

RESEARCH PAPER

# Salicylic acid improves salinity tolerance in *Arabidopsis* by restoring membrane potential and preventing salt-induced K<sup>+</sup> loss via a GORK channel

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## 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<sup>+</sup> efflux and H<sup>+</sup> influx from the mature root zone were both significantly decreased in roots pretreated with 10–500 μM SA, with strongest effect being observed in the 10–50 μM SA range. Considering temporal dynamics (0–8-h SA pretreatment), the 1-h pretreatment was most effective in enhancing K<sup>+</sup> retention in the cytosol. The pharmacological, membrane potential, and shoot K<sup>+</sup> and Na<sup>+</sup> accumulation data were all consistent with the model in which the SA pretreatment enhanced activity of H<sup>+</sup>-ATPase, decreased NaCl-induced membrane depolarization, and minimized NaCl-induced K<sup>+</sup> leakage from the cell within the first hour of salt stress. In long-term treatments, SA increased shoot K<sup>+</sup> and decreased shoot Na<sup>+</sup> accumulation. The short-term NaCl-induced K<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> efflux via GORK channels.

**Key words:** H<sup>+</sup>-ATPase, H<sup>+</sup> flux, depolarization, K<sup>+</sup> flux, membrane potential, outward-rectifying K<sup>+</sup> 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.*, 2007). Moreover, the critical role of SA in modulation of ion transport processes (e.g.  $Na^+$ ,  $K^+$ , and  $H^+$  fluxes) at the cellular level has been overlooked during salt stress. Thus, direct measurements of transport processes in short-term salt exposure studies (few minutes to hours) are critical to characterize how the specific ion transporters are modulated by exogenous SA.

The intracellular  $K^+$  decreases significantly under salinity (Carden *et al.*, 2003). The problem is intensified when  $K^+$  loss is accompanied by accumulation of  $Na^+$  inside the cytosol in the 50–200 mM range (Maathuis and Amtmann, 1999; Flowers and Hajibagheri, 2001) whereby it replaces  $K^+$  in metabolic reactions, thus impairing normal enzymatic activity and metabolism and finally causing cell death (Maathuis and 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  $^{86}Rb^+$ , 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, 2005;

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 ionic homeostasis of  $Na^+$  and  $K^+$ . 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. Heynch 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

KNO<sub>3</sub>, 0.625 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgSO<sub>4</sub>, 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.045 mM FeNaEDTA, 0.16 μM CuSO<sub>4</sub>, 0.38 μM ZnSO<sub>4</sub>, 1.8 μM MnSO<sub>4</sub>, 45 μM H<sub>3</sub>BO<sub>3</sub>, 0.015 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, and 0.01 μM CoCl<sub>2</sub>) in 1.5-ml centrifuge tubes and vernalized at 4 °C for 48 h. The bottom of the tubes was cut off and the tubes were inserted in a floater 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<sup>-2</sup> s<sup>-1</sup>. 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 used for hydroponic culture before the surface-sterilized and vernalized *Arabidopsis* seeds were placed on top. Plants were grown under a 16/8 light/dark cycle at 23 °C with an irradiance of 150 μmol m<sup>-2</sup> s<sup>-1</sup>. After 1 week, each pot was thinned to four uniform and healthy seedlings. After 3 weeks, pots were irrigated with 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 CaSO<sub>4</sub> 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<sup>+</sup> and K<sup>+</sup> concentrations

Dry *Arabidopsis* shoots were weighed and digested in 6 ml of 98% (v/v) H<sub>2</sub>SO<sub>4</sub> and 3 ml 30% (v/v) H<sub>2</sub>O<sub>2</sub> for 5 h as described by Skoog *et al.* (2000). The shoot Na<sup>+</sup> and K<sup>+</sup> 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 CaCl<sub>2</sub>, 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<sup>-2</sup> s<sup>-1</sup> 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<sup>+</sup>, K<sup>+</sup>, and Na<sup>+</sup> 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 KH<sub>2</sub>PO<sub>4</sub>, adjusted to pH 6.0 using NaOH for the H<sup>+</sup> electrodes; 0.5 M KCl for the K<sup>+</sup> electrodes; and 0.5 M NaCl for the Na<sup>+</sup> electrodes). The electrode tips were then front-filled with commercially available ion-selective cocktails (H<sup>+</sup> 95297; K<sup>+</sup> 60031; both from Sigma-Aldrich). For Na<sup>+</sup> measurements, an improved calixarene-based Na<sup>+</sup> 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<sup>+</sup> 0.25–1.5 mM; Na<sup>+</sup> 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).

#### Ion-flux measuring protocols

A seedling was placed on a glass slide; the root was immobilized horizontally by thin parafilm strips and then placed in a Petri dish with 30 ml basal salt medium (1 mM KCl, 0.1 mM CaCl<sub>2</sub>, pH 5.5) (Guo *et al.*, 2009; Jayakannan *et al.*, 2011) for 60 min before commencing MIFE measurements. For the SA treatments, plants were pretreated with different SA concentrations for specified times (1, 4, 6, or 8 h). After the specified pretreatment duration, the pretreatment solution was withdrawn and the measuring solution was introduced. Net ion fluxes were measured in the basal salt medium for 5 minutes to ensure steady initial values, then 4 M stock solution of NaCl was applied to reach a final NaCl concentration of 100 mM. Transient recordings of the flux kinetics of K<sup>+</sup>, H<sup>+</sup>, and Na<sup>+</sup> 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.

