Humic Acid Confers HIGH-AFFINITY K+ TRANSPORTER 1-Mediated Salinity Stress Tolerance in Arabidopsis

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Excessive salt disrupts intracellular ion homeostasis and inhibits plant growth, which poses a serious threat to global food security. Plants have adapted various strategies to survive in unfavorable saline soil conditions. Here, we show that humic acid (HA) is a good soil amendment that can be used to help overcome salinity stress because it markedly reduces the adverse effects of salinity on Arabidopsis thaliana seedlings. To identify the molecular mechanisms of HA-induced salt stress tolerance in Arabidopsis, we examined possible roles of a sodium influx transporter HIGH-AFFINITY K+ TRANSPORTER 1 (HKT1). Salt-induced root growth inhibition in HKT1 overexpressor transgenic plants (HKT1-OX) was rescued by application of HA, but not in wild-type and other plants. Moreover, salt-induced degradation of HKT1 protein was blocked by HA treatment. In addition, the application of HA to HKT1-OX seedlings led to increased distribution of Na⁺ in roots up to the elongation zone and caused the reabsorption of Na⁺ by xylem and parenchyma cells. Both the influx of the secondary messenger calcium and its cytosolic release appear to function in the destabilization of HKT1 protein under salt stress. Taken together, these results suggest that HA could be applied to the field to enhance plant growth and salt stress tolerance via post-transcriptional control of the HKT1 transporter gene under saline conditions.

Keywords: Arabidopsis, calcium, HKT1, humic acid, salt stress

INTRODUCTION
One of the largest global concerns is climate change, which is bringing about rapid soil erosion in agricultural lands worldwide. One of the major abiotic stresses, salinity, causes soil degradation and inhibits nutrient absorption, consequently reducing crop yields (Ashraf and Foolad, 2007; Tester and Davenport, 2003). Plants must cope with both osmotic and ionic stress under high-salinity conditions. Osmotic stress reduces water uptake and cell expansion and delays lateral bud development (Munns and Tester, 2003). Ionic stress reduces water uptake and cell expansion and delays lateral bud development (Munns and Tester, 2003). Ionic stress is induced when toxic ions such as Na⁺ accumulate at high levels in cells, specifically in leaves and shoots, leading to increased leaf mortality along with chlorosis and necrosis (Glenn et al., 1999; Yeo and Flowers, 1986). In addition, high Na⁺ concentrations interrupt the uptake of potassium.
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Humic acid (HA) improves plant development by regulating metabolic and signaling pathways by acting directly on certain targets in diverse physiological processes. HA treatment enhances the mobilization of toxic heavy metals, especially from abandoned mine tailings, indicating that HA could be utilized as a possible remedy to reduce further soil contamination. Moreover, HA has protective effects against high saline stress by inhibiting Na⁺ uptake in barley (Marketa et al., 2016), and it reduces yield losses in maize under salt stress (Masciandaro et al., 2002). However, recent extensive studies have failed to further explain the physiological and molecular mechanisms underlying how HA confers salt tolerance to plants.

In the current study, we investigated how HA increases salt tolerance in Arabidopsis seedlings. The salt-induced degradation of HKT1 was impaired by HA treatment, and HA increased the protein abundance of HKT1 in the root stele, which resulted in greater reabsorption of Na⁺ from xylem vessels into xylem parenchyma cells and, consequently, less translocation of Na⁺ to the shoot.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**  
*A. thaliana* mutant and transgenic seedlings, including wild-type (WT, Col-0), *sos1-1*, *hkt1-1*, *SOS1-OX*, and *HKT1-OX* (Ali et al., 2012; Kim et al., 2013) seedlings were surface-sterilized and grown on 1/2 MS medium under long-day conditions under a 16 h light/8 h dark photoperiod, 130 µmol m⁻² s⁻¹ light intensity at 23°C.

**Salt tolerance assay**  
Five-day-old WT Arabidopsis seedlings were transferred to...
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1/2 MS medium containing 250 mM NaCl alone or supplemented with 86 or 860 mg L\(^{-1}\) HA (Sigma-Aldrich). The fresh weight of 15 plants was measured at 7 days after treatment, with three independent replications.

