

RESEARCH PAPER

Haem oxygenase modifies salinity tolerance in *Arabidopsis* by controlling K⁺ retention via regulation of the plasma membrane H⁺-ATPase and by altering SOS1 transcript levels in roots

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Abstract

Reactive oxygen species (ROS) production is a common denominator in a variety of biotic and abiotic stresses, including salinity. In recent years, haem oxygenase (HO; EC 1.14.99.3) has been described as an important component of the antioxidant defence system in both mammalian and plant systems. Moreover, a recent report on *Arabidopsis* demonstrated that HO overexpression resulted in an enhanced salinity tolerance in this species. However, physiological mechanisms and downstream targets responsible for the observed salinity tolerance in these HO mutants remain elusive. To address this gap, ion transport characteristics (K⁺ and H⁺ fluxes and membrane potentials) and gene expression profiles in the roots of *Arabidopsis thaliana* HO-overexpressing (35S:HY1-1/2/3/4) and loss-of-function (*hy-100*, *ho2*, *ho3*, and *ho4*) mutants were compared during salinity stress. Upon acute salt stress, HO-overexpressing mutants retained more K⁺ (less efflux), and exhibited better membrane potential regulation (maintained more negative potential) and higher H⁺ efflux activity in root epidermis, compared with loss-of-function mutants. Pharmacological experiments suggested that high activity of the plasma membrane H⁺-ATPase in HO overexpressor mutants provided the proton-motive force required for membrane potential maintenance and, hence, better K⁺ retention. The gene expression analysis after 12h and 24h of salt stress revealed high expression levels of H⁺-ATPases (AHA1/2/3) and Na⁺/H⁺ antiporter [salt overly sensitive1 (SOS1)] transcripts in the plasma membrane of HO overexpressors. It is concluded that HO modifies salinity tolerance in *Arabidopsis* by controlling K⁺ retention via regulation of the plasma membrane H⁺-ATPase and by altering SOS1 transcript levels in roots.

Key words: Gene expression, H⁺-ATPase, haem oxygenase, ion fluxes, membrane potential, potassium, sodium.

Introduction

Salinity stress is the most severe environmental stress of irrigated agricultural crops, affecting at least 20% of irrigated land worldwide (Tuteja, 2007). On the other hand, dry land salinity also poses a major threat to agriculture production. For example, somewhere between 10% and 25% of currently

arable land could be out of production by 2020, and the overall cost of dryland salinity may exceed \$1 billion by 2100 (<http://www.environment.gov.au/soe/2001/publications/fact-sheets/salinity.html>). In addition, 67% of the agricultural area has a potential for 'transient salinity' (Rengasamy,

Abbreviations: AHA, H⁺-ATPase; HO, haem oxygenase; KOR channels, potassium outward rectifying channels; MS medium, Murashige and Skoog medium; NSCC, non-selective cation channel; ROS, reactive oxygen species; SOS1, salt overly sensitive1.

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2006) which may amount to \$1330 million per annum in lost opportunities (Rengasamy, 2002). Engineering salinity-tolerant cultivars through screening or through genetic manipulation is an attractive management option. For that, a thorough understanding of the target sites of salinity stress, signalling pathways, and mechanisms of salinity tolerance in plants is essential.

Extensive research on salinity tolerance conducted over the last few decades has concluded that salinity tolerance is controlled by multiple stress-responsive genes. A cross-talk between other components of stress signal transduction pathways was also found (Munns, 2005; Chen *et al.*, 2007). In this regard, reactive oxygen species (ROS) generated during salinity stress (Demidchik *et al.*, 2010) were suggested to act as messengers to cause the induction of multiple stress-responsive genes responsible for salt acclimation and repair. Intracellular ROS levels under favourable conditions are tightly regulated by numerous peroxidative and antioxidative reactions within the cell. Environmental factors may modify the equilibrium between ROS production and scavenging, resulting in a rapid rise in ROS a phenomenon known as an 'oxidative burst' (Shekhawat and Verma, 2010). Increased ROS production has been reported in response to drought, flooding, heat, cold, pathogens, wounding, ozone, heavy metals, air pollution, nutrient deprivation, excess light, UV radiation, and salinity (reviewed in Mittler and Blumwald, 2010). In order to protect a cell from oxidative damage, excess ROS should be scavenged by antioxidant mechanisms. Traditionally, this role has been allocated to a network of various enzymatic [e.g. superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidase (GPX)] and non-enzymatic antioxidants. Recently, haem oxygenase (HO; EC 1.14.99.3) has emerged as a previously unexplored, but crucial player in the plant's antioxidant network mainly because (i) HO enzyme products possess antioxidant properties (see detailed description below); (ii) if HO activity is enhanced by HO inducers, the performance of other antioxidant enzymes, namely SOD, CAT, and APX, also improved to tackle ROS stress (Ling *et al.*, 2009; Wu *et al.*, 2011); and (iii) HO is triggered by diverse stress-inducing stimuli, including salinity, hypoxia, heavy metals, UV radiation, hydrogen peroxide, and nitric oxide (reviewed in Wu *et al.*, 2011).

HO is a ubiquitous and highly active enzyme, which catalyses the stereospecific cleavage of haem into biliverdin (BV), carbon monoxide (CO), and free iron (Fe^{2+}). Both BV and iron are subsequently converted into potential antioxidants called bilirubin and ferritin, respectively (Gozzelino *et al.*, 2010). Similarly CO has a profound effect as an antioxidant and is also involved in ROS homeostasis regulation in plants (Piantadosi, 2008). Exogenous addition of CO has been shown to delay programmed cell death (Wu *et al.*, 2011), improve root elongation growth (Xuan *et al.*, 2007) and adventitious root formation (Xuan *et al.*, 2008), and enhance salinity tolerance in wheat (Xie *et al.*, 2008). As HO is the sole enzyme responsible for endogenous CO in plants, overexpressing HO may enhance plant tolerance to salinity stress. Indeed, *Arabidopsis* HO overexpression (35S:*HY1-1/2/3/4*) mutants demonstrated enhanced salinity tolerance

in *Arabidopsis* when compared with knockout (*hy1*, *ho2*, *ho3*, and *ho4*) mutants (Xie *et al.*, 2011). However, the physiological mechanisms and downstream targets responsible for the observed salinity tolerance in these HO mutants have not been elucidated.