#### Pharmacology experiment

Prior to flux measurements, *Arabidopsis* seedlings were pretreated with 1 mM sodium orthovanadate (Na<sub>2</sub>VO<sub>4</sub>; P-type H<sup>+</sup>-ATPase inhibitor) or 0.1 mM *N,N'*-dicyclohexylcarbodiimide (DCCD; H<sup>+</sup>-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

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

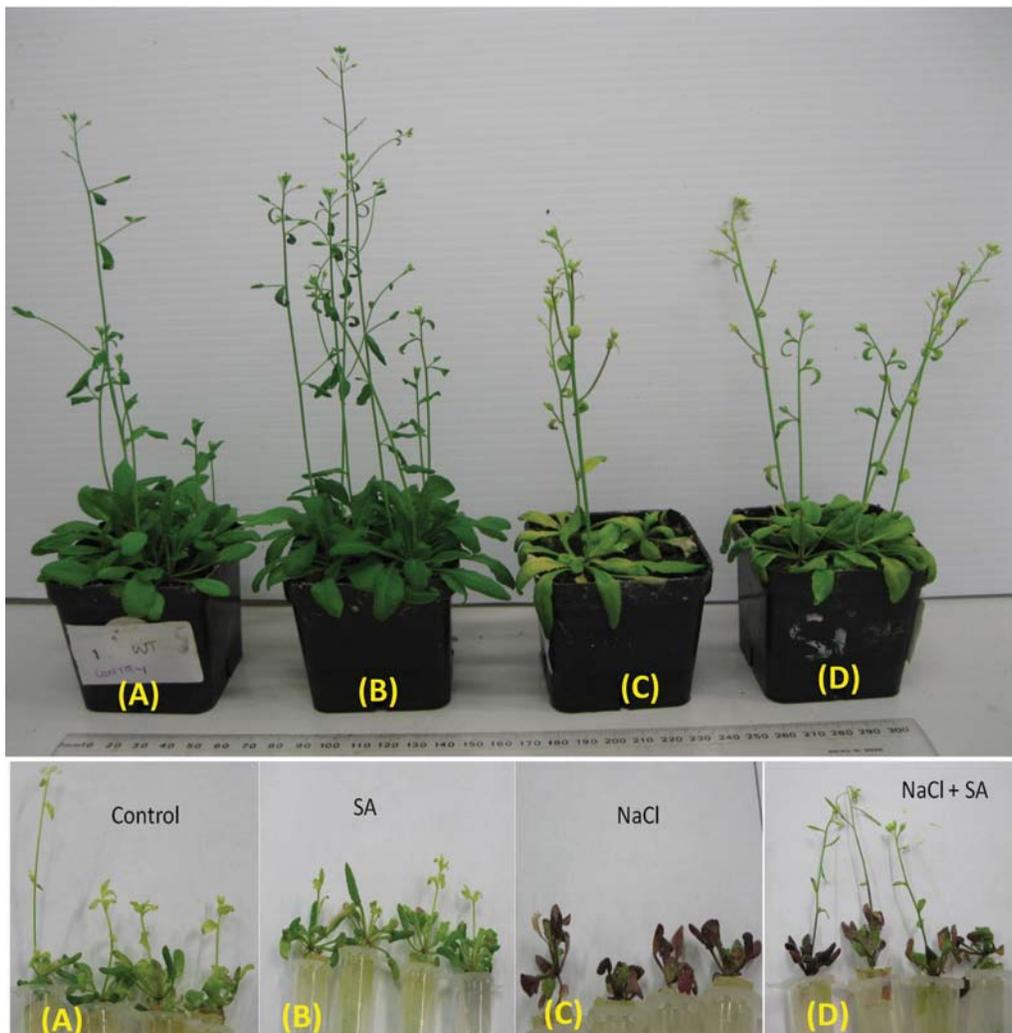
### SA pretreatment improved shoot growth and water content of *Arabidopsis* during salt stress

The 50  $\mu\text{M}$  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 $\text{K}^+$ efflux from *Arabidopsis* roots

Similarly to previous reports (e.g. Shabala *et al.*, 2005, 2006), acute NaCl stress resulted in a large  $\text{K}^+$  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  $\mu\text{M}$ ) reduced the NaCl-induced  $\text{K}^+$  efflux in a dose-dependent manner (Fig. 2A). The most beneficial SA concentrations were 10, 50, and 100  $\mu\text{M}$  (Fig. 2A inset), resulting in more than a 5-fold decrease in the magnitude of NaCl-induced  $\text{K}^+$  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  $\mu\text{M}$  SA pretreatment (1 h) without salt stress, (C) 100 mM NaCl, and (D) 50  $\mu\text{M}$  SA pretreatment (1 h) plus 100 mM NaCl. For SA pretreatment, the pots were irrigated with 50  $\mu\text{M}$  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  $\mu$ M SA (in hydroponic culture) or the pots were irrigated with 50  $\mu$ M SA (for soil culture) for 1 h. Values are mean  $\pm$  SE ( $n = 4$ ). Data with different letters are significantly different ( $P \leq 0.05$ ).

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

#### *SA pretreatment modified NaCl-induced H<sup>+</sup> fluxes from Arabidopsis roots*

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

#### *SA effects are time dependent*

To assess the time dependency of the SA action on K<sup>+</sup> and H<sup>+</sup> 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  $\mu$ M SA (i.e. no pretreatment); and (iii) 100 mM NaCl applied after 1-h pretreatment with 50  $\mu$ M SA. Simultaneous application of SA and NaCl slightly reduced K<sup>+</sup> loss during the first 3 minutes of salt stress (Fig. 3A), but the total K<sup>+</sup> loss during 1 h of salt stress was not significantly different from SA-untreated plants (Fig. 3C). Regarding H<sup>+</sup> fluxes, simultaneous application of SA and NaCl in comparison with SA-untreated plants did not change the H<sup>+</sup> flux kinetics (Fig. 3B) or total H<sup>+</sup> 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<sup>+</sup> efflux (Fig. 3A, C), the lowest net H<sup>+</sup> fluxes (Fig. 3B), and the lowest total H<sup>+</sup> 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<sup>+</sup> and H<sup>+</sup> transport systems from the cytoplasmic side.

#### *Effect of the SA pretreatment duration on NaCl-induced K<sup>+</sup> efflux*

To determine the optimal duration of SA pretreatment, *Arabidopsis* seedlings were pretreated with two SA

concentrations found to be effective (10 and 50  $\mu$ M; Fig. 2) for four different times (1, 4, 6, or 8 h). Although all the pretreatment durations significantly reduced the salt-induced K<sup>+</sup> efflux, the 1-h pretreatment resulted in the lowest K<sup>+</sup> efflux (Fig. 4A, B).

#### *SA pretreatment upregulated H<sup>+</sup>-ATPase during salt stress*

To ascertain whether NaCl-induced H<sup>+</sup> efflux (Fig. 2B) was due to enhanced H<sup>+</sup>-ATPase activity, the *Arabidopsis* seedlings were pre-incubated with 50  $\mu$ M SA in the presence of known inhibitors of H<sup>+</sup>-ATPase (1 mM vanadate or 0.1 mM DCCD). The 1-h pretreatment with SA and either vanadate or DCCD shifted H<sup>+</sup> efflux (pretreatment with SA alone) to net H<sup>+</sup> 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<sup>+</sup>-ATPase under salt stress.