**Salt sensitivity assay**
Five-day-old Arabidopsis seedlings (WT, sos1-1, hkt1-1, SOS1-OX, and HKT1-OX) were transferred to 1/2 MS containing 100 mM NaCl alone or supplemented with 860 mg L\(^{-1}\) HA. The fresh weight of shoots (indicating HA-induced recovery of fresh weight in shoots under salt stress) were measured at 8 days after treatment. The primary root length (indicating HA-induced recovery of root growth under salt treatment) was measured at 9 days after treatment and analyzed using ImageJ software (1.48v, http://imagej.nih.gov/ij).

**Visualization of Na\(^+\) ions in plant cells by fluorescent staining**
Five-day-old Arabidopsis seedlings treated with 100 mM NaCl with or without HA (860 mg L\(^{-1}\)) for 14 h were stained with 5 μM CoroNa-Green AM (Invitrogen) for 3 h in the presence of pluronic acid (Sigma-Aldrich) at a final concentration of 0.02% in the dark (Leshem et al., 2006; Mazel et al., 2006). The stained roots were examined under a confocal microscope (Olympus FV1000) at excitation and emissions wavelengths of 488 nm and 516 nm, respectively. The cell walls and dead cells were stained with 1 μg ml\(^{-1}\) propidium iodide (Invitrogen).

**Immunoblot analysis**
Nine-day-old HKT1-OX (GFP-fused) or SOS1-OX (HA-fused) seedlings were treated with 100 mM NaCl in the absence or presence of 860 mg L\(^{-1}\) HA in 1/2 MS medium for 6 and 12 h. After treatment, whole plants were immediately divided into shoots and roots and frozen at -80℃ until use. Total proteins were isolated in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% [v/v] NP-40, 1 mM PMSF, 1 μg ml\(^{-1}\) leupeptin, 1 μg ml\(^{-1}\) aprotinin, 1 μg ml\(^{-1}\) pepstatin) and separated by 10% SDS-PAGE. After blocking with 5% (w/v) skim milk in TBS-T, the membrane was incubated with the primary antibody overnight at 4℃. Immunoblot analysis was performed with α-GFP antibody (1:3,000; Abcam) for HKT1-GFP or α-HA antibody (1:2,000; Roche) for SOS1-HA, and the membrane was then incubated with α-rabbit (for HKT1-GFP: 1:3000; Thermo Scientific) or α-rat (for SOS1-HA: 1:3000; Sigma) secondary antibody. Bands were detected based on chemiluminescence using ECL-detecting reagent (Thermo Scientific).

**Expression of HKT1 transcripts using quantitative RT-PCR**
Nine-day-old HKT1-OX were treated and prepared as described above. Total RNA was extracted using TRIzol reagent (Qiagen) and synthesized cDNA using oligo dt primers and RevertAid First Strand cDNA synthesis Kit (Thermo Scientific). Equal amounts of cDNA were amplified with gene-specific forward (For) and reverse (Rev) primers for measurement of Arabidopsis HKT1 (For: 5'-TCTCCTTGAGGATGACGTG-3'; Rev: 5'-AACGATCCACCAACTTCTC-3') and AT5G12240 as an internal control (For: 5'-AGCGGCGCTGAGAAGAAAGT-3'; Rev: 5'-TCTCGAAAGCCCTTGCAAATCT-3') by TOPreal™ qPCR 2X PreMix (SYBR Green with low ROS) kit (Enzymomics) in CFX96 Touch™ Real-Time PCR detection system (Bio-Rad).

**Treatment with Ca\(^{2+}\) flux inhibitors**
Nine-day-old HKT1-OX (GFP-fused) seedlings were floated in 50 mM nicotinamide (an inhibitor of calcium release by cADPR), 1 mM GdCl3 (a calcium influx inhibitor), or 5 μM U73122 (an inhibitor of calcium release from the vacuole) in the absence or presence of 100 mM NaCl for 6 or 12 h after treatment. Immunoblot analysis was carried as described above.

