Salicylic acid improves salinity tolerance in Arabidopsis by restoring membrane potential and preventing salt-induced K\(^+\) loss via a GORK channel

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Received 20 December 2012; Revised 15 February 2013; Accepted 28 February 2013

Abstract

Despite numerous reports implicating salicylic acid (SA) in plant salinity responses, the specific ionic mechanisms of SA-mediated adaptation to salt stress remain elusive. To address this issue, a non-invasive microelectrode ion flux estimation technique was used to study kinetics of NaCl-induced net ion fluxes in Arabidopsis thaliana in response to various SA concentrations and incubation times. NaCl-induced K\(^+\) efflux and H\(^+\) influx from the mature root zone were both significantly decreased in roots pretreated with 10–500 \(\mu\)M SA, with strongest effect being observed in the 10–50 \(\mu\)M SA range. Considering temporal dynamics (0–8-h SA pretreatment), the 1-h pretreatment was most effective in enhancing K\(^+\) retention in the cytosol. The pharmacological, membrane potential, and shoot K\(^+\) and Na\(^+\) accumulation data were all consistent with the model in which the SA pretreatment enhanced activity of H\(^+\)-ATPase, decreased NaCl-induced membrane depolarization, and minimized NaCl-induced K\(^+\) leakage from the cell within the first hour of salt stress. In long-term treatments, SA increased shoot K\(^+\) and decreased shoot Na\(^+\) accumulation. The short-term NaCl-induced K\(^+\) efflux was smallest in the gork1-1 mutant, followed by the rbohD mutant, and was highest in the wild type. Most significantly, the SA pretreatment decreased the NaCl-induced K\(^+\) efflux from rbohD and the wild type to the level of gork1-1, whereas no effect was observed in gork1-1. These data provide the first direct evidence that the SA pretreatment ameliorates salinity stress by counteracting NaCl-induced membrane depolarization and by decreasing K\(^+\) efflux via GORK channels.

Key words: H\(^+\)-ATPase, H\(^+\) flux, depolarization, K\(^+\) flux, membrane potential, outward-rectifying K\(^+\) channel, potassium homeostasis.

Introduction

Salicylic acid (SA) is known as a signalling molecule that modifies plant responses to pathogen infection. The SA concentrations increase after a pathogen attack, inducing the expression of pathogen-related genes and initiating the development of systemic acquired resistance and hypersensitive response (Dempsey et al., 1999; Durrant and Dong, 2004). Necrotic damage occurs at a site of the pathogen entry during the hypersensitive response, which is usually accompanied by production of reactive oxygen species (ROS). In addition to being an important component of biotic stress tolerance mechanism, SA also regulates various aspects of plant responses to abiotic stresses through extensive signalling cross-talk with other growth hormones (Horváth et al., 2007; Tuteja and Sopory, 2008; Asensi-Fabado and Munné-Bosch, 2011). In particular, SA plays a key role in plant adaptive responses to osmotic stress, high salinity, oxidative stress, heavy metals, ozone, UV radiation, high temperatures, and chilling and drought stresses (reviewed in Horváth et al., 2007; Ashraf et al., 2010; Hayat et al., 2010). However, exact mechanisms by which SA protects plants during abiotic stresses remain obscure.
Exogenous addition of SA can ameliorate toxicity symptoms induced by salinity stress in many plant species (reviewed in Horváth et al., 2007; Ashraf et al., 2010; Hayat et al., 2010). The ability of exogenously applied SA to improve photosynthetic capacity, enhance antioxidant protection by modifying the activities of antioxidant enzymes, increase accumulation of soluble carbohydrates, increase ATP content, and maintain optimum K+/Na+ ratio under saline conditions has been suggested as potential mechanisms of salt tolerance in plants (reviewed in Horváth et al., 2007; Ashraf et al., 2010; Hayat et al., 2010). However, most of these results are from long-term (days to months) salt-exposure experiments. Hence, the above effects are likely to be indirect and strongly dependent on doses of SA used, plant species studied, and intensity and duration of salt stress (reviewed in Horváth et al., 2010). In this context, reports on the effects of exogenous SA. Amtmann, 1999; Shabala and Cuin, 2008; Demidchik et al., 2010). In this context, reports on the effects of exogenous SA on ionic relations and membrane-transport processes in plants are rare and highly controversial. Using radioisotope 86Rb+, it has been shown that SA application alone induced plasma-membrane depolarization (Glass, 1974a) and inhibited K+ uptake in barley seedlings (Glass, 1974a, b; Harper and Balke, 1981). These results are very difficult to explain given the importance of K+ retention to plant metabolism (Marschner, 1995) and the reported ameliorative effects of SA on salinity stress via increasing K+ concentration at the whole-plant level (He and Zhu, 2008; Kováčik et al., 2009). Hence, the role of K+ dynamics in the amelioration of salt stress by SA remains to be elucidated.

