). Overexpression of SOS1 has been shown to improve salt tolerance in transgenic *Arabidopsis* (Shi et al. 2003). In addition, the SOS signal-transduction pathway has also been shown to be involved in K^+ acquisition by roots (Wu et al. 1996; Zhu et al. 1998). Under NaCl conditions, *sos1* mutant plants accumulate more Na^+ and retain less K^+ (Wu et al. 1996). Details of this process are unknown.

The ability of plant cells to maintain an optimal K^+/Na^+ ratio in the cytosol is one of the key features of plant salt tolerance (Maathuis and Amtmann 1999). Salinity may reduce K^+ uptake as a result of competition between Na^+ and K^+ for plasma membrane uptake sites (Tyerman and Skerrett 1999; Maathuis and Amtmann 1999; Tester and Davenport 2003). Furthermore, salt stress causes an excessive leakage of K^+ from the cell (Shabala 2000; Shabala et al. 2003; Chen et al. 2005), primarily due to plasma membrane depolarisation (Nocito et al. 2002; Shabala et al. 2003). It is not clear which of the numerous K^+ transport systems at the plasma membrane might be affected by the SOS signalling pathway, and what are their modes of regulation.

The application of external Ca^{2+} has been shown to ameliorate salinity stress symptoms significantly in many species (Cramer et al. 1987; Rengel 1992). Multiple mechanisms have been suggested, including Ca^{2+} blockage of Na^+ uptake through non-selective cation channels (NSCC; Demidchik and Tester 2002), prevention of K^+ leak from the cell (Shabala et al. 2003, 2005), enhanced K^+/Na^+ uptake selectivity at the plasma membrane (Cramer et al. 1987) and enhanced Na^+ extrusion via the SOS1 Na^+/H^+ antiporter (Liu and Zhu 1998). Although a direct link between the role of Ca^{2+} in SOS signal-transduction pathway and the amelioratory effects of supplemental Ca^{2+} on K^+ and Na^+ homeostasis is unlikely, ameliorative effects of Ca^{2+} may vary between wild-type (WT) plants and *sos* mutants. Surprisingly, direct electrophysiological evidence is scarce. There is also a lack of *in planta* electrophysiological data about how the signalling and ion transport activities are integrated at the cellular and tissue levels, and much of the physiology regarding the actual functioning of the *SOS* genes and signal-transduction pathway elements remain to be characterised. Knowledge of the physiological roles played by the various elements is crucial for a full understanding of plant salt-tolerance mechanisms, to enable the development of salt-tolerant cultivars.

Here, we report the use of the MIFE non-invasive ion flux microelectrode technique to address some of the above issues and to shed light on how the SOS-signalling pathway controls the fluxes of various ions across the plasma membrane of *Arabidopsis* root cells. Several specific questions were addressed: (1) how do SOS mutations affect K^+ and H^+ homeostasis within plant cells? (2) what are the spatial aspects of functional expression of *sos* genes? and (3) what is the role of Ca^{2+} in controlling K^+ and H^+ homeostasis in salinised tissues? As a result, we provide an improved model of the signal transduction pathways operating in *Arabidopsis* salt tolerance.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (wild type Columbia—WT, and *sos* mutants) seeds were obtained from NASC (Nottingham, UK). Plants were grown at 22°C and 24 h fluorescent lighting (100 mmol m^{-2} s^{-1} irradiance) in sterile conditions in 90 mm Petri dishes containing 0.35% Phytagel (Sigma) with full-strength Murashige and Skoog media (Duchefa, Haarlem, The Netherlands) and 1% (w/v) sucrose (see Demidchik et al. 2002 for more details). Seeds were surface sterilized with commercial bleach and sown on the surface of the Phytogel. The dishes were sealed with Parafilm and placed on edge, 5° off the vertical, so roots grew down the Phytagel surface without penetrating it. About 6–8-day-old plants were used for measurements.

Ion flux measurements

Net fluxes of Na^+, K^+ and H^+ were measured non-invasively using the MIFE (University of Tasmania, Hobart, Australia) technique essentially as described in our previous publications (Shabala et al. 1997; Shabala 2000). Briefly, microelectrodes were pulled and salinised with tributylchlorosilane. After backfilling, electrode tips were filled with commercially available ionophore cocktails (60031 for K^+; 71176 for Na^+; 95297 for H^+; all from Fluka, Busch, Switzerland). The electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan), their tips put together and positioned 20 μm above the root surface.

About 8–10-mm long apical root segments were excised and mounted horizontally in a Perspex holder by using agar (see Babourina et al. 2000 for details). The holder was immediately placed in a 4 mL measuring chamber filled with appropriate solution (follows later). The chamber was mounted on a computer-driven 3D-manipulator (PatchMan NP2, Eppendorf, Hamburg, Germany) and left to equilibrate for 40–50 min.

Net ion fluxes were measured from the root epidermis in mature (~2–3 mm from the root tip) and meristematic (~120 μm from the tip) zones. During measurements, the MIFE software controlled the PatchMan NP2 to move the electrodes between two positions, 20 and 50 μm from the root surface in a 10-s square-wave manner. The software also recorded electric potential differences from the electrodes between the two positions using a DAS08 analogue to digital card (Computer Boards, USA) in the computer (see Shabala et al. 1997; Newman 2001 for details). Using the calibrated Nernst slope of each electrode, ion fluxes were subsequently calculated using the MIFE software for cylindrical diffusion geometry (Newman 2001).

Solutions and protocols

The basic measuring solution (BSM) was 0.5 mM KCl plus 0.1 mM $CaCl_2$, pH 5.5 (unbuffered). When transient NaCl-induced ion flux responses were measured (as in Figs. 1–3), a double stock (100 mM) NaCl solution made up in 2 mL of BSM was added to 2 mL of BSM solution in the chamber. Calcium treatment (as in Figs. 4, 5) was given as 2 mM $CaCl_2$ stock added to roots incubated for 2.5–3 h in 0.5 mM KCl + 0.1 mM $CaCl_2$ + 50 mM NaCl solution. In most protocols, net ion fluxes were measured for 10 min prior to treatment to ensure steady initial values. Then salinity or calcium treatment was given, and transient ion flux responses were measured for another 50 min. The time required for the treatment solution addition and the establishment of the diffusion gradients after mixing was ~40 s (judged from measurements of Na^+ concentrations in the bath; data not shown). Accordingly, the first 60 s after solution changes were later discarded from the analysis and appear as a gap in all figures.