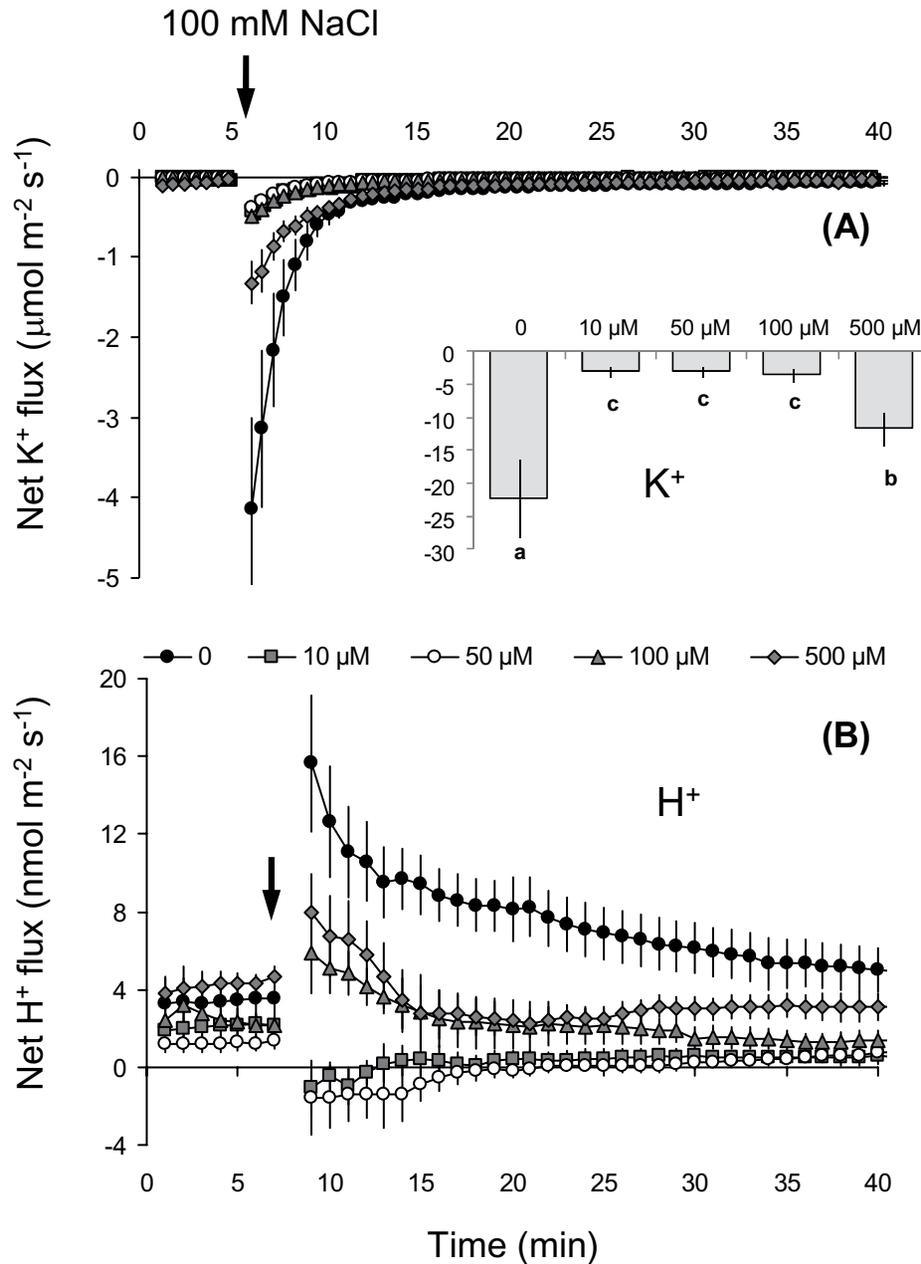
The SA pretreatment combined with either vanadate or DCCD partially reversed improved K<sup>+</sup> retention by SA-pretreated *Arabidopsis* seedlings during salt stress. Hence, a part of SA-related improvement in K<sup>+</sup> retention under salt stress is attributed to increased plasma-membrane H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

#### *SA pretreatment did not decrease Na<sup>+</sup> entry into roots but reduced Na<sup>+</sup> accumulation in the shoot*

The acute salt stress (either 50 or 100 mM NaCl) resulted in massive Na<sup>+</sup> influx into the *Arabidopsis* roots (Table 2) during the first hour. Pretreatment of seedlings with 50  $\mu$ M SA had no significant effect ( $P \leq 0.05$ ) on root Na<sup>+</sup> uptake regardless of NaCl concentration (50 or 100 mM) in the short-term MIFE experiments. In contrast, SA was effective in reducing shoot Na<sup>+</sup> 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<sup>+</sup> concentration (Table 3). Hence, net root Na<sup>+</sup> uptake appeared unaffected by SA, whereas either xylem Na<sup>+</sup> loading and/or Na<sup>+</sup> 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<sup>+</sup> channels are voltage dependent, effects of SA pretreatment on membrane potential kinetics during NaCl stress in root epidermal cells of *Arabidopsis* were tested. Pretreatment of roots in 50  $\mu$ M SA for 1 h shifted the resting potential towards more negative values compared with SA-untreated plants ( $-127 \pm 3$  and  $-105 \pm 2$  mV, respectively) under control conditions. Adding 100 mM NaCl to the bathing medium resulted in a highly significant membrane depolarization (Fig. 6); however,



**Fig. 2.** Effect of different salicylic acid concentrations (0 to 500 µM) in the 1-h pretreatment on net K<sup>+</sup> (A) and H<sup>+</sup> (B) fluxes measured at the mature root zone of 4–5-day-old *Arabidopsis thaliana* wild-type (Col-0) seedlings in response to 100mM NaCl. Insets shows total K<sup>+</sup> (nmol m<sup>-2</sup>) 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.