Overproduction of ROS during salinity stress is usually derived from impaired electron transport processes in the plasma membrane (via NADPH oxidase), chloroplasts, and mitochondria (Shabala *et al.*, 1998; Shabala, 2009; Smith *et al.*, 2009). The release of free haem from chloroplasts and mitochondria (Thomas and Weinstein, 1990) during salt stress will inflict oxidative damage on plants because (i) haem forms a stable prosthetic group for electron transport proteins, namely cytochrome *c*, cytochrome *c* oxidase, cytochrome *c* reductase, cytochrome *b5*, and cytochrome *b558*, functioning in chloroplasts and mitochondria, and NADPH oxidase; and (ii) free haem can catalyse unfettered free radical production through the Fenton reaction (reviewed in Gozzelino *et al.*, 2010). Thus, the presence of HO in chloroplasts and mitochondrial membranes is required to protect cells from oxidative damage. In fact, green fluorescent protein studies have confirmed the presence of HOs in chloroplasts (Muramoto *et al.*, 1999) and mitochondria (Silva-Filho, 2003). In addition, CO application (a by-product of HO) has resulted in down-regulation of NADPH oxidase (Ling *et al.*, 2009), suggesting the central role of HO in preventing salt-induced overproduction of ROS in plants.

From the above literature review, it is evident that previous HO studies primarily focused on only one component of salinity stress, namely salinity-induced ROS production (oxidative component). To the best of the authors' knowledge, the impact of HO on physiological traits directly affecting intracellular ionic homeostasis, such as Na^+ exclusion from uptake (Shi *et al.*, 2003; Apse and Blumwald, 2007; Cuin *et al.*, 2011) or K^+ retention in the cytosol (Chen *et al.*, 2005; Shabala and Cuin, 2008), have never been addressed in direct experiments. Thus, the aim of this study was to test how *Arabidopsis* HO mutants respond to the ionic component of salt stress. Ion transport studies using the state-of-the-art microelectrode ion flux estimation (MIFE) technique and gene expression studies revealed that HO modifies salinity tolerance in *Arabidopsis* by controlling K^+ retention and Na^+ exclusion from the cytosol via regulation of the plasma membrane H^+ -ATPases (AHAs), providing some further insights into the molecular mechanisms of salinity tolerance in plants.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* L. loss-of-function HO mutants (Fig. 1A) *hy1-100* (CS236, Col-0), *ho2* (SALK_025840, Col-0), *ho3* (SALK_034321, Col-0), and *ho4* (SALK_044934, Col-0) were obtained from the Arabidopsis Biological Resource Center (<http://www.arabidopsis.org/abrc/>), as were seeds of the wild type (Col-0). HO overexpression mutants (35S:*HY1-1*, 35S:*HY1-2*, and 35S:*HY1-4*; Fig. 1A) were constructed and multiplied in the laboratory (Xie *et al.*, 2011). Seeds were surface sterilized with commercial bleach containing 0.1% (v/v) Triton for 10 min, and washed at least three times with sterilized deionized water. Surface-sterilized

seeds were sown on the surface of 90 mm Petri dishes containing solid 0.35% (w/v) phytoagar, full-strength Murashige and Skoog (MS) medium (Sigma-Aldrich, Castle Hill, NSW, Australia), and 1% (w/v) sucrose at pH 5.7. Petri dishes containing seeds were sealed with parafilm strips, kept at 4 °C for 2 d, and then transferred into a growth chamber with 16/8 h day/night length, 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density, and 23–25 °C temperature. The Petri dishes were oriented upright, allowing the roots to grow down along the phytoagar surface without penetrating it. However, the roots were anchored in the phytoagar by root hairs.

For biomass measurements, *Arabidopsis* seedlings were grown in MS medium containing 50 mM NaCl for 20 d. At the end of the experiment, 50 seedlings were harvested for each genotype, rinsed with deionized water, and their fresh weight was assessed. In order to assess radicle emergence during salt stress, *Arabidopsis* seeds were sown on MS medium containing 100 mM NaCl. Seeds were vernalized (as above), and germination percentage was assessed after 48 h in the growth chamber. These experiments were repeated twice, with three replicates each time.

Ion flux measurements

Net fluxes of H^+ and K^+ were measured using the non-invasive MIFETM technique (ROCU, University of Tasmania, Hobart, Australia) essentially as described by Shabala *et al.* (2005) and Newman (2001). Briefly, microelectrodes were pulled from borosilicate glass capillaries (GC 150-10, Harvard Apparatus Ltd, Kent, UK), oven dried at 230 °C overnight, and silanized using tributylchlorosilane (Fluka, catalogue no. 90796). Electrode tips were optimized to obtain external tip diameters of 2–3 μm . The electrodes were back-filled with appropriate solutions (0.15 mM NaCl+0.4 mM KH_2PO_4 , and adjusted to pH 6.0 using NaOH for the H^+ electrode and 0.5 M KCl for the K^+ electrode). The electrode tips were then front-filled with ionophore cocktails (Fluka, catalogue no. 95297 for H^+ and 60031 for K^+). Prepared electrodes were calibrated in a set of standards (pH from 4.76 to 7.10; K^+ from 0.25 to 1 mM). Electrodes with Nernst slope responses of <50 mV per decade were discarded. Electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan) with their tips positioned close together, ~40 μm above the root surface. During measurement, a computer-controlled stepper motor moved the electrode between two positions (40 μm and 80 μm , respectively) from the root surface in a 10 s square-wave manner. The CHART software (see Newman, 2001) recorded the potential differences between the two positions and converted them into electrochemical potential differences using the calibrated Nernst slope of the electrode. Net ion fluxes were calculated using the MIFEFLUX software for cylindrical diffusion geometry (Newman, 2001).

Ion fluxes were measured at the distal elongation (~300 μm from root cap) and mature root zones (beyond 2 mm) of 4- to 5-day-old *Arabidopsis* seedlings as described in Bose *et al.* (2010a, b). Roots of an *Arabidopsis* seedling were gently secured in a horizontal position with a Parafilm strip on the surface of a small plastic block. The seedling was then immediately placed in a 10 ml Perspex measuring chamber filled with 7 ml of basic salt medium (BSM; 0.5 KCl mM, 0.1 CaCl_2 mM, pH 5.7 unbuffered), mounted on a computer-driven 3D-micromanipulator (MMT-5, Narishige), and allowed to equilibrate for at least 30 min. Steady-state ion fluxes were recorded over a period of 5 min. A double stock of NaCl-containing solution was then applied and mixed to reach the required final 100 mM NaCl concentration. The resulting transient H^+ and K^+ fluxes were measured for up to 20 min. The time required for stock addition, mixing, and the establishment of diffusion gradients is reported to be ~40 s (Shabala and Hariadi, 2005). Accordingly, the first 60 s after the solution change was discarded from the analysis. For the pharmacology experiment, 35S:*HY1-4* seedlings were pre-treated with a P-type H^+ -ATPase inhibitor (1 mM sodium orthovanadate) for 1 h before the salt treatment (100 mM NaCl). Fluxes of between six and nine individual seedlings were averaged for every genotype, root zone, and treatment combination.