Under salt conditions, a passive entry of Na+ ions through the plasma membrane causes a strong membrane depolarization that favours K+ leakage via depolarization-activated K+ outward-rectifying (KOR) channels (reviewed in Shabala and Cuin, 2008). The K+ can also leak from the cytosol via non-selective cation channels (NSCC), following their activation by ROS. Indeed, ROS concentrations increase significantly under saline conditions (reviewed by Miller et al., 2009), and ROS-induced activation of NSCC channels is also widely reported (Demidchik et al., 2003, 2010; Mittler et al., 2004; Demidchik and Maathuis, 2007; Miller et al., 2008). Hence, prevention of K+ loss through above channels during salt stress is critical for salt tolerance in plants. Indeed, divalent cations (Shabala et al., 2003, 2006), polyamines (Pandolfi et al., 2010) and compatible solutes (Cuin and Shabala, 2007; Cuin and Shabala, 2007) are efficient in preventing NaCl-induced K+ leakage and improving plant growth during salt stress. However, it is unknown whether SA plays the same role in preventing NaCl-induced K+ leakage.

The activation of proton pumps by salt stress (Kerkeb et al., 2001) is positively correlated with salinity tolerance, and this effect is stronger in salt-tolerant than salt-sensitive species (Niu et al., 1993; Chen et al., 2007b; Sahu and Shaw, 2009). Such an increase in H+ pumping could act in two parallel pathways. First, enhanced activity of H+-ATPase would regulate voltage-dependent outward-rectifying K+ channels and prevent K+ leakage via KOR channels (Chen et al., 2007b). Secondly, H+ pumping would provide a driving force for the plasma-membrane Na+/H+ exchanger (SOS1-Salt overly sensitive 1) to remove Na+ from the cytoplasm to the apoplast (Shi et al., 2000; Apse and Blumwald, 2007), thus reducing Na+ load in the cytoplasm. Interestingly, the recent studies on temperature stress have demonstrated that SA pretreatment increased the activity of the plasma-membrane H+-ATPase in grape and peas (Liu et al., 2008, 2009) and, hence, each of the two pathways mentioned above may be potentially affected by SA.

Thus, the working hypothesis for this study was that beneficial effects of SA during salt stress may be related to upregulation of the plasma-membrane H+-ATPase activity and consequent effects on intracellular K+ dynamics. Consequently, the aim of this work was characterization of the downstream targets of SA signalling. This was achieved by applying the non-invasive microelectrode ion flux estimation (MIFE) technique (Shabala et al., 1997; Newman, 2001) to characterize the effects of exogenous SA application on the functioning of the plasma-membrane transporters in epidermal cells of Arabidopsis roots under salt stress. This study shows that root pre-incubation with micromolar concentrations of SA leads to a significant mitigation of salt stress due to enhanced K+ retention in SA-treated roots, resulting from enhanced H+-ATPase activity and reduced salt-induced K+ loss via a GORK channel.

Materials and methods

Plant materials

Arabidopsis thaliana L. Heynich wild-type ecotype Columbia (Col-0) and rbohD (SALK_021661, Col-0) mutant seeds were obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus, OH, USA); gork1-1 (SALK_092448, Col-0) mutant seeds were a generous gift from Prof. Hervé Sentenac (ENSAM, Montpellier, France).

Growth experiments

Whole-plant responses to SA under salinity stress were studied in hydroponic and soil culture experiments.

Hydroponic culture

Arabidopsis seeds were surface sterilized with 1% (v/v) sodium hypochlorite (commercial bleach) plus 0.01% Triton for 10 min and washed thoroughly with sterilized deionized water. Seeds were then sown on 0.8% (w/v) agar containing nutrient solution (1.25 mM...
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KNO₃, 0.625 mM KH₂PO₄, 0.5 mM MgSO₄, 0.5 mM Ca(NO₃)₂, 0.045 mM FeNaEDTA, 0.16 μM CuSO₄, 0.38 μM ZnSO₄, 1.8 μM MnSO₄, 45 μM H₂BO₃, 0.015 μM (NH₄)₂MoO₄·2H₂O, and 0.01 μM CoCl₂) in 1.5 ml centrifuge tubes and sterilized at 4 °C for 48 h. The bottom of the tubes was cut off and the tubes were inserted in a float with holes and suspended over aerated nutrient solution. The seedlings were grown in a controlled growth room under a 16/8 light/dark cycle at 23 °C with an irradiance of 150 μmol m⁻² s⁻¹. After 1 week, seedlings were thinned to one healthy seedling in each centrifuge tube. After 3 weeks, seedlings were kept in nutrient solution supplemented with 50 μM SA for 1 h. Then, the SA-pretreated seedlings were transferred to nutrient solution containing 100 mM NaCl. The salt treatment continued for 2 weeks. A randomized complete block design was used with four replications (four pots, each pot containing four centrifuge tubes) for each treatment. Nutrient solutions were replaced every 2 days to ensure a relatively constant ion composition.