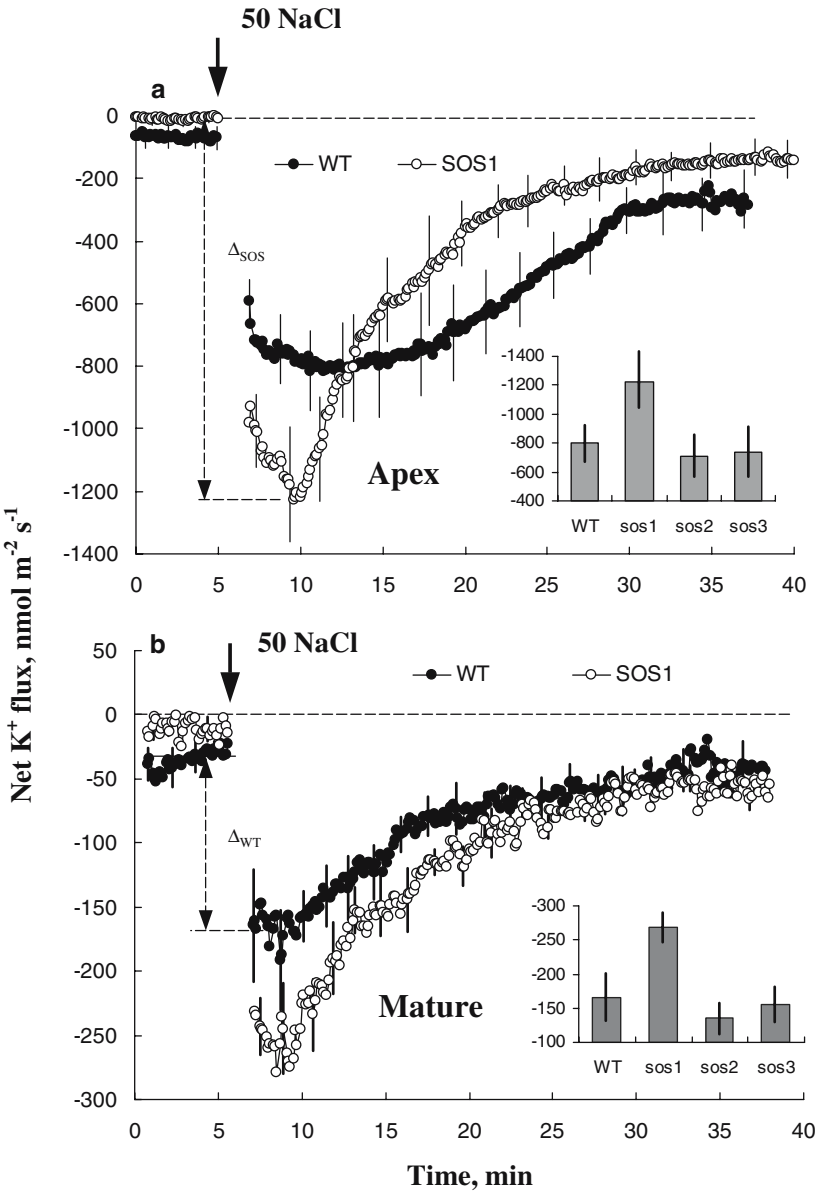

Fig. 1 Effect of salinity (50 mM NaCl) on net K^+ fluxes (influx positive) measured from *Arabidopsis* root apex (**a**) and mature zone (**b**). Fluxes are shown for WT (Columbia) and *sos1* roots for each zone. Responses from *sos2* and *sos3* roots were qualitatively similar to those shown for *sos1* and WT. Means ± SE (n = 5–7). *Insets* in each panel show the peak K^+ efflux change (Δ), measured as depicted in the *main panel*, for each of the genotypes studied

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 of epidermal cells in mature (~2–3 mm from the root tip) and meristematic (~120 μm from the tip) zones. Apical root segments were excised and mounted in the holder as described earlier and left to equilibrate in either BSM or the NaCl-containing solution for 50–60 min. Steady-state membrane potential values were measured from at least five individual plants for each treatment and each section of root, with not more than three measurements taken from any one root. Measurements were always made within 80 min of the excision and mounting. Membrane potentials were recorded for 1.5–2 min after the potential stabilised following cell penetration. Transient changes in membrane potential of root epidermal cells were measured in the mature zone, ~2–3 mm from the root tip. Once impaled, cell membrane potential was recorded for at least 2 min in control (BSM) conditions to ensure the seal stability. Then 50 mM NaCl was added, and membrane potential kinetics were recorded for another 20–40 min.

Results

NaCl-induced ion flux responses

Similar to our previous observations on barley (Shabala et al. 2003) and corn (Shabala 2003) roots, salinity stress (50 mM NaCl) caused significant changes in net ion