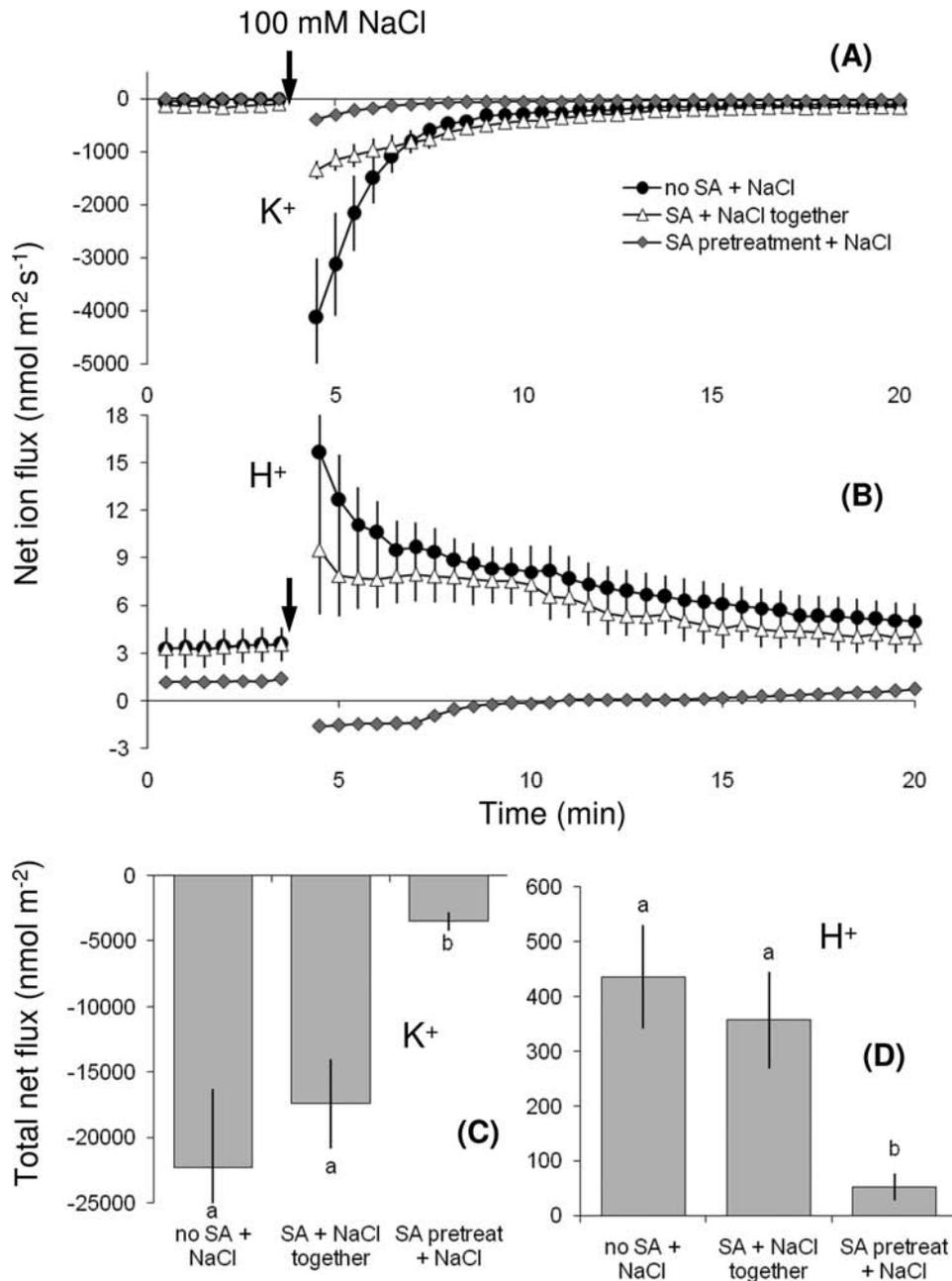
a 20 mV difference between SA-untreated and -pretreated plants was maintained throughout the measurement period (Fig. 6).

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

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

To delineate between those channels, NaCl-induced K<sup>+</sup> 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<sup>+</sup> efflux was smallest in *gork1-1*, followed by *rbohD*, and was highest in the wild type (Fig. 7A, C). The SA pretreatment significantly



**Fig. 3.** (A, B) Net fluxes of K<sup>+</sup> (A) and H<sup>+</sup> (B) were compared for the mature root zones of 4–5-day-old *Arabidopsis thaliana* wild-type (Col-0) seedlings not pretreated with salicylic acid (SA) or pretreated with 50  $\mu$ M SA for 1 h prior to the addition of 100 mM NaCl (salt stress) or simultaneously treated with 50  $\mu$ M SA and 100 mM NaCl. (C, D) Total fluxes of K<sup>+</sup> (C) and H<sup>+</sup> (D) during 1 h of salt treatment. Values are mean  $\pm$  SE ( $n = 7$  seedlings). Different letters in (C) and (D) indicate significant differences.

decreased NaCl-induced K<sup>+</sup> 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<sup>+</sup> loss by modulating K<sup>+</sup> conductance through KOR (GORK) channels.