**Soil culture**

The pots containing a peat/perlite/vermiculate soil mixture (1:1:1, v/v) was drenched overnight with the same growth nutrient solution supplemented with 50 μM SA for an hour and then exposed to salt stress by adding nutrient solution containing 100 mM NaCl. The salt treatment was maintained for 2 weeks by watering with nutrient solution containing 100 mM NaCl every 3 days. Control plants were irrigated with the nutrient solution without NaCl. A randomized complete block design was used, with four replicate pots for each treatment.

**Biomass**

Plants in both hydroponic and soil experiments were harvested after 2 weeks of NaCl treatment (at the age of 5 weeks). Shoots were thoroughly rinsed with ice-cold 0.5 mM CaSO₄ solution, excess water was removed by blotting shoots with paper towels, and fresh weight was measured immediately. Plants were then dried at 65 °C for 2 d in a Unitherm Dryer (Birmingham, UK) and weighed. Shoot water content (%) was calculated as the difference between fresh and dry weight of the biomass.

**Shoot Na⁺ and K⁺ concentrations**

Dry Arabidopsis shoots were weighed and digested in 6 ml of 98% (v/v) H₂SO₄ and 3 ml 30% (v/v) H₂O₂ for 5 h as described by Skoog et al. (2000). The shoot Na⁺ and K⁺ concentrations were determined by a flame photometer.

**Electrophysiology**

For the MIFE and membrane potential experiments, surface-sterilized Arabidopsis seeds were placed in 90-mm Petri dishes containing agar (0.8% w/w) media with 1 mM KCl plus 0.1 mM CaCl₂, pH 5.5 (Guo et al., 2009, Jayakannan et al., 2011). Seeds were vernalized at 4 °C for 2 days in the dark and then transferred into a growth chamber under a 16/8 day/night cycle at 23 °C with the irradiance of 150 μmol photons m⁻² s⁻¹ during the day. Petri dishes were positioned vertically to prevent roots penetrating into the agar. Four to five-day-old seedlings were used for ion flux and membrane potential measurements. All the measurements were made at the mature root zone (>2 mm from the root tip).

**Ion-flux measurements**

Net fluxes of H⁺, K⁺, and Na⁺ were measured non-invasively using ion-selective vibrating microelectrodes (University of Tasmania, Hobart, Australia) as described previously (Shabala et al., 1997, 2003). Briefly, microelectrodes were pulled from borosilicate glass capillaries (GC 150-10; Harvard Apparatus, Kent, UK), oven-dried at 230 °C overnight and silanized using tributylchlorosilane (no. 90796; Fluka). Electrode tips were broken to obtain external tip diameters of 2–3 μm. The tips of blank electrodes were back-filled with appropriate solutions (0.15 mM NaCl + 0.4 mM KH₂PO₄, adjusted to pH 6.0 using NaOH for the H⁺ electrodes; 0.5 M KCl for the K⁺ electrodes; and 0.5 M NaCl for the Na⁺ electrodes). The electrode tips were then front-filled with commercially available ion-selective cocktails (H⁺ 95297, K⁺ 606031; both from Sigma- Aldrich). For Na⁺ measurements, an improved calixarene-based Na⁺ cocktail was used (see Jayakannan et al., 2011 for details). Prepared electrodes were calibrated in a set of standards (pH 4.76–7.10; K⁺ 0.25–1.5 mM; Na⁺ 5–150 mM). Electrodes with the Nernst slope responses of less than 50 mV per decade were discarded. Electrodes were mounted on a manually operated 3D-micromanipulator (MMT-5; Narishige, Toyko, Japan) and their tips were aligned and positioned 40 μm away from the root surface. During the measurements, a computer-controlled stepper motor moved electrodes in a slow (10-s cycle, 40-μm amplitude) square-wave between the two positions, close to (40 μm) and away from (80 μm) the root surface. CHART software (Shabala et al., 1997, Newman, 2001) recorded the potential difference between two positions and converted it to electrochemical potential difference using the calibrated Nernst slope of the electrode. Net ion fluxes were calculated using the MIFEFLUX software for cylindrical diffusion geometry (Newman, 2001).