Membrane potential (E_m) measurements

The roots of an intact 4- to 5-day-old *Arabidopsis* seedling were immobilized and pre-conditioned as described above. E_m measurements were made using conventional KCl-filled Ag/AgCl microelectrodes (Cuin and Shabala, 2005; Bose *et al.*, 2010a). In brief, borosilicate glass microelectrodes (Harvard Apparatus Ltd) with tip diameter ~0.5 μm were filled with 1 M KCl, connected to the MIFE electrometer (Newman, 2001) via an Ag/AgCl half-cell, and impaled into the epidermal cells of the distal root elongation zone with a manually operated micromanipulator (MMT-5, Narishige). E_m was monitored continually using CHART software (for details, see Newman, 2001). Once a stable E_m measurement was obtained for 1 min, salt treatment (100 mM NaCl) was imposed. E_m measurements were continued up to 15–20 min after treatment. E_m values of 6–8 individual seedlings were averaged for every genotype and treatment combination.

SOS1 and AHA expression analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Real-time quantitative reverse transcription-PCRs (RT-PCRs) were performed using a Mastercycler[®] ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR[®] Premix Ex TaqTM (TaKaRa Bio Inc., China) according to the manufacturer's instructions. Using specific primers (Supplementary Table S1 available at JXB online), expression levels of *salt overly sensitive1* (*SOS1*) and *AHA1/2/3* genes were presented as values relative to corresponding control samples at the indicated times or under the indicated conditions, after normalization to *actin2/7* (accession no. NM_121018) transcript levels.

Statistical analysis

Data are means \pm SE. Statistical significance of mean values was determined using the standard LSD test at the $P \leq 0.05$ level.

Results

HO improves radicle emergence and growth during salinity stress

Salinity stress severely affects radicle emergence and growth of diverse plant species. Radicle emergence (Fig. 1B) and fresh weight (Fig. 1C) of *Arabidopsis* HO gain- and loss-of-function mutants (Fig. 1A) were assessed under 100 mM and 50 mM NaCl stress, respectively. As expected, HO gain-of-function mutants (35s:*HY1-1/2/4*) showed the highest percentage (55–100%) of radicle emergence, compared with <20% in HO loss-of-function mutants (*hy1-100*, *ho2*, and *ho3*) (Fig. 1B). A similar trend was observed for fresh weight data, with overexpressing lines having fresh weight in the range of 0.22–0.28 g per pot at 50 mM NaCl stress compared with 0.07–0.16 g per pot for knockout lines (Fig. 1C). In both cases, the wild-type Col-0 plants were in between these two groups (22% radicle emergence and 0.17 g of fresh weight per pot).

HO induces H^+ efflux and improves K^+ retention during salinity stress

Salinity stress (100 mM NaCl) caused significant changes in the transport of H^+ (Figs 2, 3) and K^+ (Fig. 4) ions in both