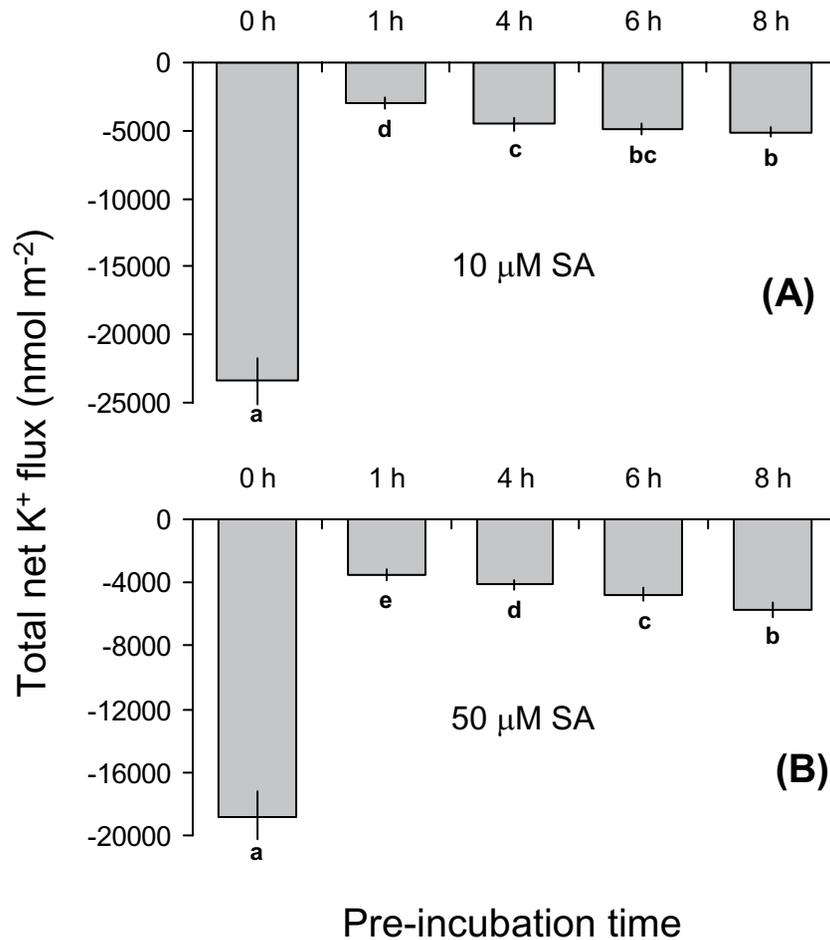
Consistent with previous results (Figs. 2B and 3B), the SA pretreatment either decreased net H<sup>+</sup> influx or even induced H<sup>+</sup> efflux in all three genotypes during salt stress (Fig. 8). Thus, the SA pretreatment enhanced H<sup>+</sup>-ATPase activity in all genotypes (Fig. 8), but the beneficial effects on retention of K<sup>+</sup> 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<sup>+</sup> efflux from Arabidopsis roots*

Ion transport processes, particularly K<sup>+</sup> homeostasis maintenance during salt stress, has emerged as a fundamental component of salt tolerance mechanism (Maathuis and



**Fig. 4.** Total amount of K<sup>+</sup> 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) μM 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.

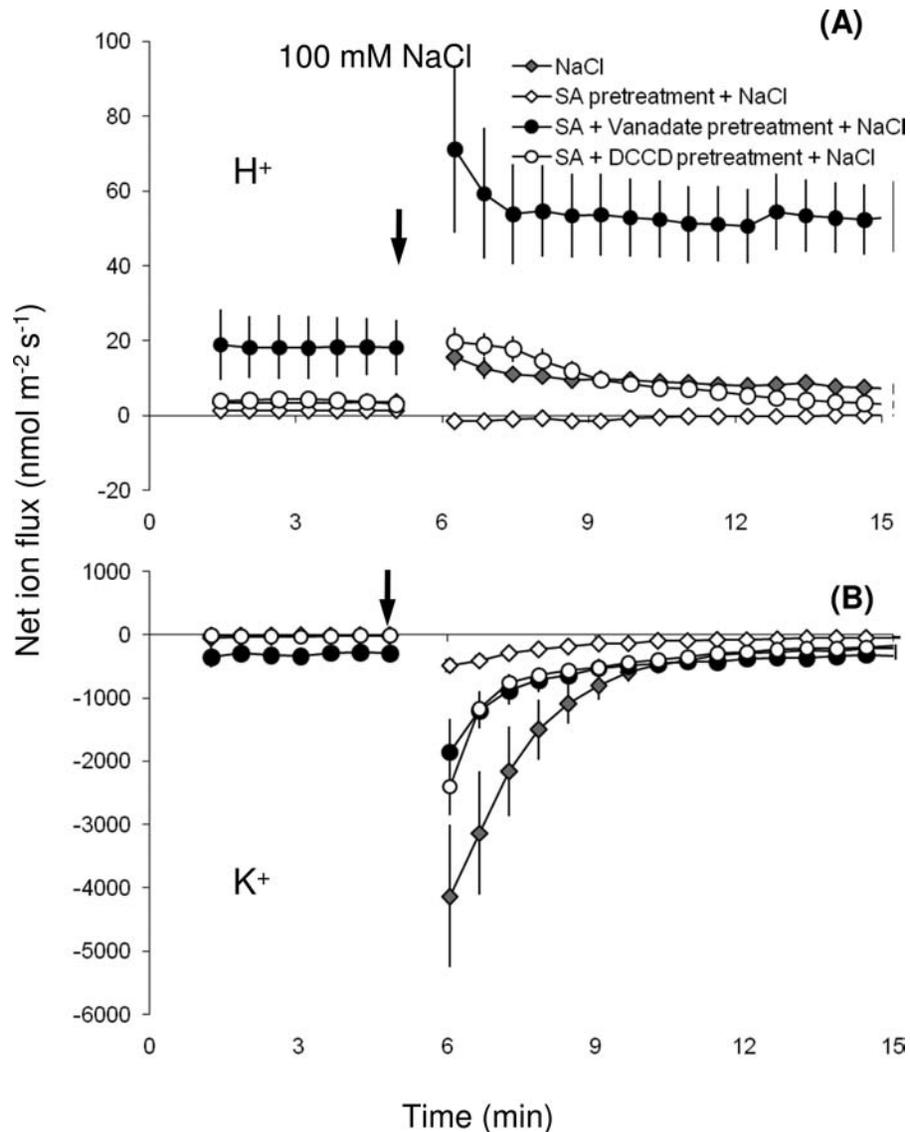
Amtmann, 1999; Shabala and Cuin, 2008; Demidchik et al., 2010). Indeed, a strong positive correlation between the ability of roots to retain K<sup>+</sup> and salt tolerance was previously reported in barley (Chen et al., 2005, 2007a,b), wheat (Cuin et al., 2008), lucerne (Smethurst et al., 2008), and *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<sup>+</sup> concentration in roots (e.g. He and Zhu, 2008; Kováčik et al., 2009), it is unclear whether enhanced K<sup>+</sup> uptake or prevention of K<sup>+</sup> loss played a major role in this ameliorative effect.