**Pharmacology experiment**

Prior to flux measurements, Arabidopsis seedlings were pretreated with 1 mM sodium orthovanadate (Na₃VO₄; P-type H⁺-ATPase inhibitor) or 0.1 mM N,N′-dicyclohexylcarbodiimide (DCCD; H⁺-ATPase inhibitor) for 1 h. Both chemicals were purchased from Sigma-Aldrich.

**Membrane-potential measurements**

The root of an Arabidopsis seedling was immobilized and preconditioned as described above. The membrane potential measurements were made as described by Bose et al. (2010a, b). Conventional microelectrode (Harvard Apparatus) with a tip diameter of ~0.5 μm was filled with 1 mM KCl and connected to a MIFE electrometer (Newman, 2001) via an Ag-AgCl half-cell. Then, the mounted electrode was impaled into the epidermal cells of mature root zone with a manually operated 3D-micromanipulator. The membrane potential was monitored continually using CHART software (Newman, 2001). Once a stable membrane potential value was obtained for 1 minute, salt treatment (100 mM NaCl) was applied to reach a final NaCl concentration of 100 mM. Transient recordings of the flux kinetics of K⁺, H⁺, and Na⁺ were measured for specified times. The time required for the stock addition and the establishment of the diffusion gradients is reported to be about 40 s (Shabala and Hariadi, 2005). Accordingly, the data for the first 60 s after the solution change were discarded from the analysis, creating a gap in all figures.
imposed. The transient membrane potential kinetics was recorded up to 30 minutes after treatment commencement. The membrane potential values of eight individual seedlings were averaged for every treatment combination.

Statistical analysis
Statistical significance of mean and standard error values was determined using the standard least significant difference test at $P \leq 0.05$.

Results

SA pretreatment improved shoot growth and water content of Arabidopsis during salt stress

The 50 μM SA root pretreatment for 1 h in both soil and hydroponic experiments did not affect shoot growth, biomass, or water content under control conditions (Fig. 1 and Table 1). In contrast, salt stress reduced shoot growth (Fig. 1), biomass, and water content (Table 1) in both SA-untreated and -pretreated plants. However, salt-induced negative effects were significantly diminished in the SA-pretreated plants (Fig. 1 and Table 1).

SA pretreatment minimized salt-induced K$^+$ efflux from Arabidopsis roots

Similarly to previous reports (e.g Shabala et al., 2005, 2006), acute NaCl stress resulted in a large K$^+$ efflux (Fig. 2A) from the mature root zone of Arabidopsis. A 1-h pretreatment of Arabidopsis roots in various concentrations of SA (10, 50, 100, or 500 μM) reduced the NaCl-induced K$^+$ efflux in a dose-dependent manner (Fig. 2A). The most beneficial SA concentrations were 10, 50, and 100 μM (Fig. 2A inset), resulting in more than a 5-fold decrease in the magnitude of NaCl-induced K$^+$ efflux compared with controls not pretreated with SA.

Fig. 1. Effect of salicylic acid (SA) pretreatment on growth of Arabidopsis thaliana wild-type (Col-0) plants under control or saline (100 mM NaCl for 2 weeks) conditions. Plants were grown in either soil (top panel) or a hydroponic system (bottom panel) for 3 weeks before pretreatment and treatment. The treatments lasted for 2 weeks. (A) Control, (B) 50 μM SA pretreatment (1 h) without salt stress, (C) 100 mM NaCl, and (D) 50 μM SA pretreatment (1 h) plus 100 mM NaCl. For SA pretreatment, the pots were irrigated with 50 μM SA for 1 h.
Table 1. Effect of salicylic acid on shoot growth and water content of *Arabidopsis thaliana* grown under saline conditions (100 mM NaCl) for 2 weeks. Wild-type (Col-0) plants were grown either in hydroponic or soil culture until 3 weeks old before the treatment was applied. For salicylic acid (SA) pretreatment, seedlings in centrifuge tubes were kept in nutrient solution supplemented with 50 μM SA (in hydroponic culture) or the pots were irrigated with 50 μM SA (for soil culture) for 1 h. Values are mean ± SE (n = 4). Data with different letters are significantly different (P ≤ 0.05).