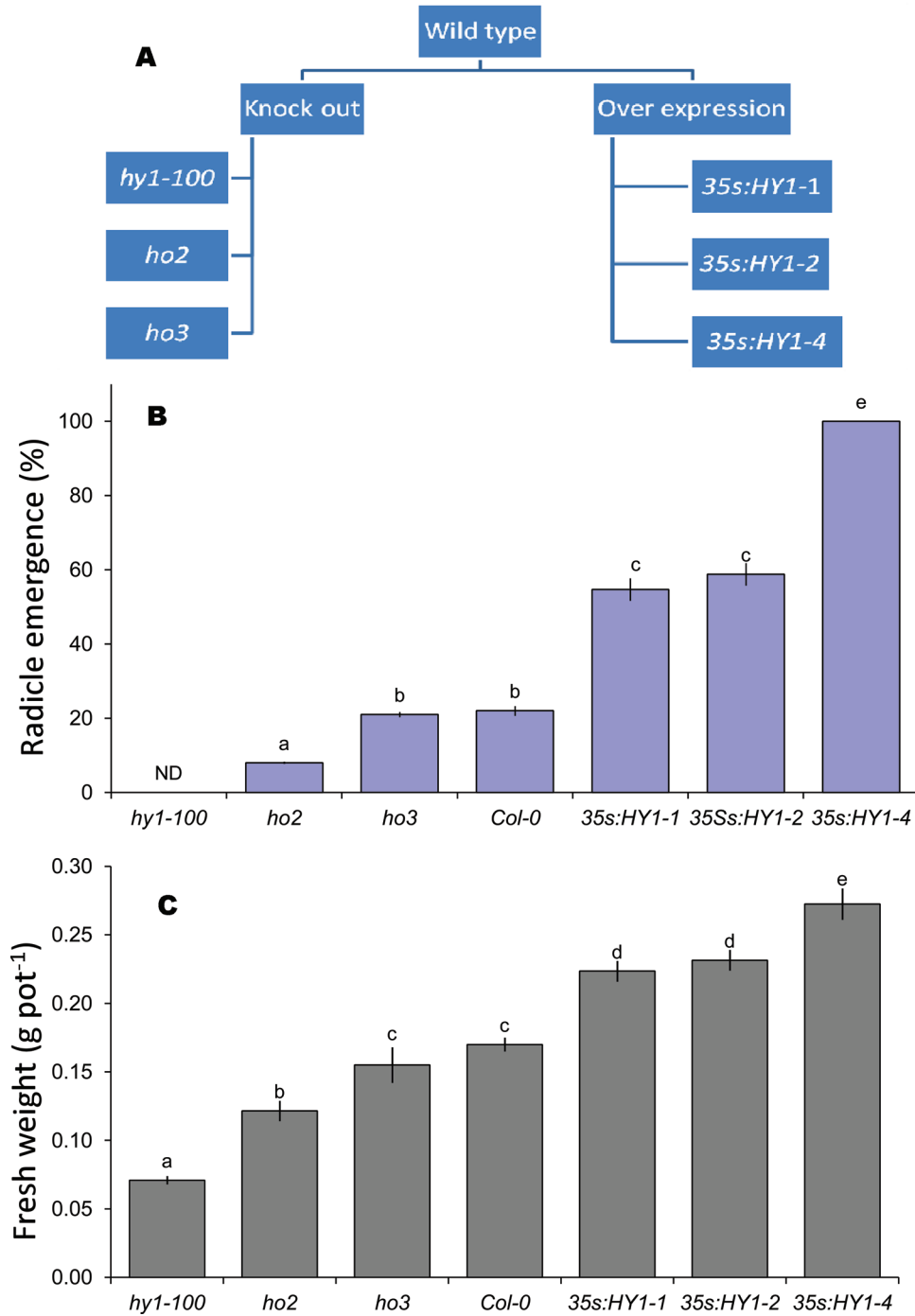


Fig. 1. (A) Overview of the haem oxygenase (HO) knockout and overexpression *Arabidopsis thaliana* lines used in this study. (B) Percentage of radicle emergence in 100mM NaCl agar medium 48h after sowing. (C) Fresh weight of *Arabidopsis* seedlings grown in 50mM NaCl agar medium. In B and C, each bar represents the mean \pm SE of two independent experiments. Bars sharing common letters are not significantly different by LSD test at $P \leq 0.05$. (This figure is available in colour at JXB online.)

the mature and elongation root zones of all the *Arabidopsis* genotypes tested.

Under control conditions, net H^+ flux from the mature root zone did not differ among genotypes (Fig. 2). Application of acute salt stress induced significant H^+ efflux from all the genotypes tested. However, salt-induced H^+ efflux was least in HO loss-of-function mutants (*hy1-100*, *ho2*, and *ho3*)

when compared with the wild type and HO gain-of-function mutants (*35s:HY1-1/2/4*) (Fig. 2).

Given the qualitative similarity of both ion flux (Fig. 2) and growth (Fig. 1) responses within the two groups, one HO-overexpressing (*35s:HY1-4*) and one knockout (*hy1-100*) line were selected for further studies. Similar to the mature zone, salinity treatment caused the highest net H^+ efflux from

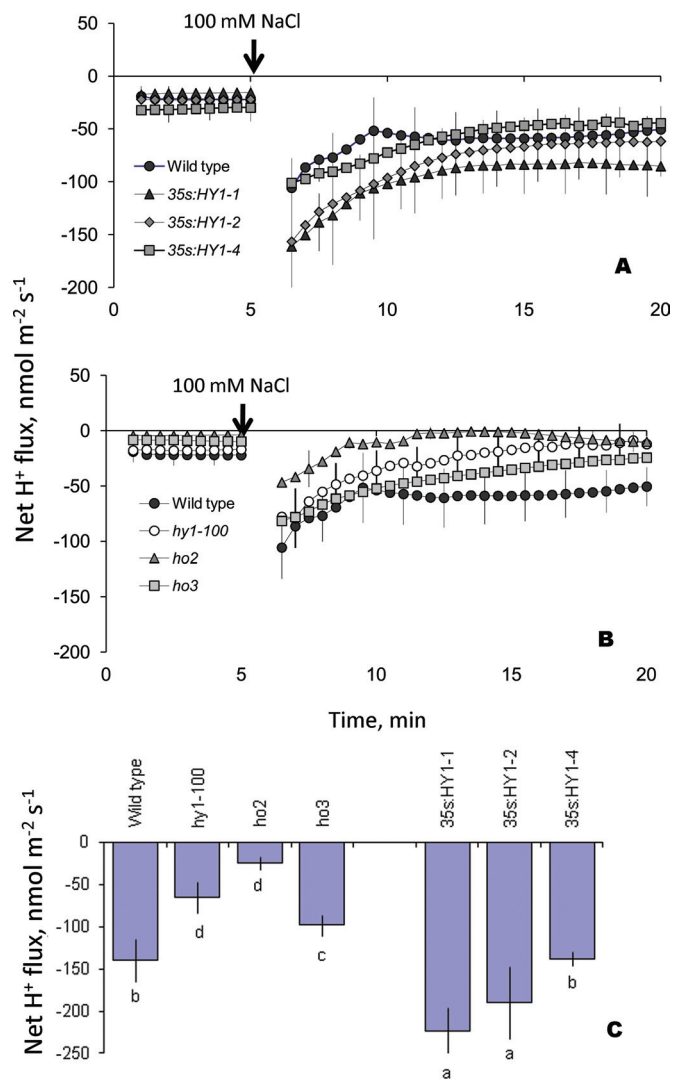


Fig. 2. Effect of 100 mM NaCl stress on H⁺ fluxes measured at the mature root zone of 4- to 5-day-old *Arabidopsis* seedlings. (A) Comparison between the wild type and HO overexpressor mutants. (B) Comparison between the wild type and HO knockout mutants. (C) Average H⁺ extrusion during 1 h of 100 mM NaCl stress. Each point or bar represents the mean \pm SE ($n=6-9$ seedlings). In C, bars sharing common letters are not significantly different by LSD test at $P \leq 0.05$. (This figure is available in colour at JXB online.)

the elongation root zone of HO gain-of-function mutant *35s:HYP1-4* when compared with the wild type and the HO knockout mutant (*hy1-100*) (Fig. 3). Pre-treating seedling of the *35s:HYP1-4* mutant with P-type H⁺-ATPase inhibitor (1 mM sodium orthovanadate) resulted in the abolishment of salt-induced H⁺ efflux in this mutant (Fig. 4A), indicating that the observed HO-induced stimulation of net H⁺ efflux was mediated by a P-type H⁺-ATPase.

Similar to previous observations (e.g. Shabala *et al.*, 2005, 2006), 100 mM NaCl induced K⁺ efflux from the mature and elongation zone of intact *Arabidopsis* roots (Fig. 5). Peak K⁺ efflux was measured between 2 min and 5 min after salt

application. After reaching peak K⁺ efflux, the K⁺ flux recovered gradually but not fully. Moreover, the salinity-induced peak K⁺ efflux measured at the elongation zone was at least 10-fold higher than at the mature root zone. The above result confirmed that the root elongation zone is more sensitive to salt treatment than the mature root zone. In both root zones, the HO gain-of-function mutant *35s:HYP1-4* recorded the least K⁺ efflux, followed by the wild type, while the highest K⁺ efflux was measured in the HO knockout mutant (*hy1-100*) (Fig. 5). Better K⁺ retention in the *35s:HYP1-4* mutant was partly reversed (Fig. 4B) by sodium orthovanadate (P-type H⁺-ATPase inhibitor) pre-treatment, suggesting that a substantial part of the K⁺ retention through HO overexpression may be attributed to increased plasma membrane H⁺-ATPase activity.