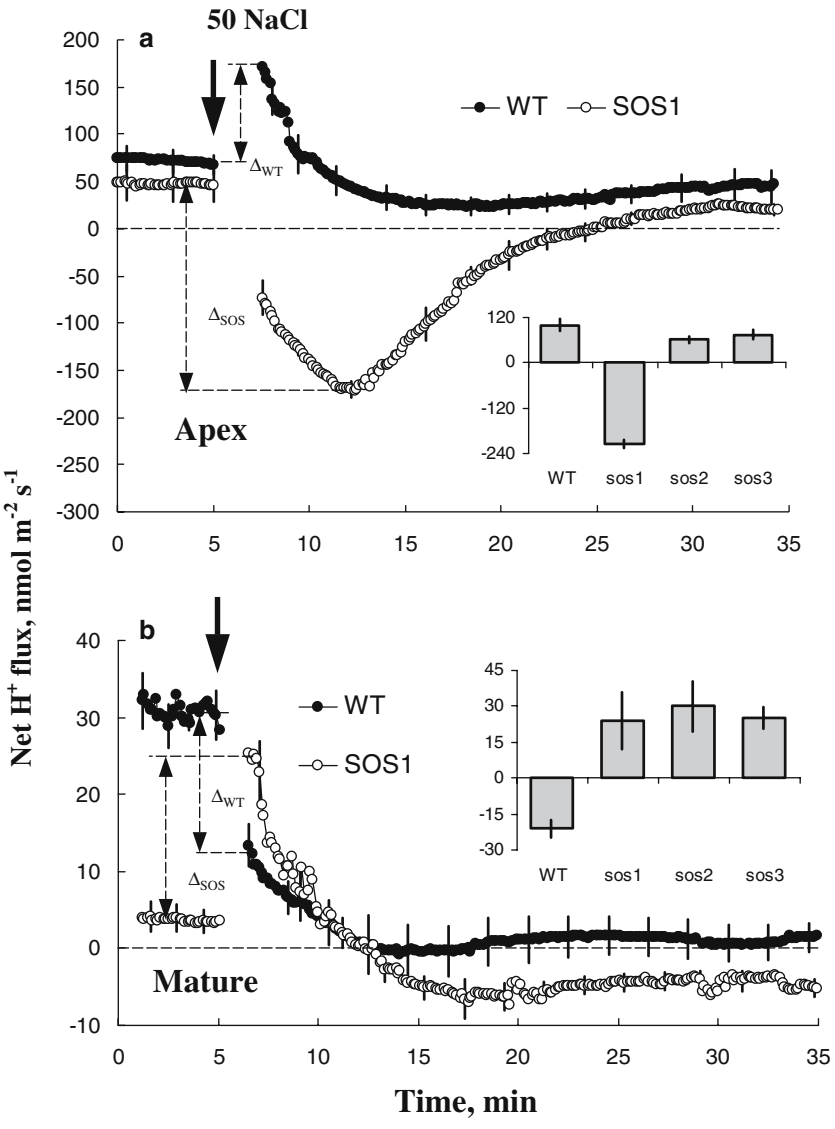

Fig. 2 Effect of salinity (50 mM NaCl) on net H^+ fluxes measured from *Arabidopsis* root apex (**a**) and mature zone (**b**). Fluxes are shown for WT (Columbia) and *sos*1 roots for each zone. Responses from *sos2* and *sos3* roots were qualitatively similar to those shown for WT plants in the root apex (*panel a*) and to those from *sos1* plants in the mature zone (*panel b*). Means ± SE (n = 4–7). *Insets* in each panel show the peak H^+ flux change (Δ), measured as depicted in the *main panel*, for each of the genotypes studied

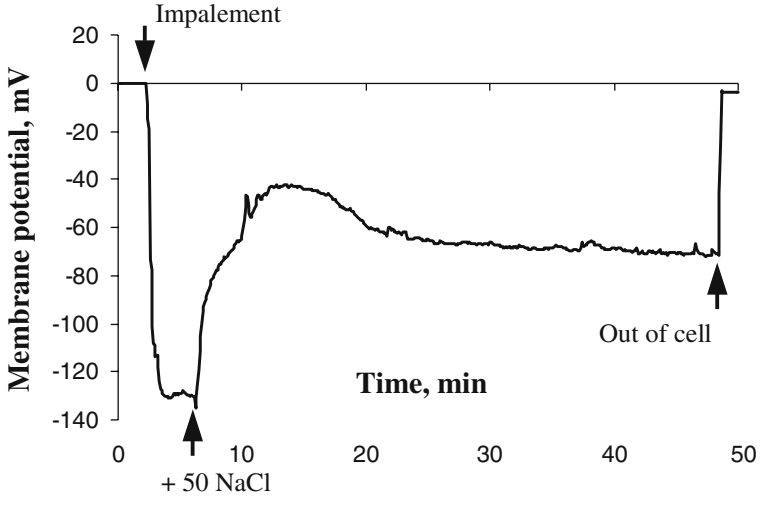

Fig. 3 NaCl-induced changes in the membrane potential of epidermal cells of *Arabidopsis* (WT Columbia) roots. Measurements were taken in mature zone, 2–3 mm from the root tip. One (of six) representative example is shown

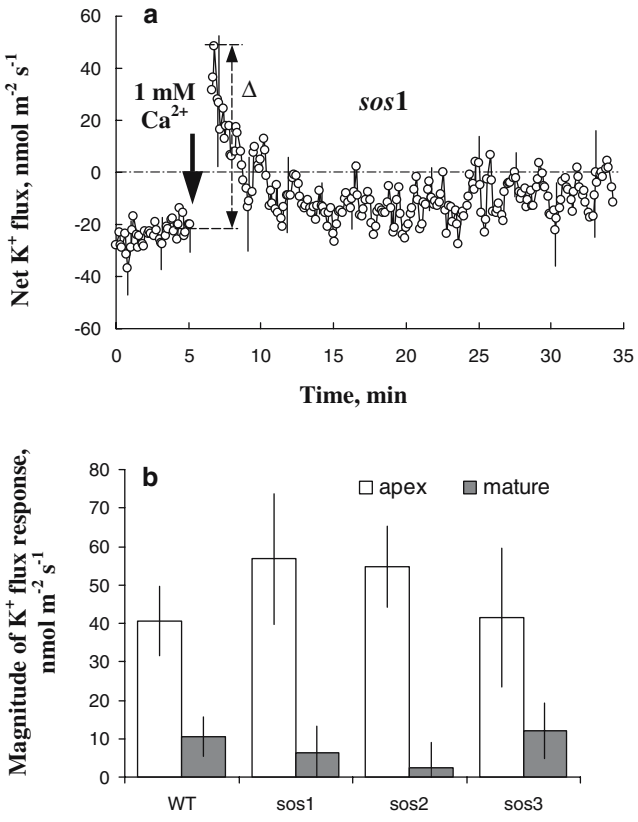

Fig. 4 Effect of supplemental Ca^{2+} on net K^+ fluxes from *Arabidopsis* roots. Plants were incubated in low Ca^{2+} (0.1 mM) solution in the presence of 50 mM NaCl for 2.5–3 h. (**a**) K^+ flux response to 1 mM $CaCl_2$ (added at *arrow*) for the apical zone of *sos1* roots. Means ± SE (n = 6). Qualitatively similar responses were obtained for all genotypes, from both mature zone and root apex. (**b**) Magnitude of Ca^{2+}- induced K^+ flux change (depicted as Δ in *panel a*), measured from apical and mature root zones of WT Columbia and *sos* mutants. Means ± SE (n = 4–7)

fluxes from both mature and apical meristem zones of *Arabidopsis* roots (Figs. 1, 2).