| Treatment | Freshweight (mg/pot) | Dryweight (mg/pot) | Water content (% w/w) |
|-----------|----------------------|--------------------|-----------------------|
| Control   | 620 ± 40a            | 123 ± 9a           | 84.3 ± 1.1a           |
| SA        | 618 ± 62a            | 122 ± 4a           | 83.3 ± 1.3a           |
| NaCl      | 186 ± 15c            | 61 ± 4c            | 69.7 ± 2.2c           |
| NaCl + SA | 319 ± 36b            | 93 ± 10b           | 74.2 ± 0.9b           |

SA pretreatment modified NaCl-induced H⁺ fluxes from *Arabidopsis* roots

Acute salt stress (100 mM NaCl) induced H⁺ influx in SA-untreated plants. In comparison, SA-pretreated plants showed either lower H⁺ influx (pretreatment with 100 or 500 μM SA) or had net H⁺ flux around zero (pretreatment with 10 or 50 μM SA) during salt stress (Fig. 2B).

SA effects are time dependent

To assess the time dependency of the SA action on K⁺ and H⁺ transport systems, the following treatments were compared: (i) salt stress applied in the absence of SA; (ii) 100 mM NaCl was applied together with 50 μM SA. Simultaneous application of SA and NaCl slightly reduced K⁺ loss during the first 3 minutes of salt stress (Fig. 3A), but the total K⁺ loss during 1 h of salt stress was not significantly different from SA-untreated plants (Fig. 3C). Regarding H⁺ fluxes, simultaneous application of SA and NaCl in comparison with SA-untreated plants did not change the H⁺ flux kinetics (Fig. 3B) or total H⁺ flux (Fig. 3D) during salt stress.

Compared to treatments with no SA addition or simultaneous SA and NaCl addition, the 1-h pretreatment with SA resulted in the lowest K⁺ efflux (Fig. 3A, C), the lowest net H⁺ fluxes (Fig. 3B), and the lowest total H⁺ influx during salt stress (Fig. 3D). Given that the SA action is dependent on the plant exposure to SA prior to exposure to NaCl, the results suggest that SA needs to be taken up across the plasma membrane to exert control over K⁺ and H⁺ transport systems from the cytoplasmic side.

Effect of the SA pretreatment duration on NaCl-induced K⁺ efflux

To determine the optimal duration of SA pretreatment, *Arabidopsis* seedlings were pretreated with two SA concentrations found to be effective (10 and 50 μM; Fig. 2) for four different times (1, 4, 6, or 8 h). Although all the pretreatment durations significantly reduced the salt-induced K⁺ efflux, the 1-h pretreatment resulted in the lowest K⁺ efflux (Fig. 4A, B).

SA pretreatment upregulated H⁺-ATPase during salt stress

To ascertain whether NaCl-induced H⁺ efflux (Fig. 2B) was due to enhanced H⁺-ATPase activity, the *Arabidopsis* seedlings were pre-incubated with 50 μM SA in the presence of known inhibitors of H⁺-ATPase (1 mM vanadate or 0.1 mM DCCD). The 1-h pretreatment with SA and either vanadate or DCCD shifted H⁺ efflux (pretreatment with SA alone) to net H⁺ influx, which was particularly high with the SA + vanadate pretreatment (Fig. 5A). These results suggest that the partial reversal of the NaCl-induced influx (Fig. 3B, D) by SA pretreatment is due to SA enhancing the activity of H⁺-ATPase under salt stress.

The SA pretreatment combined with either vanadate or DCCD partially reversed improved K⁺ retention by SA-pretreated *Arabidopsis* seedlings during salt stress. Hence, a part of SA-related improvement in K⁺ retention under salt stress is attributed to increased plasma-membrane H⁺-ATPase activity.

SA pretreatment did not decrease Na⁺ entry into roots but reduced Na⁺ accumulation in the shoot

The acute salt stress (either 50 or 100 mM NaCl) resulted in massive Na⁺ influx into the *Arabidopsis* roots (Table 2) during the first hour. Pretreatment of seedlings with 50 μM SA had no significant effect (P ≤ 0.05) on root Na⁺ uptake regardless of NaCl concentration (50 or 100 mM) in the short-term MIFE experiments. In contrast, SA was effective in reducing shoot Na⁺ concentration in the long-term experiments (100 mM NaCl for 2 weeks) (Table 3). Moreover, the SA pretreatment had a beneficial effect on shoot K⁺ concentration (Table 3). Hence, net root Na⁺ uptake appeared unaffected by SA, whereas either xylem Na⁺ loading and/or Na⁺ retrieval from the shoot were decreased by SA. This issue needs to be followed up in a separate study.