The entry of positively charged Na<sup>+</sup> ions through the plasma membrane (Table 2) resulted in 70–90-mV depolarization during 100 mM NaCl stress (Fig. 9). During this depolarization, K<sup>+</sup> uptake through K<sup>+</sup> inward rectifying (KIR) channels is thermodynamically impossible; at the same time, depolarization would favour increased K<sup>+</sup> leakage through depolarization-activated KOR channels (Shabala and Cuin, 2008). Indeed, the SA-untreated plants exhibited relatively large K<sup>+</sup> efflux upon exposure to acute salt stress (Fig. 2A). The 1-h pretreatment of *Arabidopsis* in physiologically relevant concentrations (10–500 μM) of SA decreased

NaCl-induced K<sup>+</sup> efflux from roots (resulting in enhanced K<sup>+</sup> 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<sup>+</sup> 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; Hosy 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<sup>+</sup> efflux between wild type, *gork1-1*, and *rbohD* (Fig. 7) suggested that about 25% of the K<sup>+</sup> efflux was mediated by ROS-activated channels and remaining 75% was mediated by depolarization-activated KOR during salt



**Fig. 5.** Beneficial effects of salicylic acid (SA) during 100 mM NaCl stress are diminished by root exposure to H<sup>+</sup>-ATPase pump inhibitors. NaCl-induced net H<sup>+</sup> (A) and K<sup>+</sup> fluxes (B) were measured at the mature root zone of 4- to 5-day-old *Arabidopsis thaliana* wild-type (Col-0) pretreated with 50  $\mu$ M SA for 1 h in the presence of either 1 mM vanadate or 0.1 mM *N,N'*-dicyclohexylcarbodiimide (DCCD). Values are mean  $\pm$  SE ( $n = 7$  seedlings).

stress (Fig. 7A, C). Pretreatment with SA did not provide any additional decrease in NaCl-induced K<sup>+</sup> efflux from the *gork1-1* mutant (Fig. 7B, C). In contrast, the SA pretreatment decreased NaCl-induced K<sup>+</sup> 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<sup>+</sup> efflux through depolarization-activated GORK channels is the key mechanism in SA-mediated salt tolerance in *Arabidopsis*.

**Table 2.** Effect of 1 h pretreatment with 50  $\mu$ M salicylic acid (SA) on net root Na<sup>+</sup> uptake in 4-day-old *Arabidopsis thaliana* wild-type (Col-0) seedlings exposed to various salinities. Values are mean  $\pm$  SE ( $n = 7$ ). Data with different letters are significantly different ( $P \leq 0.05$ ).

Treatment	Net root uptake ( $\mu$ mol Na <sup>+</sup> m <sup>-2</sup> )
50 mM NaCl	73.8 $\pm$ 1.5 <sup>a</sup>
50 mM NaCl + SA	74.2 $\pm$ 1.6 <sup>a</sup>
100 mM NaCl	137.3 $\pm$ 1.8 <sup>b</sup>
100 mM NaCl + SA	136.2 $\pm$ 1.9 <sup>b</sup>

*SA action is dependent on SA dose and pretreatment duration*

The diminishing effect of exogenously applied SA on both NaCl-induced K<sup>+</sup> efflux and H<sup>+</sup> influx was not obvious when 50  $\mu$ M 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

**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  $\mu$ M SA (for hydroponic culture) or pots were irrigated with 50  $\mu$ M SA (for soil culture) for 1 h. Values are mean  $\pm$  SE ( $n = 4$ ). Data with different letters are significantly different ( $P \leq 0.05$ ).

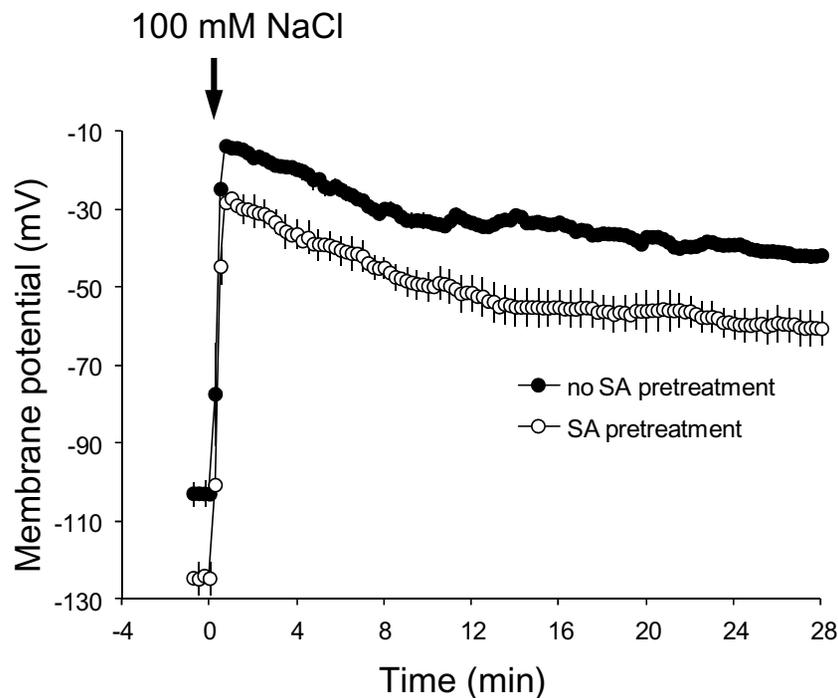
Treatment	Shoot Na (g (kg DW) <sup>-1</sup> )	Shoot K (g (kg DW) <sup>-1</sup> )
Control	0.83 $\pm$ 0.05 <sup>a</sup>	19.2 $\pm$ 0.05 <sup>a</sup>
SA	0.91 $\pm$ 0.09 <sup>b</sup>	19.6 $\pm$ 1.42 <sup>a</sup>
NaCl	37.1 $\pm$ 2.2 <sup>d</sup>	9.4 $\pm$ 0.48 <sup>b</sup>
NaCl + SA	24.2 $\pm$ 1.8 <sup>c</sup>	13.1 $\pm$ 0.69 <sup>c</sup>

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  $\leq 100$   $\mu$ M was effective in decreasing K<sup>+</sup> leakage from cells, whereas 500  $\mu$ M 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