**Measuring Na\(^+\) ion concentrations in plants**
Three-week-old WT plants were transferred to 1/2 MS plates containing 100 mM NaCl with or without 860 mg L\(^{-1}\) HA and grown for an additional 2 days. The plants were rinsed with deionized water and dried at 65℃ for 2 days. The dry tissues were ground using a mortar and pestle, and 100 mg of dry tissue was extracted with 10 ml of HClO\(_4\):H\(_2\)O:H\(_2\)SO\(_4\) (9:5:1, v/v/v) on a heating block with a gradual increase in temperature from 100℃ to 320℃. After digestion, the samples were diluted to a final volume of 100 ml with deionized water and filtered through filter paper (Whatman No. 2). The Na\(^+\) ion content was analyzed using Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS, Perkin Elmer Optima 2200 DV).

**RESULTS**

**HA confers enhanced salt tolerance in Arabidopsis**
The protective effect of HA in plants under salt stress has primarily been demonstrated in cereals such as maize and wheat (Aydin et al., 2012; Khaled and Fawy, 2011). Very recently, we also reported that HA increases seed germination rates in Arabidopsis in a dose-dependent manner and that the application of 86 mg L\(^{-1}\) HA confers salt stress tolerance to this plant under excessive salt concentrations (250 mM NaCl) (Cha et al., 2017). In the current study, to determine whether the use of HA at concentrations greater than 86 mg L\(^{-1}\) would cause a dramatic increase in salt tolerance, we performed a salt tolerance assay in which WT Arabidopsis seedlings were grown on 1/2 MS agar plates under a fixed concentration of NaCl (250 mM) and various concentrations of HA (0, 86, and 860 mg L\(^{-1}\)). In the absence of HA, the seedlings were nearly dead when grown on salt stress medium, with chlorosis observed in shoots. However, seedlings grown on 86 or 860 mg L\(^{-1}\) HA medium plus 250 mM NaCl had green shoots (Fig. 1A). We measured the fresh weight of plants, finding that increasing the concentration of HA in seedlings under salinity stress increased salt stress tolerance in a dose-dependent manner (Fig. 1B). In tomato, the application of HA to the soil increased plant growth up to a concentration of 1 g kg\(^{-1}\), but the effects were reduced at 2 g kg\(^{-1}\) (Türkmen et al., 2004). HA has a positive effect on various plants, but how HA promotes plant growth and salt tolerance has remained elusive.
HA can bind to and chelate Na. Thus, we performed ICP-MS analysis to determine whether or without 250 mM NaCl in the absence or presence of HA (86 or 860 mg L\(^{-1}\)) and grown for another 7 days. (A) Pictures taken at 7 days after treatments. (B) Fresh weight of plants shown in A was measured and relatively calculated compared to the fresh weight of plants grown in the absence of NaCl. Data are means ± SE (n = 3). Significant differences are shown as asterisks (Student’s t-test, *P < 0.05; **P < 0.01; ***P < 0.001).

Fig. 1. HA confers salt stress tolerance in a dose-dependent manner. Five-day-old WT seedlings were transferred to 1/2 MS with or without 250 mM NaCl in the absence or presence of HA (86 or 860 mg L\(^{-1}\)) and grown for another 7 days. (A) Pictures taken at 7 days after treatments. (B) Fresh weight of plants shown in A was measured and relatively calculated compared to the fresh weight of plants grown in the absence of NaCl. Data are means ± SE (n = 3). Significant differences are shown as asterisks (Student’s t-test, *P < 0.05; **P < 0.01; ***P < 0.001).