SA pretreatment decreased the extent of plasma-membrane depolarization during salt stress

Keeping in mind that many plasma-membrane K⁺ channels are voltage dependent, effects of SA pretreatment on plasma membrane potential kinetics during NaCl stress in root epidermal cells of *Arabidopsis* were tested. Pretreatment of roots in 50 μM SA for 1 h shifted the resting potential towards more negative values compared with SA-untreated plants (–127 ± 3 and –105 ± 2 mV, respectively) under control conditions. Adding 100 mM NaCl to the bathing medium resulted in a highly significant membrane depolarization (Fig. 6); however,
a 20 mV difference between SA-untreated and -pretreated plants was maintained throughout the measurement period (Fig. 6).

**SA pretreatment decreased NaCl-induced K⁺ efflux by modulating K⁺ conductance via depolarization-activated KOR channels**

The SA pretreatment might have prevented K⁺ loss during salt stress by modulating K⁺ conductance via either depolarization-activated KOR and/or ROS-activated NSCC channels.

To delineate between those channels, NaCl-induced K⁺ fluxes were measured in two *Arabidopsis* mutants, viz. (i) gork1-1 mutant that lacks depolarization-activated KOR channels in the root epidermal cells (Ivashikina *et al*., 2001; Hosy *et al*., 2003; Demidchik *et al*., 2010) and (ii) rbohD mutant that cannot produce ROS through NADPH oxidase during salt stress (Xie *et al*., 2011; Ma *et al*., 2012), but has a fully functional GORK channel.

Without the SA pretreatment, NaCl-induced K⁺ efflux was smallest in gork1-1, followed by rbohD, and was highest in the wild type (Fig. 7A, C). The SA pretreatment significantly

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**Fig. 2.** Effect of different salicylic acid concentrations (0 to 500 μM) in the 1-h pretreatment on net K⁺ (A) and H⁺ (B) fluxes measured at the mature root zone of 4–5-day-old *Arabidopsis thaliana* wild-type (Col-0) seedlings in response to 100 mM NaCl. Insets show total K⁺ (nmol m⁻²) leaked out (negative flux) during 1 h of salt treatment. Values are mean ± SE (n = 7 seedlings). Different letters in the inset indicate significant differences.
decreased NaCl-induced K⁺ efflux in the wild type and the rbohD mutant, but not in the gork1-1 mutant (Fig. 7B, C). Thus, these results suggest the SA pretreatment decreased K⁺ loss by modulating K⁺ conductance through KOR (GORK) channels.

Consistent with previous results (Figs. 2B and 3B), the SA pretreatment either decreased net H⁺ influx or even induced H⁺ efflux in all three genotypes during salt stress (Fig. 8). Thus, the SA pretreatment enhanced H⁺-ATPase activity in all genotypes (Fig. 8), but the beneficial effects on retention of K⁺ in the cells were not observed in the gork1-1 mutant (Fig. 7C) because it lacks voltage-dependent (depolarization-activated) KOR channels.

**Discussion**

SA pretreatment decreased NaCl-induced K⁺ efflux from Arabidopsis roots

Ion transport processes, particularly K⁺ homeostasis maintenance during salt stress, has emerged as a fundamental component of salt tolerance mechanism (Maathuis and...
Indeed, a strong positive correlation between the ability of roots to retain K\(^+\) and salt tolerance was previously reported in barley (Chen et al., 2005, 2007a,b), wheat (Cuin et al., 2008), lucerne (Smethurst et al., 2008), and Arabidopsis (Shabala et al., 2005, 2006). Although exogenous application of SA ameliorated detrimental effects of salinity in many plant species (see Horváth et al., 2007; Ashraf et al., 2010; Hayat et al., 2010) and resulted in increased K\(^+\) concentration in roots (e.g. He and Zhu, 2008; Kováčik et al., 2009), it is unclear whether enhanced K\(^+\) uptake or prevention of K\(^+\) loss played a major role in this ameliorative effect.

The entry of positively charged Na\(^+\) ions through the plasma membrane (Table 2) resulted in 70–90-mV depolarization during 100 mM NaCl stress (Fig. 9). During this depolarization, K\(^+\) uptake through K\(^+\) inward rectifying (KIR) channels is thermodynamically impossible; at the same time, depolarization would favour increased K\(^+\) leakage through depolarization-activated KOR channels (Shabala and Cuin, 2008). Indeed, the SA-untreated plants exhibited relatively large K\(^+\) efflux upon exposure to acute salt stress (Fig. 2A). The 1-h pretreatment of Arabidopsis in physiologically relevant concentrations (10–500 \(\mu\)M) of SA decreased NaCl-induced K\(^+\) efflux from roots (resulting in enhanced K\(^+\) retention). Moreover, SA-pretreated plants decreased the extent of depolarization by about 20 mV during salt stress (Fig. 9). Hence, these results suggest that SA-pretreated roots maintained a lower depolarized membrane potential under salt stress, thereby decreasing NaCl-induced depolarization-activated K\(^+\) efflux through KOR channels.