Measurement of Na^+ flux was complicated by poor Na^+ liquid ion exchanger (LIX) selectivity, which was shown to be sensitive to both K^+ and Ca^{2+} (Carden et al. 2001; Chen et al. 2005). Accordingly, these results are not reported and discussed in this paper. Instead, the focus is made on measured K^+ and H^+ fluxes.

Acute salt stress also caused significant K^+ efflux from *Arabidopsis* roots (Fig. 1). The peak K^+ efflux was reached several minutes after NaCl application, followed by a gradual recovery of K^+ flux (although it remained negative, i.e., net efflux). About a five-fold difference (significant at P = 0.01) was found between the peak K^+ efflux measured from mature and apical root zones in each genotype (Fig. 1), with K^+ fluxes in root meristem being more sensitive to salt treatment. Among the genotypes, significantly (P = 0.05) higher magnitude of K^+ flux response was measured from *sos1* roots in both the apical and mature zones (1226 ± 183 and 268 ± 22 nmol m^{-2} s^{-1}, respectively), while WT, *sos2* and *sos3* roots showed much smaller NaCl-induced K^+ efflux (750–800 and 130–160 nmol m^{-2} s^{-1} for root apex and mature zones, respectively).

Salinity-induced H^+ flux kinetics also showed some genotypic specificity (Fig. 2). In the root apex, 50 mM NaCl induced qualitatively similar H^+ flux responses in WT, *sos2* and *sos3* roots (Fig. 2a). One minute after salt addition, a significant (P = 0.05) increase in net H^+ influx was measured, as illustrated for WT roots in Fig. 2a (closed symbols). The magnitude of NaCl-induced H^+ influx was between 90 and 120 nmol m^{-2} s^{-1} and was not significantly (P = 0.05) different between these genotypes (Fig. 2a inset). In contrast, for *sos1* roots, the salt stress caused significant (P = 0.01) H^+ efflux (peak –215 ± 12; Fig. 2a).

The H^+ flux "story" was rather different in the mature root zone (Fig. 2b). Here, no significant difference was found between H^+ flux responses among the *sos* mutants (Fig. 2b inset). At the same time, NaCl treatment caused qualitatively different types of H^+ flux response from WT, with a NaCl-induced move towards H^+ efflux (Fig. 2b). This difference is significant at P = 0.05. Interestingly, the mature zone showed a steady pre-treatment net H^+ influx that was significantly higher (P = 0.05) in WT roots than in the *sos* mutants (Table 1).

NaCl-induced membrane potential responses

Membrane potentials (MP) in the mature zones of *Arabidopsis* roots were not significantly different between the various genotypes under control conditions (Table 2). At the same time, MP in the apical zones of *sos1* plants (–101 ± 3 mV) was significantly (P = 0.05) smaller than in WT (–121 ± 7 mV). Exposure to 50 mM NaCl for 1 h caused all mutants, in both zones, to depolarise to less negative MP than the WT (Table 2). *sos1* roots were depolarised to less negative values than the other genotypes, both in mature zone and in the root apex (Table 1). The difference in steady-state MP between WT and *sos1* roots under saline conditions was 12 ± 1.6 and 25 ± 2.0 mV for mature zone and root apex, respectively (both significant at P = 0.001; Table 1).

In another set of experiments, transient kinetics of NaCl-induced membrane potential changes were measured. The time course of MP changes (Fig. 3) was very similar to that of NaCl-induced K^+ flux (Fig. 1b), with the maximum membrane depolarisation observed 4–5 min after NaCl treatment, roughly at the same time as the peak K^+ efflux. This was followed by a substantial (15–20 mV) membrane repolarisation, with MP reaching new steady-state values 20–30 min after salt application.

Effects of supplemental Ca^{2+}

As SOS mutations are known to be associated with significant alterations in intracellular Ca^{2+} signalling