HO increases the rate of plasma membrane repolarization during salinity stress

No significant difference in E_m values was found in the root elongation zone among genotypes (*35s:HYP1-4*, wild type, and *hy1-100*) under control conditions (-132 ± 4 mV; Fig. 6). Adding 100 mM NaCl to the bath depolarized the E_m values to -22 ± 2 mV within 1.5 min of salt application in all genotypes. However, the rate of E_m repolarization was significantly more pronounced in the HO gain-of-function mutant *35s:HYP1-4*, followed by the wild type. E_m values remained the least negative (most depolarized) in the HO loss-of-function mutant *hy1-100* (Fig. 6). Fifteen minutes after NaCl treatment, E_m values of the *35s:HYP1-4* mutant repolarized to -54 ± 3.2 mV, but only to -31 ± 2 mV in the *hy1-100* mutant. The E_m values of the wild-type plants were between those of the *35s:HYP1-4* and *hy1-100* mutants (-41 ± 2 mV).

HO up-regulates the relative expression of plasma membrane SOS1 and AHA1/2/3

The activity of the plasma membrane H⁺ATPase is essential not only for the maintenance of negative membrane potential under saline conditions but also to provide a driving force for Na⁺ exclusion via the Na⁺/H⁺ exchanger (SOS1). Hence, relative gene expression of *SOS1* and *AHA1/2/3* was quantified at three time points (0, 12, and 24 h) after application of 100 mM NaCl to roots (Fig. 7). The *SOS1* expression was increased 5- and 10-fold in the *35s:HYP1-4* mutant after 12 h and 24 h of salt application, respectively. Although the wild type also showed an increase in *SOS1* expression, the expression level remained within 2- to 4-fold. In contrast, *SOS1* expression remained unchanged in the *hy1-100* mutant.

The relative expression levels of *AHA1/2* peaked at 12 h, whereas *AHA3* expression peaked 24 h after salt application in the *35s:HYP1-4* mutant. Although the wild type showed a similar trend, the expression levels were smaller than in the *35s:HYP1-4* mutant. Interestingly, *AHA1/2/3* expression levels gradually decreased during salt treatment in the *hy1-100* mutant. The above results confirm the role of HO in the functioning of AHAs and SOS1 as Na⁺ exclusion mechanisms in plants.

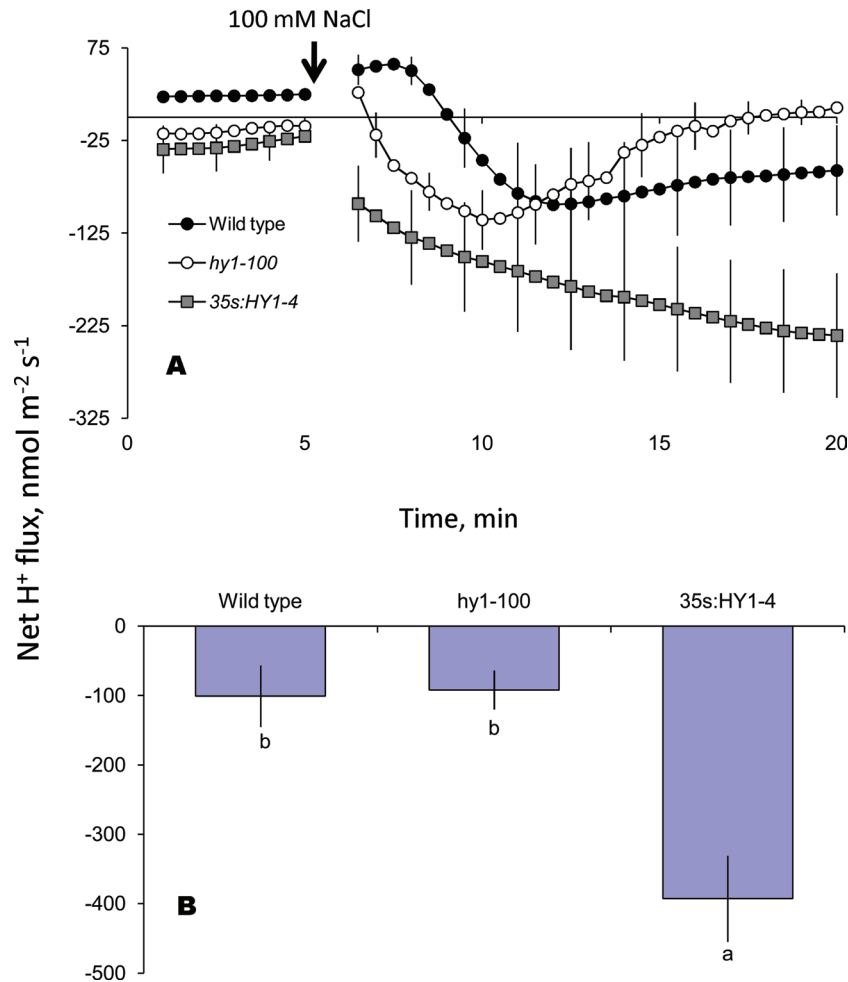


Fig. 3. Effect of 100 mM NaCl stress on H⁺ fluxes measured at the elongation root zone of 4- to 5-day-old *Arabidopsis* seedlings. (A) Transient H⁺ flux comparison between the wild type, and HO overexpressor and suppressor mutants. (B) Average H⁺ extrusion during 1 h of 100 mM NaCl stress. Each point or bar represents the mean \pm SE ($n=6-9$ seedlings). In B, bars sharing common letters are not significantly different by LSD test at $P \leq 0.05$. (This figure is available in colour at JXB online.)

Discussion

Under saline conditions, Na⁺ enters into root epidermal cells through non-selective cation channels (NSCCs; Demidchik and Tester, 2002) and high-affinity K⁺ transporters (HKTs) (Laurie *et al.*, 2002). Such an entry of positively charged Na⁺ ions causes dramatic depolarization of the plasma membrane, leading to immediate K⁺ leak via depolarization-activated outward rectifying K⁺ (KOR) channels (Shabala *et al.*, 2006; Chen *et al.*, 2007). This depletes the cytosolic K⁺ pool, impairing cell metabolism (reviewed in Shabala and Cuin, 2008) and potentially leading to programmed cell death under saline conditions (Shabala, 2009; Demidchik *et al.*, 2010). Since K⁺ loss occurs through voltage-dependent KOR channels, maintenance of a more negative potential during salt stress will help the plants to retain enough K⁺ in the cytosol. Indeed, *Thellungiella halophila*, a salt-tolerant relative of *A. thaliana*, was able to maintain a more negative potential and K⁺ retention during 100 mM NaCl stress (Volkov and Amtmann, 2006). A similar relationship was also established between

barley genotypes with contrasting salt tolerance (Chen *et al.*, 2007). In this study, an ~ 23 mV depolarization difference was found between a HO gain-of-function (-54 ± 3.2 mV) and a knockout (-31 ± 2 mV) mutant within 10 min of 100 mM NaCl stress (Fig. 6). This potential difference was reflected in the magnitude of K⁺ loss during salt stress in both the root zones; the loss is least in the 35s:HY1-4 mutant and highest in the hy1-100 mutant (Fig 4). The above results suggest that HO prevents K⁺ loss through KOR channels during salinity stress by increasing the rate of membrane potential repolarization.