HA reduces salt sensitivity in the shoots of hypersensitive mutants

HA possesses many ionizable sites that allow it to bind to and chelate various cations, including Na\(^{+}\) (Tunstall, 2005). Thus, we performed ICP-MS analysis to determine whether HA can bind to and chelate Na\(^{+}\) ions in agar medium. Interestingly, plants grown on medium containing either NaCl or HA plus NaCl absorbed similar amounts of Na\(^{+}\) ions, suggesting that HA does not inhibit the uptake of NaCl by plants in culture (Supplementary Fig. S1). To explore the molecular mechanism by which HA confers salt tolerance in Arabidopsis, we investigated the possible roles of two major sodium transporters, SOS1 and HKT1, using overexpression and mutant plants. Loss-of-function mutants of sos1-1 and hkt1-1 displayed opposite phenotypes, i.e., hypersensitivity and increased tolerance to salt, respectively. In addition, overexpression of SOS1(SOS1-OX) in Arabidopsis displayed salt-tolerance while that of HKT1(HKT1-OX) was sensitive to salt (Ali et al., 2012; Kim et al., 2013). We transferred 5-day-old seedlings to MS agar plates containing 100 mM NaCl (for the salt sensitivity assay) with or without 860 mg L\(^{-1}\) HA and photographed them at 8 days after transfer to salt medium. Previous studies showed that the shoots of sos1-1 and HKT1-OX plants are extremely sensitive to 100 mM NaCl treatment (Ali et al., 2012; Kim et al., 2013). Consistent with previous reports, both the sos1-1 mutant and HKT1-OX showed highly chlorotic leaves under salt stress compared to WT and the other lines (Fig. 2A), as well as a significant reduction in the fresh weight of shoots under salt treatment (Fig. 2B). However, when HA was provided to the medium together with salt, the salt sensitivity of HKT1-OX was highly recovered, especially in shoots (Fig. 2A) and the fresh weight of shoots of HKT1-OX was 2.3-fold higher than that treated with salt alone (Fig. 2B). However, the reduced fresh weight treated by salt alone in other seedlings (WT, SOS1-OX, sos1-1, and hkt1-1) was recovered in a range of 1.36 to 1.56-fold by HA supplementation to salt medium (Fig. 2B).

HA rescues salt-induced root growth inhibition in HKT1-OX

HA promotes lateral root formation by exhibiting auxin-like activity in maize, tomato, and Arabidopsis (Dobbss et al., 2007; Trevisan et al., 2010; Zandonadi et al., 2007). By contrast, salt stress reduces root growth, including primary and lateral root growth, although primary root growth is more severely affected than lateral root growth (Julkowska et al., 2014). In addition, we have previously reported that primary root growth of sos1-1 and HKT1-OX was dramatically reduced by salt stress (Ali et al., 2012; Kim et al., 2013). To determine whether salt stress-induced inhibited root growth could be rescued by the application of HA, we transferred 5-day-old WT, SOS1-OX, sos1-1, HKT1-OX, and hkt1-1 seedlings to MS medium containing 100 mM NaCl with or without 860 mg L\(^{-1}\) HA and grew the plants vertically for an additional 9 d. Primary root growth by HA treatment in all plants did not show significant differences compared to that under control condition (Fig. 3). Both sos1-1 and HKT1-OX roots were hypersensitive to 100 mM NaCl, whereas WT, SOS1-OX, and hkt1-1 roots were less sensitive to this treatment (Fig. 3A). The root length of HKT1-OX increased 1.5-fold in the presence of HA plus NaCl compared to salt treatment alone, whereas the root lengths of the remaining plants did not significantly differ between treatments (Fig. 3B). Root and shoot tissues from both sos1-1 and HKT1-OX exhibited different levels of salt sensitivity under HA application: the salt sensitivity in roots was not highly influenced by HA application, whereas the salt sensitivity in shoots was markedly reduced by this treatment (shoot vs. root in sos1-1, x1.56 vs. x0.94; in HKT1-OX, x2.3 vs. x1.5) (Figs. 2B and 3B). These results suggest that HA improves salt tolerance by excluding Na\(^{+}\) from the shoot, while the excess Na\(^{+}\) ions still remain in the root. Indeed, the ability to exclude Na\(^{+}\) from the shoot has a major effect on salinity tolerance in several crop plants (Meller and Tester, 2007; Munns, 1993; 2002; Tester and Davenport, 2003). In addition, these results suggest that the Na\(^{+}\) transporter AtHKT1:1 plays a role in increasing salt tolerance in response to HA treatment.
HA increases the efficiency of Na\(^+\) reabsorption from xylem vessel to xylem parenchyma cells in the root stelae.