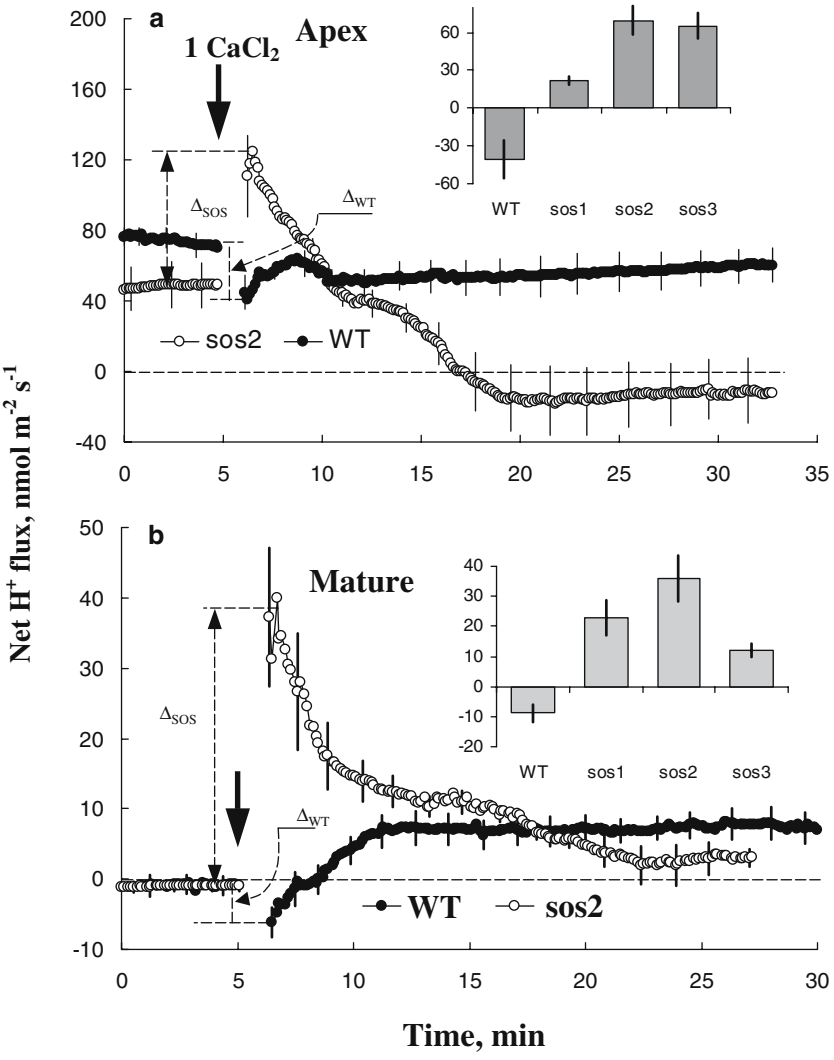

Fig. 5 Effect of supplemental Ca^{2+} on net H^+ fluxes from *Arabidopsis* roots. Plants were incubated in low Ca^{2+} (0.1 mM) solution in the presence of 50 mM NaCl for 2.5–3 h, and then 1 mM $CaCl_2$ was added (as indicated by *arrows*). Fluxes are for (**a**) root apex and (**b**) mature root zone of WT (Columbia) and *sos2* roots. Means ± SE (n = 6–10). Responses from *sos1* and *sos3* roots were qualitatively similar to those shown for *sos2*. *Insets* in each panel show the peak H^+ flux change (Δ), depicted as shown in the *main panels*, for each of the genotypes studied. Means ± SE (n = 6–10)

(Halfter et al. 2000; Elphick et al. 2001), we compared effects of supplemental Ca^{2+} on net K^+ and H^+ flux responses from *Arabidopsis* roots, pre-treated for several hours in 50 mM NaCl with low (0.1 mM) Ca^{2+}.

Immediately upon addition of 1 mM Ca^{2+}, significant ($P = 0.05$) K^+ influx was measured (Fig. 4a). No significant (P = 0.05) genotypical difference was found for the K^+ fluxes at one minute (Fig. 4b). Apical tissues were more sensitive, with the magnitude of Ca^{2+}-induced K^+ flux response at the meristem being 5–6-fold higher than at the mature zone (Fig. 4b).

Addition of supplemental Ca^{2+} to salinised *sos* mutants also caused an immediate and very dramatic H^+ uptake, not observed for WT (Fig. 5). In the apex, *sos2* and *sos3* roots were the most sensitive (significant compared with *sos1* or WT at P = 0.05; Fig. 5a inset). In the mature zone, all *sos* roots responded to added Ca^{2+} by increased net H^+ uptake (Fig. 5b inset). At the same time, qualitatively different H^+ flux responses were measured from WT roots, with a pronounced shift towards net H^+ efflux within the first several minutes after Ca^{2+} application in both zones (Fig. 5a, b). In general, Ca^{2+}-induced H^+ flux responses were larger in the root apex than in the mature zone (Fig. 5).

Discussion

SOS mutations affect the function of the entire root

SOS1, the final element of the SOS signal-transduction chain has been found predominantly in the root tip (Shi et al. 2002). Consequently, it was assumed that this is the region, where this gene is functionally expressed (Zhu 2003). Here, we have shown that the function of the *entire root* has been altered in *sos* (and particularly *sos1*) mutants (as evident by results from the mature root zone; Figs. 1, 2). This strongly suggests that location of the gene expression does not always portray the location of gene function. The possible explanation for this fact may be that measuring the promoter activity (reported by Shi et al.

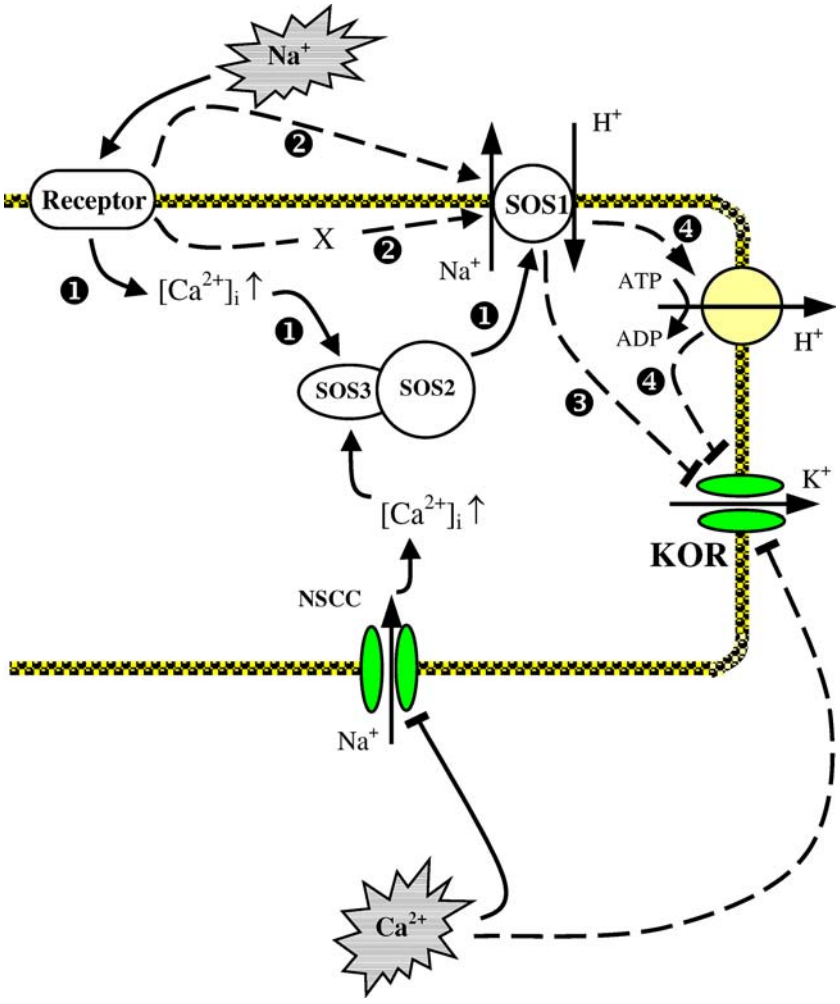

Fig. 6 An improved model (based on Zhu 2002, 2003) depicting SOS signalling in *Arabidopsis*. *Solid lines* in this scheme represent signalling components previously suggested in the literature. *Punctuated lines* are added based on our findings. In this model, the SOS1 activity is regulated either by the SOS2/SOS3 complex (as suggested in the literature; (❶) or by-passing it (❷). The latter can be done either by direct effect of external Na^+ on SOS1 activity (consistent with the idea of SOS1 being a putative Na^+ sensor; Zhu 2002) or via some alternative intracellular signalling pathway (from some hypothetical Na^+ receptor via anas yet unidentified signalling component X). In addition to being involved in active Na^+ extrusion from the cytosol, SOS1 also regulates K^+ transport at the plasma membrane. This effect is independent of the SOS2/SOS3 complex and may be due either to a direct blockage of a DAPC channel (❸) or, more likely, mediated by SOS1 control of the H^+-ATPase (❹)