As the plasma membrane Na⁺/H⁺ exchanger is electroneutral, its activity cannot be responsible for the pronounced repolarization of the membrane voltage in the HO gain-of-function mutant (Fig. 6). On the other hand, plasma membrane H⁺-ATPase activity is essential for maintenance of the membrane potential (Palmgren and Nissen, 2010) and was shown to determine the genotypic difference in salinity tolerance in some species (e.g. barley; Chen *et al.*, 2007). This also appears to be the case here. Indeed, the higher rate of

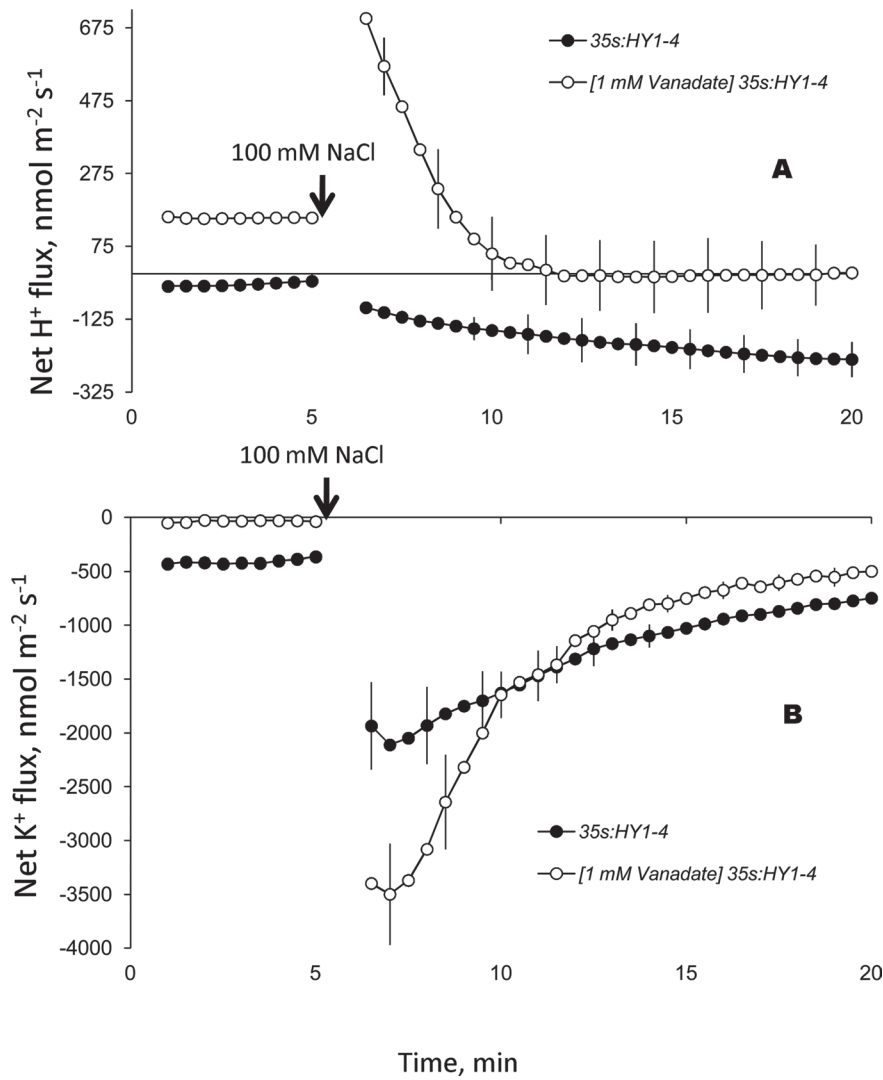


Fig. 4. Effect of 100 mM NaCl treatment on transient (A) H⁺ and (B) K⁺ flux kinetics measured at the elongation zone of the *35s:HY1-4* mutant in the presence and absence of the H⁺-pump inhibitor vanadate. Each point represents the mean \pm SE ($n=6-9$ seedlings).

membrane potential repolarization in *35s:HY1-4* was closely associated with a higher H⁺ efflux from root epidermal cells (Fig. 3). Likewise, this highest H⁺ efflux resulted in the least NaCl-induced K⁺ efflux in the *35s:HY1-4* mutant (Fig. 5). This H⁺ efflux was strongly affected by sodium orthovanadate, a known inhibitor of P-type ATPases (Fig. 6). Vanadate treatment also augmented the NaCl-induced K⁺ efflux (Fig. 5). Thus, the above results indicate that HO up-regulates H⁺-ATPase activity, thereby resulting in a higher rate of membrane potential repolarization and enabling better K⁺ retention during salt stress. This notion is further supported by the H⁺ flux data from the mature root zone (Fig. 2) wherein all HO gain-of-function mutants (*35s:HY1/2/4*) showed higher H⁺ efflux compared with HO knockout mutants (*hy1-100*, *ho2*, *ho3*).

Molecular analysis of H⁺-ATPases (*AHA1/2/3*) revealed no difference in expression levels in any of transcripts between the wild type and any of mutants studied (Fig. 7). This suggests that the observed up-regulation of H⁺-ATPase activity

in gain-of-function HO mutants occurred at the physiological (post-translational) level. At the same time, long-term salinity exposure resulted in increased AHA transcript levels in the wild type and especially in the HO gain-of-function mutant (Fig. 7), while in the HO knockout this up-regulation was absent. Thus, it appears that HO production is also essential to confer a salinity-induced increase in H⁺-ATPase expression at the transcriptional level. Further studies are needed to reveal specific details of this process.