GORK channels are downstream targets of salicylic acid

To prove the notion of the KOR channels being downstream targets of SA action, additional experiments were conducted involving two Arabidopsis mutants, namely gork1-1, lacking depolarization-activated KOR (Ivashikina et al., 2001; Hossy et al., 2003; Demidchik et al., 2010), and rbohD, lacking the capacity to produce ROS via NADPH oxidase and thus without the capacity to activate ROS-dependent channels (Xie et al., 2011; Ma et al., 2012). Comparison of NaCl-induced K\(^+\) efflux between wild type, gork1-1, and rbohD (Fig. 7) suggested that about 25% of the K\(^+\) efflux was mediated by ROS-activated channels and remaining 75% was mediated by depolarization-activated KOR during salt stress.

Fig. 4. Total amount of K\(^+\) leaked from the mature root zone of 4–5-day-old Arabidopsis thaliana wild-type (Col-0) seedlings exposed to 100 mM NaCl for 1 h. Seedlings were pre-incubated in either 10 (A) or 50 (B) \(\mu\)M salicylic acid (SA) for various periods of time (0, 1, 4, 6, or 8 h). Values are mean ± SE (n = 8 seedlings). Different letters indicate significant differences.
Salicylic acid in salinity tolerance in plants

Pretreatment with SA did not provide any additional decrease in NaCl-induced K⁺ efflux from the gork1-1 mutant (Fig. 7B, C). In contrast, the SA pretreatment decreased NaCl-induced K⁺ efflux in the wild type and the rbohD mutant to make it similar to the gork1-1 mutant (Fig. 7B, C). Thus, these results provide explicit evidence that a decrease in K⁺ efflux through depolarization-activated GORK channels is the key mechanism in SA-mediated salt tolerance in Arabidopsis.

SA action is dependent on SA dose and pretreatment duration

The diminishing effect of exogenously applied SA on both NaCl-induced K⁺ efflux and H⁺ influx was not obvious when 50 μM SA was supplied simultaneously with 100 mM NaCl, but reached maximum when SA was applied as 1-h pretreatment before the salt addition (Fig. 3). This suggests transport of SA and build up of optimal SA concentration inside the root tissue is critical during salt stress. Reaching the threshold...
SA concentration inside the root tissue is dependent on several factors, such as external concentration of SA, the mode of plasma-membrane transport, and duration of the pretreatment. The experiments with various concentrations of exogenous SA showed that SA at ≤100 μM was effective in decreasing K⁺ leakage from cells, whereas 500 μM SA (Fig. 2) and higher concentrations (1 mM and above in experiments reported in the literature, Norman et al., 2004) are not as effective and can even be detrimental. Moreover, Norman et al. (2004) showed that a 6–10-fold increase in cytosolic SA concentration is achieved within 0.5–1 h of SA pretreatment, and that elevated cytosolic SA concentrations dropped sharply after 4 h of pretreatment. Hence, it is suggested here that 1 h pretreatment is the optimum duration to reach the desired cytosolic SA concentrations and get the most beneficial effects during salt stress (Fig. 4). The slightly diminished SA effects in longer pretreatment durations (4, 6, and 8 h; Fig. 4) can be explained by the metabolic conversion of free SA into inactive SA forms and subsequent transport and storage in the vacuole (Dean et al., 2005; Dempsey et al., 2011).

**SA pretreatment enhanced H⁺-ATPase activity during salt stress**

The SA pretreatment shifted NaCl-induced H⁺ influx towards zero net H⁺ flux (Figs. 2B, 3B, 5A, and 8). This effect was diminished in roots pretreated with SA and vanadate or DCCD, two known metabolic inhibitors of H⁺-ATPase activity (Fig. 5A), giving a strong evidence for the ameliorating effect of SA on the H⁺-ATPase pump during salt stress. Similarly, the SA pretreatment shifted the resting membrane potential towards more negative values by about −20 mV; the same potential difference between SA-pretreated and SA-untreated roots was maintained during salt stress (Fig. 6). Hence, these results demonstrate that SA pretreatment enhanced H⁺-ATPase activity under salt stress, which in turn minimized the extent of plasma-membrane depolarization and thus decreased NaCl-induced K⁺ efflux via depolarization-activated KOR channels. Even though SA was found to enhance the H⁺-ATPase activity in pea and grape leaves under temperature stress (Liu et al., 2008, 2009), the present paper is the first report, as far as is known, on the role of enhanced H⁺-ATPase activity in the SA-mediated salinity tolerance in plants.