2002) does not take into account post-transcriptional or post-translational regulation. Our results are consistent with those by Dennison et al. (2001) on Arabidopsis *akt* mutants, who reported that promoter activity does not always match electrophysiologically-assessed function of K^+ transport. This should be kept in mind and taken into account when constructing salt-tolerant genotypes by molecular genetics tools. To our knowledge, there are no reports of differences in the actual activity of the SOS1 Na^+/H^+ exchanger, between different root zones, which would correlate expression of this gene with its functional activity. Thus our results represent the first analysis of the functioning of the SOS1 Na^+/H^+ antiporter within different sections of the root and they highlight the importance of physiological studies when assigning the actual function of transporter genes in relation to their expression characteristics.

SOS signalling pathway is highly branched

Responses of K^+ (Fig. 1) and H^+ (Fig. 2) fluxes and cell membrane potential (Table 1) were significantly altered by SOS mutations. For H^+ the change in the flux (Fig. 2) is hardly surprising since SOS1 is a putative plasma membrane Na^+/H^+ transporter (Shi et al. 2000). For the large differences in the K^+ fluxes, however (Fig. 2), the connection with the SOS1 Na^+/H^+ exchanger and the SOS signal-transduction pathway is less obvious. The SOS1 Na^+/H^+ antiporter is a specific Na^+ exchanger and has been reported to be unable to transport K^+ (Qiu et al. 2002, 2003). Thus, some other K^+ transport system was affected by the *sos1* mutation (as discussed latter).

Table 1 Steady-state H^+ flux (nmol m^{-2} s^{-1}) values measured from *Arabidopsis* root surface in the root apex (meristem) and mature zones in control (no salt) conditions. Means ± SE (*n*)

Genotype	Apex	Mature
WT (Col)	70 ± 12 (5)	32 ± 3.7 (6)
sos1	47 ± 18 (4)	3.5 ± 1.8 (5)*
sos2	62 ± 17 (7)	−1.6 ± 1.5 (5)*
sos3	85 ± 8 (4)	2.3 ± 2.5 (5)*

* Significant compared with control (WT Columbia) at $P = 0.001$; Student's "*t*"-test

Furthermore, the different *sos* mutants show different magnitudes of ion flux responses after exposure to salinity (Figs. 1, 2). If the SOS signal-transduction pathway was operating on a single, direct pathway, in which all components were necessary to activate the end response as has been proposed (Zhu 2003), one would expect that the removal of any one part of the chain would result in the same response, no matter which part of the chain was removed. This is clearly not the case. This suggests that there are additional components involved, and a much more complicated signal-transduction pathway than has so far been presented.

SOS mutation affects H^+ transport in the absence of salt stress

Significant ($P = 0.05$) differences in the pre-treatment *steady state* H^+ fluxes were observed between the WT and the *sos* mutants in the mature zone (Table 1). This must imply that all the *sos* mutations affect the activity of plasma membrane H^+ transporters (either H^+-pumping ATPase or some secondary active H^+-co-transport system) in this zone, even without imposed NaCl. This is in contrast to reports reviewed by Zhu (2002), where the effects of the mutations were seen only after the imposition of NaCl. The reason for this discrepancy could be the indirect way that SOS1 exchange activity was measured in the above studies (using purified membrane vesicles; Qiu et al. 2003) rather than the direct *in planta* method employed in our study.

MIFE results support the functional role of SOS1 as a Na^+/H^+ antiporter

In the root apex, NaCl induced qualitatively similar H^+ flux responses in WT, *sos2* and *sos3*, with an initial increase in the H^+ influx into the root. In contrast, the response of H^+ fluxes in *sos1* was qualitatively different (immediate efflux followed by a gradual recovery). This can be interpreted as increased activity of Na^+/H^+ antiport (extruding Na^+ from the cell in exchange for H^+ influx) not only in WT but also in *sos2* and *sos3* plants. Only the *sos1* mutants failed to show that activity. Strong H^+ efflux measured from *sos1* plants may be a result of NaCl activation of the H^+-ATPase pump, reported for a range of species (Nakamura et al. 1992; Ayala et al. 1996; Vera-Estrella et al. 1999). Such activation in WT, *sos2* and *sos3* plants is presumably masked by increased Na^+/H^+ antiport activity.

Na^+ effect on SOS1 by-passes the SOS2/SOS3 complex in the root apex

The fact that *sos2* and *sos3* mutants showed increased H^+ influx (although slightly smaller than in WT; Fig. 2a) in response to NaCl treatment, indicates that the activity of SOS1 Na^+/H^+ exchanger is preserved in these mutants, albeit at a reduced rate. This means that SOS1 is constitutively expressed in the apex, and therefore, SOS2 and SOS3 do not play an exclusive role in controlling the H^+/Na^+ exchange activity. Thus there is a certain degree of "by-passing" of the SOS2/SOS3 complex in signal transduction to the SOS1 Na^+/H^+ exchanger. This is in contrast to the linear SOS signal-transduction chain proposed in reports from Zhu's laboratory (Zhu 2000, 2003; Chinnusamy et al. 2005).