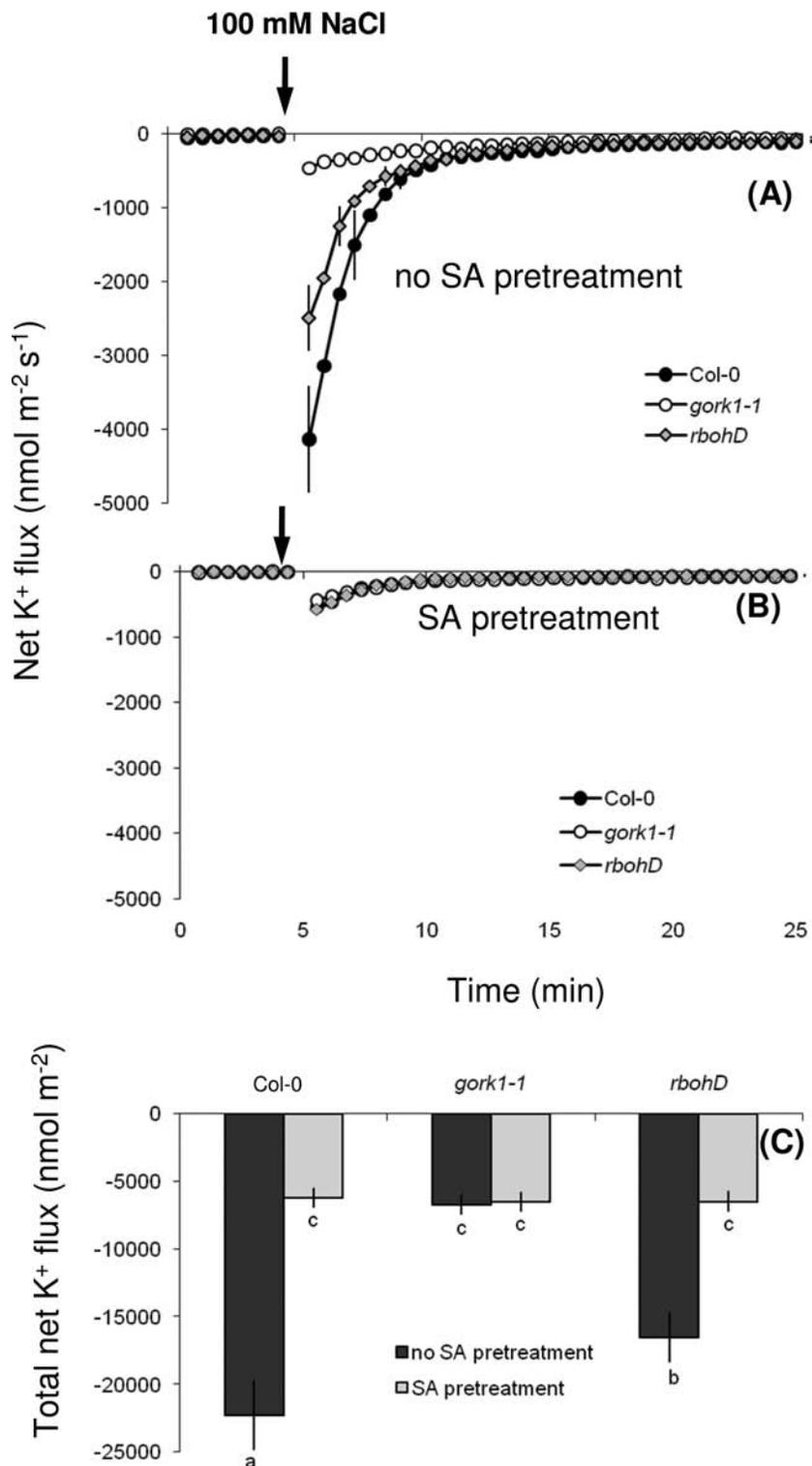
that 1 h pretreatment is the optimum duration to reach the desired cytosolic SA concentrations and get the most beneficial effects during salt stress (Fig. 4). The slightly diminished SA effects in longer pretreatment durations (4, 6, and 8 h; Fig. 4) can be explained by the metabolic conversion of free 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<sup>+</sup>-ATPase activity during salt stress

The SA pretreatment shifted NaCl-induced H<sup>+</sup> influx towards zero net H<sup>+</sup> 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<sup>+</sup>-ATPase activity (Fig. 5A), giving a strong evidence for the ameliorating effect of SA on the H<sup>+</sup>-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<sup>+</sup>-ATPase activity under salt stress, which in turn minimized the extent of plasma-membrane depolarization and thus decreased NaCl-induced K<sup>+</sup> efflux via depolarization-activated KOR channels. Even though SA was found to enhance the H<sup>+</sup>-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<sup>+</sup>-ATPase activity in the SA-mediated salinity tolerance in plants.



**Fig. 6.** Effect of 1-h pretreatment with 50  $\mu$ M 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  $\pm$  SE ( $n = 8$  seedlings).