### Table 3. Effect of salicylic acid (SA) pretreatment on shoot ion content of Arabidopsis thaliana grown under saline (100 mM NaCl) conditions for 2 weeks. Wild-type (Col-0) plants were grown either in hydroponic or soil culture until 3 weeks old before the treatment was applied. For salicylic acid (SA) pretreatment, seedlings in centrifuge tubes were kept in nutrient solution supplemented with 50 μM SA (for hydroponic culture) or pots were irrigated with 50 μM SA (for soil culture) for 1 h. Values are mean ± SE (n = 4). Data with different letters are significantly different (P ≤ 0.05).

| Treatment | Shoot Na (g (kg DW)⁻¹) | Shoot K (g (kg DW)⁻¹) |
|-----------|------------------------|------------------------|
| Control   | 0.83 ± 0.05ᵃ           | 19.2 ± 0.05ᵃ           |
| SA        | 0.91 ± 0.09ᵇ           | 19.6 ± 1.42ᵇ           |
| NaCl      | 37.1 ± 2.2ᶜ            | 9.4 ± 0.48ᶜ            |
| NaCl + SA | 24.2 ± 1.8ᵈ            | 13.1 ± 0.69ᵈ           |

**Fig. 6.** Effect of 1-h pretreatment with 50 μM salicylic acid (SA) on transient plasma-membrane potential changes in the mature root zone of 4–5-day-old Arabidopsis thaliana wild-type (Col-0) seedlings during acute 100 mM NaCl treatment. Values are means ± SE (n = 8 seedlings).
SA decreased root-to-shoot transport of Na⁺ during salt stress

Electrophysiological and molecular studies (reviewed in Apse and Blumwald, 2007) suggest that Na⁺ uptake into the root tissue occurs predominantly through NSCC and high-affinity K⁺ transporter-like proteins (Fig. 9). In this study, SA had no impact on net Na⁺ uptake by roots during 1-h-long salt stress (Table 2), suggesting SA would not modulate Na⁺ entry pathways. On the other hand, shoot Na⁺ content was significantly...
lower in SA-pretreated in comparison with SA-untreated plants (Table 3), implying SA decreased xylem loading of Na⁺ in roots and/or enhanced Na⁺ retrieval from the shoot during long-term salt stress. The decreased shoot Na⁺ concentration was also accompanied by higher K⁺ concentration (Table 3), suggesting that shoots of SA-pretreated plants were better protected from prolonged salt stress. Indeed, the SA-pretreated plants showed marked improvement over SA-untreated plants in shoot growth and water content during long-term salt exposure (Table 1).

In summary, these whole-plant ion flux, membrane potential, and pharmacological experiments suggest the following mechanism (Fig. 9). Under saline conditions, Na⁺ enters into the cytosol (via either NSCC or high-affinity K⁺ transporter, or both), causing significant depolarization of the plasma membrane. As a result, K⁺ leaks through depolarization-activated KOR channels. Pretreatment with SA enhances the H⁺-ATPase activity under salt stress in a time- and concentration-dependent manner. Enhanced H⁺-ATPase activity assists in maintaining membrane potential at more negative values, decreasing K⁺ leakage through depolarization-activated KOR channels during acute salt stress, resulting in better plant growth under long-term stress. In addition, at the whole plant

Fig. 8. Salinity (100 mM NaCl)-induced net H⁺ fluxes at the mature root zone of 4–5-day-old seedlings of Arabidopsis thaliana wild type (Col-0), gork1-1, and rbohD mutants without (A) and with (B) 1-h 50 μM salicylic acid (SA) pretreatment. (C) The total H⁺ flux during 1-h NaCl stress. Values are means ± SE (n = 8 seedlings). Different letters in (C) indicate significant differences.
level, SA also exerts beneficial effects by decreasing Na⁺ transport to the shoot. Hence, compared with SA-untreated plants, the SA-pretreated plants have the higher cytosolic K⁺/Na⁺ ratio required for normal cell functioning under salt stress.

Acknowledgements

MJ is a recipient of a Australian Postgraduate Award and a University of Western Australia Postgraduate Award. This work was supported by an ARC grant to SS and ZR.

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