H^+ pump may be a target of SOS signalling

In the mature zone, no significant differences in net H^+ fluxes between the *sos* mutants were found (Fig. 2b), with all of them responding to NaCl treatment with an initial increase in net H^+ influx. This is in contrast to WT plants, which responded to NaCl by an immediate decrease in net H^+ influx (Fig. 2b). If the measured H^+ fluxes reflect only the activity of the SOS1 Na^+/H^+

Table 2 Steady-state values of membrane potential of *Arabidopsis* root epidermal cells in control and after 1 h of 50 mM NaCl treatment. Means ± SE (*n*)

Genotype	Mature zone		Root apex	
	Control	50 mM NaCl	Control	50 mM NaCl
WT (Col)	−128 ± 3.4 (33)	−64 ± 1.9 (41)	−121 ± 6.9 (16)	−69 ± 2.8 (24)
sos1	−126 ± 2.6 (25)	−52 ± 1.3 (33)^c	−101 ± 3.2 (23)^a	−46 ± 1.3 (24)^c
sos2	−122 ± 3.0 (22)	−59 ± 1.6 (24)	−119 ± 2.0 (24)	−50 ± 2.0 (24)^c
sos3	−120 ± 2.5 (24)	−58 ± 1.2 (24)^a	−110 ± 2.7 (24)	−54 ± 1.1 (24)^c

Significance of difference from WT Columbia (at appropriate treatment) at $P =$ ^{a}0.05, ^{b}0.01 and ^{c}0.001; Student's "*t*"-test

exchanger, the results are the reverse of those expected because they would represent *increased* Na^+/H^+ exchange activity in the *sos* mutants, with *decreased* Na^+/H^+ exchange activity in the WT plants. More likely, the initial increase in the H^+ influx in the mature zone is the result of a decrease in the H^+ extrusion-pumping activity in all the *sos* mutants. This interpretation of Fig. 2b H^+ fluxes is supported by observations that NaCl-induced H^+ flux responses are stimulated by fusicoccin (Babourina et al. 2000) and suppressed by vanadate (Shabala 2000). Thus the SOS signal-transduction pathway appears to exert some control over H^+ pumping within the mature zone of the root.

SOS1 mutation affects intracellular K^+ homeostasis

The K^+ efflux from *sos1* roots was greater than that seen in either *sos2*, *sos3* or the WT plants in both the apical and mature regions. This indicates that SOS signal transduction does have an effect on K^+ homeostasis in the roots, and that this effect is linked with SOS1, independently of SOS2 and SOS3. Indeed, if the SOS2/SOS3 complex were to mediate this effect, the magnitude of the NaCl-induced K^+ efflux would be the same for all *sos* mutants. This is clearly not the case. Our results are consistent with those reported by Qi and Spalding (2004), who also observed impairment by Na^+ of K^+ permeability in *sos1* but not in *sos2* or *sos3* roots. Our results support their suggestion that a basal level of SOS1 activity, sufficient to maintain K^+ permeability of root cells, persists in the absence of the SOS2/SOS3 regulatory systems. At the same time, most published models (Yokoi et al. 2002; Zhu 2002; Xiong and Zhu 2002) suggest that K^+ transport in cells is controlled by the *SOS2* gene. Again, this discrepancy appears to arise from the different experimental techniques used (i.e., direct in vivo electrophysiology versus in vitro membrane vesicles) and once again highlights the importance of *in planta* studies to understand gene function.

Supplemental Ca^{2+} ameliorates Na^+-induced K^+ leakage

The Na^+ hypersensitivity of *sos3* plants has been shown to be reduced by increased external Ca^{2+} (Liu and Zhu 1997, 1998; Elphick et al. 2001) alongside an improvement in the K^+/Na^+ selective uptake (Liu and Zhu 1997). In this study, we showed the ameliorative effect of supplemental Ca^{2+} to restrict the NaCl-induced K^+ leak in WT and all *sos* mutant plants (Fig. 4). Specific details of this process remain to be found. However, the qualitative similarity of changes in K^+ fluxes in response to supplemental Ca^{2+} (Fig. 4b) indicates that this effect is either downstream from, or relatively independent of, the SOS signal-transduction pathway. As for effects of supplemental Ca^{2+} on H^+ flux from salinised *Arabidopsis* roots, the higher magnitude of response in *sos2* and *sos3* plants in the root apex (Fig. 5a) is consistent with idea of Ca^{2+} binding to the SOS2/SOS3 complex (Zhu 2003).

Complexity of SOS signalling: an improved model

Our results suggest that SOS signal transduction is more complicated than has so far been portrayed. In the improved model we present (Fig. 6), SOS1 does represent a Na^+/H^+ exchanger as previously proposed (Shi et al. 2000, 2002), which operates under the regulation of the SOS2/SOS3 complex (Zhu 2000, 2002, 2003) (labelled ❶ in Fig. 6). However, it appears that Na^+ can act directly upon SOS1 (as evidenced by H^+ flux data from the root apex; Fig. 3a). This pathway, labelled ❷ in Fig. 6, is consistent with the suggestion of SOS1 being a Na^+ sensor (Zhu 2002) and is reflected in the model as a by-pass of the SOS2/SOS3 complex in the conventional signal-transduction pathway (Halfter et al. 2000; Ishitani et al. 2000). Also, the fact that K^+ fluxes were greatly modified in *sos1*, compared with WT and other *sos* mutants, (Fig. 2) suggests a signal (labelled ❸ in Fig. 6) from SOS1 directly to some putative K^+ transporter by-passing SOS2 and SOS3. Alternatively, SOS1 control over K^+ transport may be mediated by the H^+-pump (labelled ❹ in our model). Pharmacological data (Shabala et al. 2005), general similarity between MP (Fig. 3) and K^+ flux (Fig. 1) changes and the fact that the *sos1* apex showed the strongest membrane depolarisation under salinity (Table 2) all suggest that the most likely targets for SOS1 control over K^+ homeostasis are depolarisation-activated outward-rectifying K^+ channels (DAPC; Maathuis and Sanders 1995). Specific mechanisms of such regulation remain to be found.