Exclusion of Na⁺ by active efflux systems back to the soil solution is critical for salinity tolerance in plants (reviewed in Munns and Tester, 2008). So far, only one plasma membrane-localized Na⁺/H⁺ exchanger encoded by the *SOS1* gene has been identified (Wu *et al.*, 1996) and measured for Na⁺ efflux under physiological conditions in plants (Zhu, 2003; Apse and Blumwald, 2007; Cuin *et al.*, 2011). Overexpression of *SOS1* reduced Na⁺ accumulation and improved salt tolerance in *Arabidopsis* (Shi *et al.*, 2003), and the opposite was the case in *SOS1* knockout mutants (Wu *et al.*, 1996; Shi *et al.*,

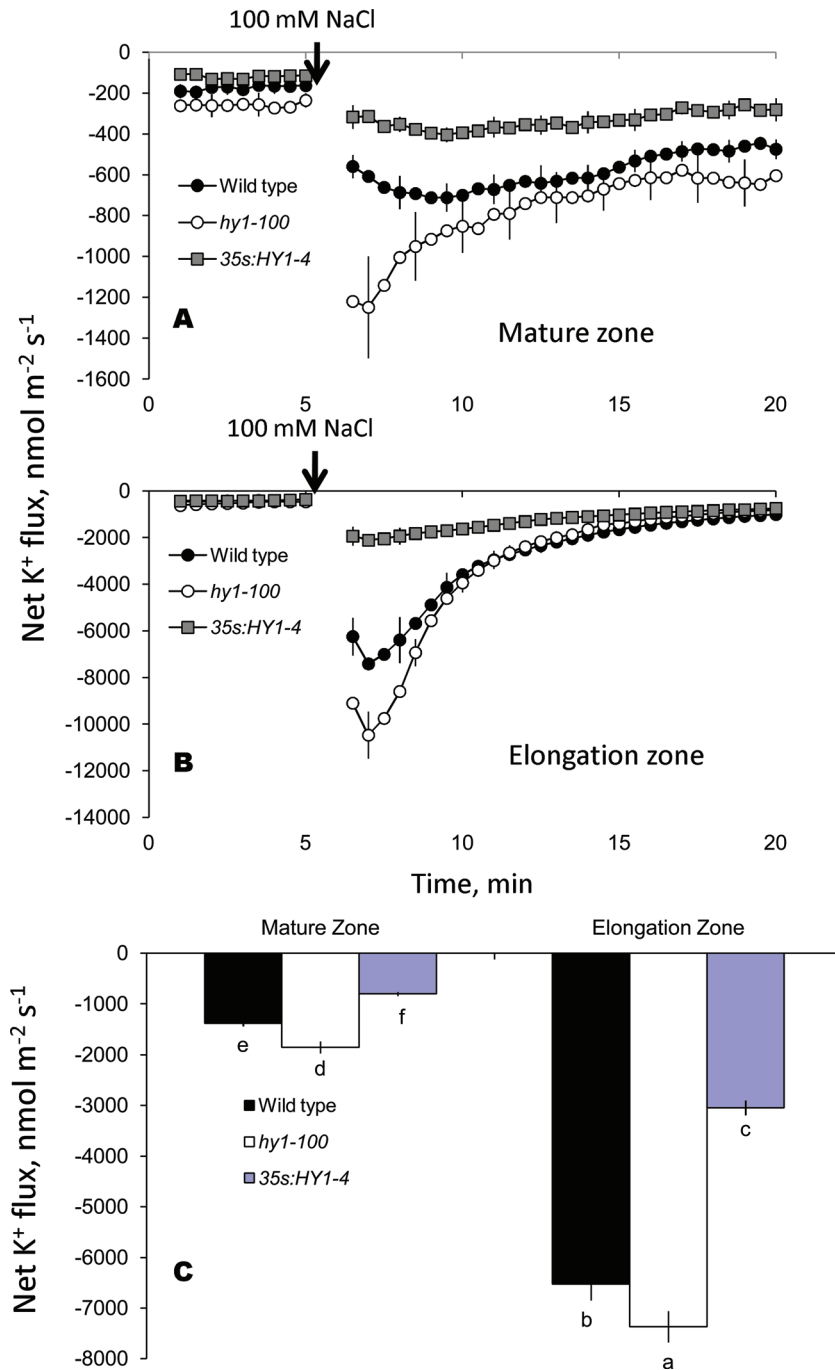


Fig. 5. Effect of 100 mM NaCl stress on transient K⁺ efflux measured at (A) the mature and (B) the elongation root zone of 4- to 5-day-old *Arabidopsis* seedlings. (C) Average K⁺ extrusion during 1 h of 100 mM NaCl stress. Each point or bar represents the mean \pm SE ($n=6-9$ seedlings). In C, bars sharing common letters are not significantly different by LSD test at $P \leq 0.05$. (This figure is available in colour at *JXB* online.)

2000). Such energy-dependent Na⁺ transport ultimately relies on a proton-motive force generated by H⁺-ATPase activity (reviewed in *Cuin et al., 2011*). X-ray micrographs of rice (*Oryza sativa*) roots treated with haemoglobin (an inducer of HO) and wheat (*Triticum aestivum*) seedlings treated with CO (a by-product of HO) revealed that their roots were able to maintain a high K⁺/Na⁺ ratio by excluding Na⁺ from the cortex (*Xie et al., 2008; Xu et al., 2011*). This would

be possible only if *SOS1* was operating in the root cortex. Indeed, here the evidence is presented that *SOS1* expression was up-regulated in a HO gain-of-function mutant but not in a knockout mutant (*Fig. 7*). Moreover, up-regulation of *AHA1/2/3* in a HO gain-of-function mutant provides the proton-motive force required for successful operation of *SOS1*. The absence of a statistically significant difference in the *SOS1* and *AHA1/2/3* expression level between HO loss- and