Na\(^+\) ions that reach the xylem by passing through several barriers in the root under salinity stress are transported to the shoot. Altering specific Na\(^+\) transport processes in specific cell types can reduce Na\(^+\) accumulation in the shoot, which is quite harmful to higher plants (Møller et al., 2009). To examine how HA enhances salinity stress tolerance in the shoot under salt stress conditions, we monitored the long-distance transport of Na\(^+\) in the root after short-term salinity treatment (Fig. 4). Five-day-old WT seedlings were treated with 100 mM NaCl in the absence or presence of HA (860 mg L\(^{-1}\)) for 14 h. We visualized the distribution of sodium using the fluorescent, sodium-specific dye CoroNa-Green AM. After salt treatment, the intensity of fluorescent staining in WT roots became stronger than in the non-treated control, as shown previously (Oh et al., 2010) (Fig. 4A). In WT, when HA was added to salt-containing medium, the intensity of
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Fig. 4. HA increases the Na$^+$ reabsorption into xylem parenchyma cells by HKT1 under salinity stress. Five-day-old WT, HKT1-OX, and hkt1-1 seedlings were treated with 100 mM NaCl with or without 860 mg L$^{-1}$ HA for 14 h. Seedling roots were stained with 5 μM CoroNa-Green AM (green) and 1 mg ml$^{-1}$ propidium iodide (red). The meristematic root tip region (A) and root elongation region (B) were observed under a confocal microscope. Arrows in right side of (B) indicate the direction of water transport from roots (R) to shoots (S). More than five plants were included for one treatment. Propidium iodide stains the cell wall and damaged cells. Abbreviations: EP, epidermis; CO, cortex; XYP, xylem parenchyma cell. Bars = 50 μm.

fluorescent staining moved upward to the elongation zone, while the intensity in the root-tip zone was reduced (Fig. 4A). Like WT, the intensity of fluorescent staining in the root tip of HKT1-OX plants also decreased, whereas in hkt1-1, there was no difference in staining pattern between the NaCl and HA + NaCl treatment groups (Fig. 4A). We then monitored fluorescent staining in the root elongation zone, finding that HA treatment increased the intensity of this staining in WT (Fig. 4B). These results suggest that HA affects Na$^+$ absorption in the root elongation zone. HKT1-type transporters can reabsorb Na$^+$ ions from the xylem stream to xylem parenchyma in the root elongation zone, resulting in reduced Na$^+$ transport to the shoot (Rubio et al., 1995). Therefore, we investigated whether the accumulation of Na$^+$ in the root elongation zone upon HA treatment functions through HKT1. No significant difference in fluorescence intensity was detected between the tip vs. elongation zone of the root in the hkt1-1 root stele in the absence or presence of HA (Fig. 4). However, HA treatment increased the level of fluorescence from CoroNa-Green in the root elongation zone of HKT1-OX seedlings, especially in xylem parenchyma cells (Fig. 4B). These results suggest that HKT1 is required to increase HA-mediated Na$^+$ accumulation in the root elongation zone.

HA blocks salt-mediated HKT1 protein degradation
To investigate the possibility that HA enhances the unloading of Na$^+$ to xylem parenchyma cells, we first examined whether HA affects HKT1 protein levels using HKT1-OX plants (GFP-fused). Endogenous HKT1 mRNA is circadian clock-controlled and diurnally oscillating with peak during the day and trough during the night (Supplementary Fig. 52). HKT1