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References

Ayala F, O'Leary JW, Schumaker KS (1996) Increased vacuolar and plasma membrane H^+-ATPase activities in *Salicornia bigelovii* Torr in response to NaCl. J Exp Bot 47:25–32

Babourina O, Leonova T, Shabala S, Newman I (2000) Effect of sudden salt stress on ion fluxes in intact wheat suspension cells. Ann Bot 85:759–767

Carden DE, Diamond D, Miller AJ (2001) An improved Na^+-selective microelectrode for intracellular measurements in plant cells. J Exp Bot 52:1353–1359

Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S (2005) Screening plants for salt tolerance by measuring K+ flux: a case study for barley. Plant, Cell Environ (in press)

Chinnusamy V, Jagendorf A, Zhu JK (2005) Understanding and improving salt tolerance in plants. Crop Sci 45:437–448

Cramer GR, Lynch J, Lauchli A, Epstein E (1987) Influx of Na^+, K^+, and Ca^{2+} into roots of salt-stressed cotton seedlings. Effects of supplimental Ca^{2+}. Plant Physiol 83:510–516

Demidchik V, Bowen HC, Maathuis FJM, Shabala SN, Tester MA, White PJ, Davies JM (2002) *Arabidopsis thaliana* root

non-selective cation channels mediate calcium uptake and are involved in growth. Plant J 32:799–808
Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from *Arabidopsis* roots. Plant Physiol 128:379–387
Dennison KL, Robertson WR, Lewis BD, Hirsch RE, Sussman MR, Spalding EP (2001) Functions of AKT1 and AKT2 potassium channels determined by studies of single and double mutants of *Arabidopsis*. Plant Physiol 127:1012–1019
Elphick CH, Sanders D, Maathuis FJM (2001) Critical role of divalent cations and Na^+ efflux in *Arabidopsis thaliana* salt tolerance. Plant, Cell Environ 24:733–740
Halfter U, Ishitani M, Zhu JK (2000) The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc Nat Acad Sci USA 97:3735–3740
Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Ann Rev Plant Physiol Plant Mol Biol 51:463–499
Ishitani M, Liu JP, Halfter U, Kim CS, Shi WM, Zhu JK (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. Plant Cell 12:1667–1677
Knight H, Trewavas AJ, Knight MR (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. Plant J 12:1067–1078.997
Liu JP, Zhu JK (1997) An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. Proc Nat Acad Sci USA 94:14960–14964
Liu JP, Zhu JK (1998) A calcium sensor homolog required for plant salt tolerance. Science 280:1943–1945
Liu J, Ishitani M, Halfter U, Kim C-S, Zhu JK (2000) The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. Proc Nat Acad Sci USA 97:3730–3734
Maathuis FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann Bot 84:123–133
Maathuis FJM, Sanders D (1995) Contrasting roles in ion transport of two K^+-channel types in root cells of *Arabidopsis thaliana*. Planta 197:456–464
Nakamura Y, Kasamo K, Shimosato N, Sakata M, Ohta E (1992) Stimulation of the extrusion of protons and H^+-ATPase activities with the decline in pyrophosphatase activity of the tonoplast in intact mung bean roots under high-NaCl stress and its relation to external levels of Ca^{2+} ions. Plant Cell Physiol 33:139–149
Newman IA (2001) Ion transport in roots: measurements of fluxes using ion-selective microelectrodes to characterize transporter function. Plant, Cell Environ 24:1–14
Nocito FF, Sacchi GA, Cocucci M (2002) Membrane depolarization induces K^+ efflux from subapical maize root segments. New Phytologist 154:45–51
Qi Z, Spalding EP (2004) Protection of plasma membrane K^+ transport by the *salt overly sensitive1* Na^+-H^+ antiporter during salinity stress. Plant Physiol 136:2548–2555
Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na+/H+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc Nat Acad Sci USA 99:8436–8441
Qiu QS, Barkla BJ, Vera-Estrella R, Zhu JK, Schumaker KS (2003) Na^+/H^+ exchange activity in the plasma membrane of *Arabidopsis*. Plant Physiol 132:1041–1052
Rengel Z (1992) The role of calcium in salt toxicity. Plant, Cell Environ 15:625–632
Sanders D (2000) Plant biology: the salty tale of *Arabidopsis*. Curr Biol 10:R486–R488
Shabala S (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. Plant, Cell Environ 23:825–837
Shabala S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. Ann Bot 92:627–634
Shabala SN, Lew RR (2002) Turgor regulation in osmotically stressed *Arabidopsis* epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. Plant Physiol 129:290–299
Shabala S, Newman I (2000) Salinity effects on the activity of plasma membrane H^+ and Ca^{2+} transporters in bean leaf mesophyll: masking role of the cell wall. Ann Bot 85:681–686
Shabala SN, Newman IA, Morris J (1997) Oscillations in H^+ and Ca^{2+} ion fluxes around the elongation region of corn roots and effects of external pH. Plant Physiol 113:111–118
Shabala S, Shabala L, Van Volkenburgh E (2003) Effect of calcium on root development and root ion fluxes in salinised barley seedlings. Funct Plant Biol 30:507–514
Shabala S, Shabala L, Van Volkenburgh E, Newman I (2005) Effect of divalent cations on ion fluxes and leaf photochemistry in salinised barley leaves. J Exp Bot 56:1369–1378
Shi HZ, Ishitani M, Kim CS, Zhu JK (2000) The *Arabidopsis thaliana* salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. Proc Nat Acad Sci USA 97:6896–6901
Shi HZ, Quintero FJ, Pardo JM, Zhu JK (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. Plant Cell 14:465–477
Shi H, Wu S-J, Zhu JK (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter improves salt tolerance in Arabidopsis. Nat Biotechnol 21:81–85
Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 91:503–527
Tyerman SD, Skerrett IM (1999) Root ion channels and salinity. Scientia Horticulturae 78:175–235
Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O (1999) Salt stress in *Mesembryanthemum crystallinum* L cell suspensions activates adaptive mechanisms similar to those observed in the whole plant. Planta 207:426–435
Wu SJ, Ding L, Zhu JK (1996) sos1, a genetic locus essential for salt tolerance and potassium acquisition. Plant Cell 8:617–627
Xiong L, Zhu JK (2002) Molecular and genetic aspects of plant responses to osmotic stress. Plant, Cell Environ 25:131–139
Yokoi S, Bressan RA, Hasegawa PM (2002) Salt stress tolerance of plants. JIRCAS Working Report, pp25–33
Zhu J-K (2000) genetic analysis of plant salt tolerance using *Arabidopsis*. Plant Physiol 124:941–948
Zhu JK (2002) Salt and drought stress signal transduction in plants. Ann Rev Plant Biol 53:247–273
Zhu JK (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441–445
Zhu JK, Liu J, Xiong L (1998) Genetic analysis of salt tolerance in *Arabidopsis thaliana*: evidence of a critical role for potassium nutrition. Plant Cell 10:1181–1192