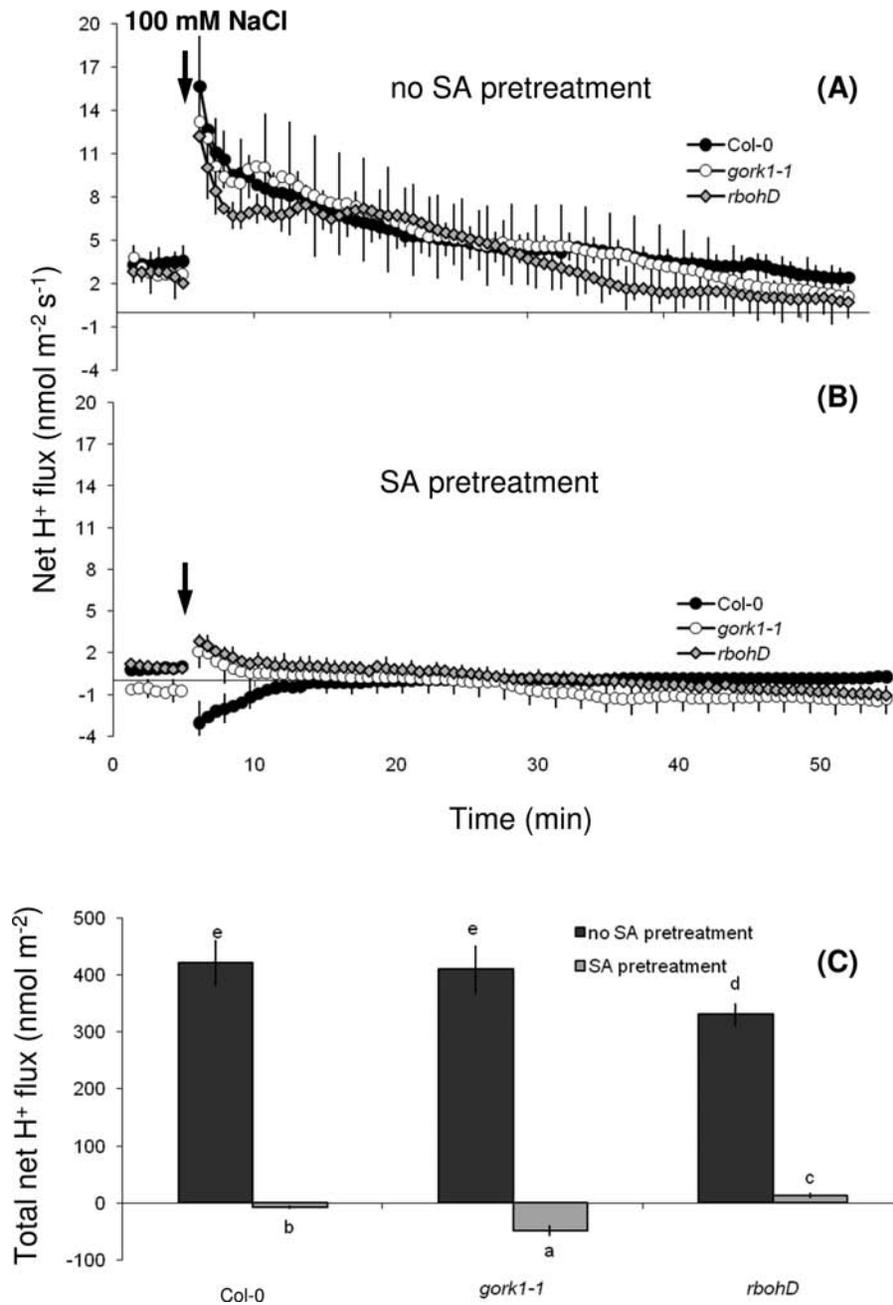


**Fig. 7.** Salinity (100 mM NaCl)-induced net K<sup>+</sup> fluxes at the mature root zone of 4–5-day-old seedlings of *Arabidopsis thaliana* wild type (Col-0), *gork1-1*, and *rbohD* mutants without (A) and with (B) 1-h 50  $\mu$ M salicylic acid (SA) pretreatment. (C) The total amount of K<sup>+</sup> leaked during 1-h NaCl stress. Values are means  $\pm$  SE ( $n = 8$  seedlings). Different letters in (C) indicate significant differences.

SA decreased root-to-shoot transport of Na<sup>+</sup> during salt stress

Electrophysiological and molecular studies (reviewed in Apse and Blumwald, 2007) suggest that Na<sup>+</sup> uptake into the root

tissue occurs predominantly through NSCC and high-affinity K<sup>+</sup> transporter-like proteins (Fig. 9). In this study, SA had no impact on net Na<sup>+</sup> uptake by roots during 1-h-long salt stress (Table 2), suggesting SA would not modulate Na<sup>+</sup> entry pathways. On the other hand, shoot Na<sup>+</sup> content was significantly

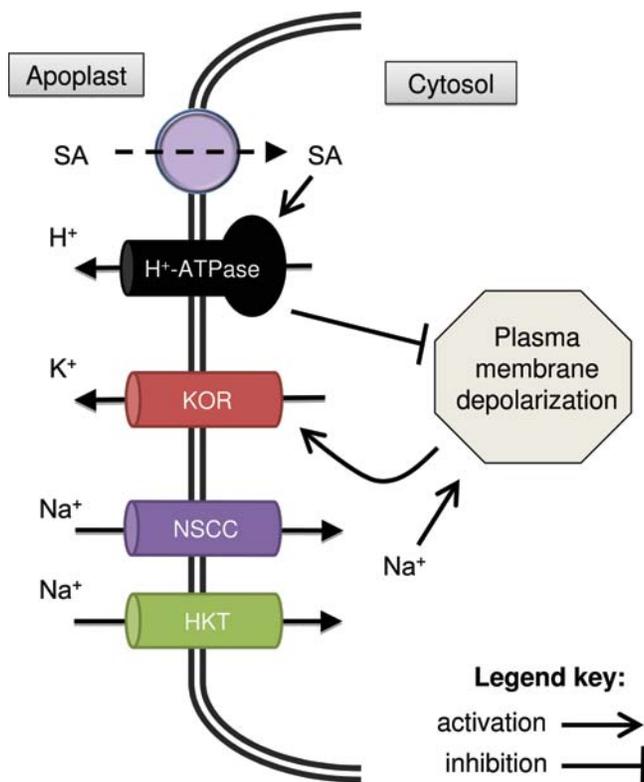


**Fig. 8.** Salinity (100 mM NaCl)-induced net H<sup>+</sup> fluxes at the mature root zone of 4–5-day-old seedlings of *Arabidopsis thaliana* wild type (Col-0), *gork1-1*, and *rbohD* mutants without (A) and with (B) 1-h 50  $\mu$ M salicylic acid (SA) pretreatment. (C) The total H<sup>+</sup> flux during 1-h NaCl stress. Values are means  $\pm$  SE ( $n = 8$  seedlings). Different letters in (C) indicate significant differences.

lower in SA-pretreated in comparison with SA-untreated plants (Table 3), implying SA decreased xylem loading of Na<sup>+</sup> in roots and/or enhanced Na<sup>+</sup> retrieval from the shoot during long-term salt stress. The decreased shoot Na<sup>+</sup> concentration was also accompanied by higher K<sup>+</sup> 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<sup>+</sup> enters into the cytosol (via either NSCC or high-affinity K<sup>+</sup> transporter, or both), causing significant depolarization of the plasma membrane. As a result, K<sup>+</sup> leaks through depolarization-activated KOR channels. Pretreatment with SA enhances the H<sup>+</sup>-ATPase activity under salt stress in a time- and concentration-dependent manner. Enhanced H<sup>+</sup>-ATPase activity assists in maintaining membrane potential at more negative values, decreasing K<sup>+</sup> 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. 9.** Schematic diagram depicting the beneficial effects of salicylic acid to cytosolic K<sup>+</sup> homeostasis in plant roots. HKT, high-affinity K<sup>+</sup> transporter; KOR, K<sup>+</sup> outward-rectifying channels; NSCC- non-selective cation channels; SA, salicylic acid.

level, SA also exerts beneficial effects by decreasing Na<sup>+</sup> transport to the shoot. Hence, compared with SA-untreated plants, the SA-pretreated plants have the higher cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio required for normal cell functioning under salt stress.

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