Fig. 5. HA positively regulates the stability of HKT1 protein under salinity stress. Nine-day-old HKT1-OX (GFP fused) plants were treated with 100 mM NaCl and/or 860 mg L$^{-1}$ HA. Whole plant, shoot and root parts were harvested separately at 0, 6 and 12 h after treatments. HKT1 protein from total extracts was evaluated by immunoblot analysis with anti-GFP antibody. A loading control is shown using comassie-brilliant blue staining (CBB) or immunoblot analysis by anti-HEAT-SHOCK PROTEIN90 (HSP90) antibody (lower panel). Experiments were repeated three times with similar results.
protein in whole plant of HKT1-OX was also accumulated during the day (6 h and 12 h) under control condition (Fig. 5, top panel). However, HKT1 protein in whole plants was rapidly degraded by NaCl, and its destabilization was fully blocked when HA was added to the medium together with NaCl. Interestingly, HA did not affect to transgene HKT1 transcripts in HKT1-OX with increase either in NaCl or HA+NaCl treatment, suggesting that HA stabilizes HKT1 protein in post-transcriptional levels (Supplementary Fig. S3). Second, we investigated whether HKT1 protein levels are differentially regulated by NaCl and/or HA in shoots and roots. In both plant parts, the HKT1 levels were dramatically reduced by 6 h and/or 12 h of 100 mM salt treatment, whereas the salt-induced degradation of HKT1 was impaired by HA treatment (Fig. 5). The root is the first organ to absorb Na+ ions from the soil. These results suggest that salt triggers the degradation of HKT1 in whole roots and shoots, but HA-induced stabilization of HKT1 causes Na+ to be unloaded from xylem to parenchyma cells in the root elongation zone, which consequently stabilizes HKT1 in the shoot. We also examined the effects of HA on SOS1 protein stability, and result showed that HA does not affect to SOS1 protein level (Supplementary Fig. S4). Therefore, HA-induced salt tolerance appears to be related to the modulation of HKT1 activity in roots.

HA is a component of humus. This heterogeneous, relatively large, high molecular weight organic complex, which ranges in color from brown to black, is amorphous, hydrophilic, molecularly flexible, and composed of polyelectrolyte compounds. HS contains a large number of complex humate molecules. Humate can bind to positive metal cations such as iron (Fe2+), copper (Cu2+), zinc (Zn2+), calcium (Ca2+), manganese (Mg2+), and magnesium (Mg2+) (Tunstall, 2005). Salinity (NaCl) stress induces Ca2+ influx: the elevated levels of cytosolic free Ca2+ serve as a second messenger (Tracy et al., 2008). To investigate the positive effects of HA on HKT1 stability/activity due to the Ca2+ chelating effect of humate on plants under salt stress conditions, we used various pharmacological agents to inhibit Ca2+ release and flux (Fig. 6). Nicotinamide inhibits cyclic ADP-ribose (cADPR), a potent calcium release agent, while GdCl3 blocks stretch-activated cation channels, thereby functioning as a Ca2+ influx inhibitor, and U73122 inhibits phospholipase C, thus acting as an inhibitor of Ca2+ efflux (Dodd et al., 2007; Tracy et al., 2008). In the absence of salt, HKT1 protein abundance increased by nicotinamide, U73122, or GdCl3 treatment compared to the control condition (Fig. 6). In the presence of 50 mM nicotinamide or 1 mM GdCl3, HKT1 was stabilized against NaCl-induced degradation under salt stress conditions. However, treatment with 5 μM U73122 (to inhibit vacuolar calcium release) failed to restore HKT1 protein to normal levels in the presence of NaCl-induced degradation (Fig. 6). These results suggest that the increase in cytosolic Ca2+ levels plays a role in NaCl-mediated HKT1 protein destabilization upon salt stress.

**DISCUSSION**

In this study, we demonstrated that HA treatment improves plant growth and reduces plant sensitivity to salinity stress. HA can function as a growth regulator by regulating hormone levels, plant growth, and stress responses (Piccolo et al., 1992; Serenella et al., 2002). HA treatment reduces the toxicity of salt in strawberry, maize, and garden cress seedlings (Masciandaro et al., 2002; Pilanal and Kaplan, 2003; Türkmen et al., 2004). Here, we showed that HA application also increases plant growth and enhances salt stress tolerance in Arabidopsis (Figs. 1-3; Cha et al., 2017).