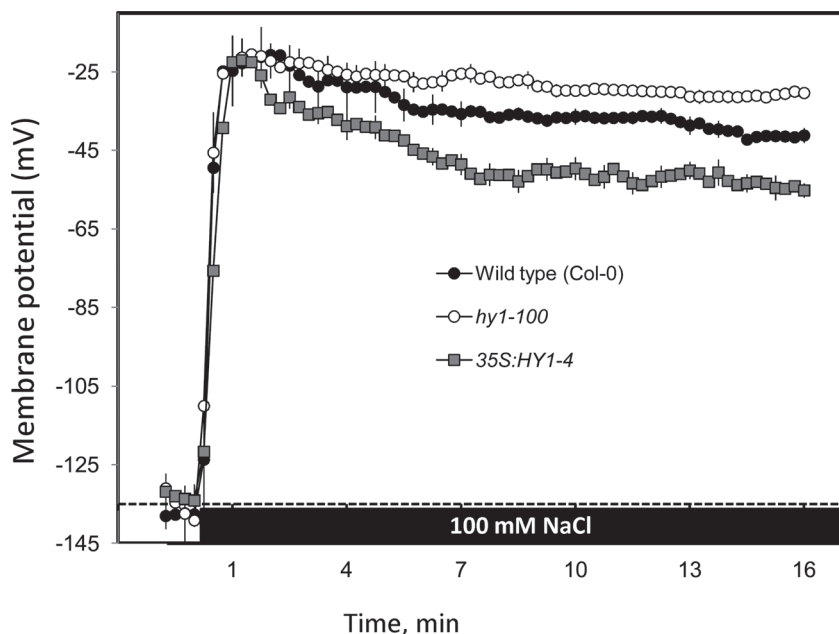


Fig. 6. Effect of 100 mM NaCl treatment on transient membrane potential kinetics measured at the elongation root zone of 4- to 5-day-old *Arabidopsis* seedlings. Each point or bar represents the mean \pm SE ($n=6-9$ seedlings).

gain-of-function mutants under control conditions (Fig. 7) suggests that the difference in the Na^+ exclusion rate may be due to post-transcriptional modification by HO.

Based on the present results, a model is proposed describing how HO gain- and loss-of-function mutants respond to the ionic component of a salt stress (Fig. 8). Application of salt stress leads to the passive entry of Na^+ ions into the cytoplasm through NSSCs, which results in depolarization

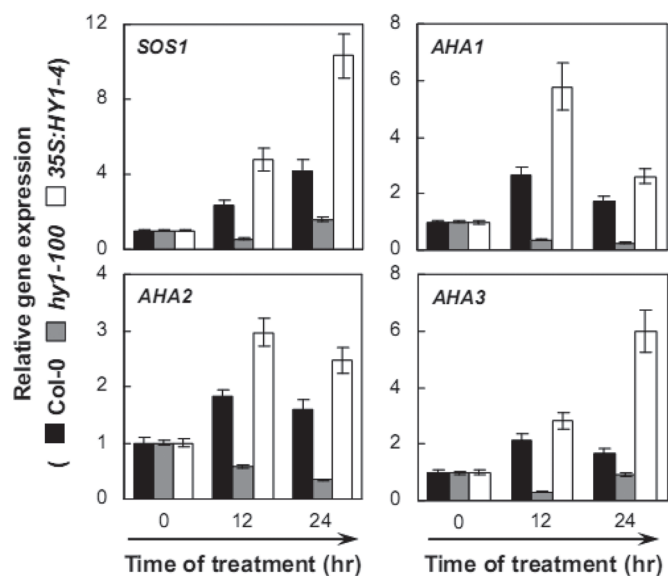


Fig. 7. Effect of 100 mM NaCl treatment on relative *SOS1* and *AHA1/2/3* expression measured in roots of 4- to 5-day-old *Arabidopsis* seedlings. Each bar represents the mean \pm SE ($n=6-9$ seedlings for each treatment and time combination obtained in three independent trials).

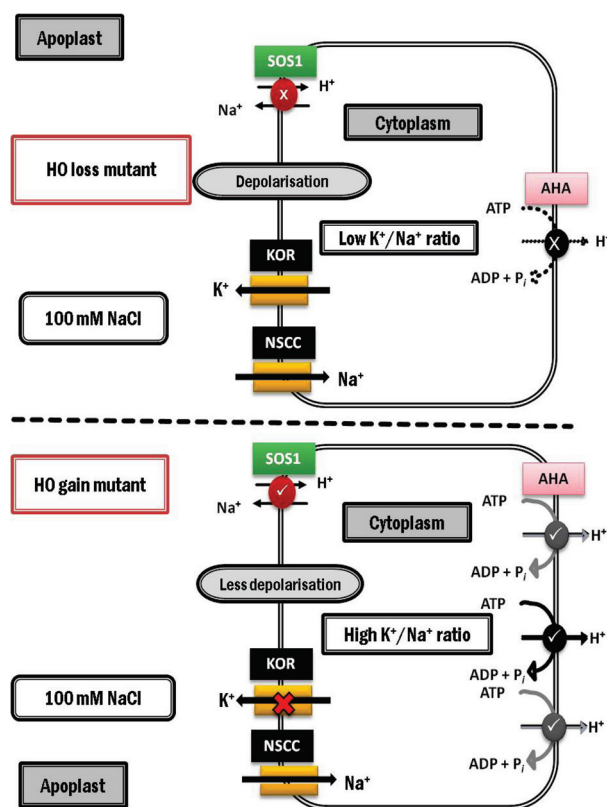


Fig. 8. Schematic diagram comparing *Arabidopsis thaliana* haem oxygenase (HO) loss- and gain-of-function mutants during salinity stress. *SOS1*, salt overly sensitive1 Na^+/H^+ exchanger; *KOR*, potassium outward rectifying channels; *NSSC*, non-selective cation channels; *AHA*, P-type ATPases. AHAs in shades of grey are additional copies after transcriptional regulation. Ticks denote up-regulation and crosses denote down-regulation of a respective transporter. (This figure is available in colour at *JXB* online.)

of the plasma membrane in both gain- and loss-of-function HO mutants. High activities of the plasma membrane H⁺-ATPases (*AHA1/2/3*) in HO-overexpressing mutants provide a proton-motive force required for (i) membrane potential repolarization (explaining more negative E_m values in the HO-overexpressing lines), thereby preventing K⁺ loss via depolarization-activating KOR channels; and (ii) Na⁺ exclusion through enhanced *SOS1* activity. HO-induced up-regulation of H⁺-ATPase activity occurs at both physiological (increased H⁺ pumping activity) and transcriptional (e.g. a large number of AHA transcripts) levels. By these mechanisms, the gain-of-function HO mutant is able to maintain a higher K⁺/Na⁺ ratio in the cytosol compared with the HO knockout during salt stress. The overall benefit of having a high K⁺/Na⁺ ratio was reflected in enhanced germination (Fig. 1B) and growth (Fig. 1C) in the overexpressing line. In contrast, the inability of the HO knockout mutant to prevent K⁺ loss and exclude Na⁺ resulted in a low K⁺/Na⁺ ratio in the cytosol, and thus poor germination (Fig. 1B) and growth (Fig. 1C) in a saline environment.

In conclusion, this work emphasizes the beneficial role of HO in K⁺ retention and Na⁺ exclusion during salt stress in plants. Thus, HO can be targeted in future breeding programmes to impart salt tolerance to crop plants.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. The sequences of PCR primers used for real-time PCR.

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