Treatment with 1 g L−1 HA has a positive effect on plant growth under saline soil conditions (Türkmen et al., 2004), which is consistent with our observation that 860 mg L−1 HA caused a significant increase in seedling survival, even under saline conditions (Fig. 1). David et al. (1994) reported that HS promotes plant growth and mineral nutrient uptake due to improved root system development. In addition, HS influences protein synthesis in higher plants (Carletti et al., 2008). Na+ strongly accumulated in both shoots and roots after the addition of NaCl, which is consistent with the findings for various barley cultivars exposed to 150 mM NaCl (Kamboj et al., 2015). HKT transporters are thought to be intricately involved in Na+ uptake and salt toxicity in plants (Ali et al., 2012; Misier et al., 2002a; 2002b; Uozumi et al., 2000; Xue et al., 2011). AtHKT1.1 localized to the plasma membrane of xylem parenchyma cells mediates the removal of Na+ from xylem vessels during salinity stress (Sunarpi et al., 2005).
When AtHKT1;1 was overexpressed in the root stele, including pericycle and xylem parenchyma cells, by the enhancer trap method, inward movement of Na\(^+\) increased in the targeted cells, resulting in improved salinity tolerance (Møller et al., 2009). All of these findings are consistent with our hypothesis that HA enhances the reabsorption of Na\(^+\) into xylem parenchyma cells by HKT1;1 and reduces the net flow of Na\(^+\) into the shoot (Fig. 4). Therefore, HA functions as a biostimulant that could potentially be used as a genetic and agricultural tool to improve the stability of HKT1 under salt stress conditions.

HA treatment improves ion uptake and mineral nutrition in plants (Trevisan et al., 2010). Asik et al. (2009) determined that both soil and foliar application of small amounts of HS increase nutrient uptake in wheat under salt stress conditions. Murat et al. (2011) reported that adding humus to the soil increases nutrient uptake in plants under 45 and 60 mM NaCl treatment. Indeed, the protective effect of HA on plants under salt stress has been demonstrated in many cereals, such as maize and wheat (Aydin et al., 2012; Khaled and Fawzy, 2011). In the current study, we showed that HA treatment relieved the growth inhibition induced by NaCl via the stabilization of HKT1 protein (Fig. 5).

Higher calcium levels in soil protect the cell membrane from the negative effects of salinity (Busch, 1995). Kwon et al. (2009) demonstrated that the addition of 60 mM NaCl to growth medium increases Na\(^+\) uptake in plants, as expected, but supplemental Ca\(^+\) reverses this effect. Ca\(^+\) also reduces the translocation of Na\(^+\) to the shoot and retains this ion in the roots. Under particular conditions, HS can stimulate plant growth, including increased plant height and dry/fresh weight (Blanchet, 1958; Guminiski, 1968). These findings are consistent with our hypothesis that influx of the secondary messenger Ca\(^+\) and cytosolic Ca\(^{2+}\) participate in NaCl-mediated destabilization of HKT1 protein under salt stress (Fig. 6). Several studies have confirmed the hypothesis that HS has a direct effect on plant physiology, specifically concerning lateral root development (Canellas et al., 2002; Carletti et al., 2008; Zandonadi et al., 2007). More recently, the auxin-like activity of HS in promoting lateral root development was investigated in the model plant Arabidopsis using a combination of genetic and molecular approaches (Trevisan et al., 2009).

In conclusion, this study demonstrates that HA plays an important role in improving salt tolerance by regulating the sodium transporters HKT1 in post-transcriptional levels. It is difficult to monitor changes in protein abundance after HA treatment due to the complex network of signaling pathways. To the best of our knowledge, HKT1 is the first protein whose levels were found to change in Arabidopsis after exposure to HA under salt stress treatment. Further research is needed to elucidate the specific functions and regulatory mechanisms underlying the effects of HA on HKT transporters and its role in salinity tolerance.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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