Molecules and Cells

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, 2008). 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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(K^+) and inhibit the activity of several enzymes (Murguia et al., 1995; Wu et al., 1996). High cytosolic K^+/Na^+ ratios in the shoot are critical for salt tolerance in glycophytes, which can only tolerate relatively low salt concentrations (Blumwald, 2000; Gorham et al., 1987; 1990; Hauser and Horie, 2010; Ren et al., 2005; Sunarpi et al., 2005; Yamaguchi and Blumwald, 2005).

When a Na^+ ion enters the plant root, it can be selectively transported through three independent biological membranes: the plasma membrane in epidermal cells, the vacuolar membrane in root and shoot cells, and the plasma membrane in xylem parenchyma cells (Horie et al., 2012). Accumulated cytosolic Na^+ can be removed by efflux systems such as Na^+/H^+ antiporters, which transport Na^+ across the plasma membrane (Apse et al., 1999; Blumwald, 2000; Pardo et al., 2006), as well as the Salt-Overly Sensitive (SOS) pathway (Park et al., 2016). This pathway is composed of three SOSs, including the calcium binding protein SOS3, which senses salt-triggered increases in calcium levels and binds to the Ser/Thr protein kinase SOS2. The SOS2:SOS3 complex is translocated to the plasma membrane through the N-terminal myristoylation of SOS3, where it activates the Na^+/H^+ antiporter SOS1 by phosphorylating its C-terminus (Guo et al., 2001; 2004; Halfter et al., 2000; Ishitani et al., 2000; Liu and Zhu, 1998; Liu et al., 2000; Qiu et al., 2002). SOS3 possessing three potential Ca^{2+}-binding sites that recognize the cytosolic calcium ion (Ca^{2+}) signal elicited by salt stress (Ishitani et al., 2000; Liu and Zhu, 1998; Moncrief et al., 1990). Exogenous application of calcium (Ca^{2+}) enhances salt tolerance in glycophytic plants (Läuchli, 1990), likely due to the SOS3-mediated activation of the SOS pathway at the epidermis.

Na^+ that enters the root cell and is transported to leaf tissue must be compartmentalized in the vacuole to avoid the cytosolic accumulation of toxic Na^+. This process is mediated by the vacuolar Na^+/H^+ antiporter, NHX, which moves Na^+ into the vacuole in exchange for H^+ (Blumwald et al., 2000). This process might also be regulated by the SOS signaling pathway (Qiu et al., 2004).

Na^+ reabsorption occurs from the xylem stream to the surrounding tissues. This process, which reduces the net flow of Na^+ into shoots (Läuchli, 1984; Lacan and Durand, 1996), is mediated by H^+ influx carriers, particularly HIGH-AFFINITY POTASSIUM (K^+) TRANSPORTER (HKT) family members (Horie et al., 2001). HKTs are involved in the retrieval of Na^+ from the xylem to reduce its transport to/accumulation in the shoot in several plant species (Davenport et al., 2007; Mäser et al., 2002a; Ren et al., 2005; Sunarpi et al., 2005). Plant HKTs, which function as Na^+ influx transporters, are divided into two subclasses based on protein sequence and ion selectivity (Mäser et al., 2002b). In rice, a Ser residue with high selectivity for Na^+ over K^+ at the first pore-loop domain is conserved in class 1 (HKT1) family members, while a Gly at the same position in class 2 (HKT2) members is permeable to both Na^+ and K^+ (Horie et al., 2001). *Arabidopsis thaliana* contains a single copy of *HKT1;1*, encoding a class 1-type protein that exhibits high specificity to Na^+ when expressed in *Xenopus* oocytes and yeast (Uozumi et al., 2000; Xue et al., 2011). HKT1;1 plays an important role in limiting Na^+ accumulation in shoot tissues by governing the net flow of Na^+ through long-distant transport *via* the stele at the plasma membranes of xylem parenchyma cells (Berthomieu et al., 2003; Davenport et al., 2007; Horie et al., 2006; Møller et al., 2009; Sunarpi et al., 2005). Total Na^+ levels in roots increase due to an increase in Na^+ retrieval from the transpiration stream through enhanced expression of *AtHKT1;1* in stelar root cells (Møller et al., 2009). Thus, HKT1 is an important but elusive target of genetic engineering to regulate Na^+ levels in plants.

Humin, which is composed of humic and fulvic acids (commonly known as humic substances [HS]), is a complex supramolecular association of abiotically transformed biomolecules that are released into soils after cell lysis (Orsi, 2014). Humin influences plant growth both directly and indirectly by functioning as a major source of organic compounds in soil (Sangeetha et al., 2006). These substances can improve soil properties such as aggregation, aeration, permeability, water holding capacity, micronutrient transport, and availability. Furthermore, the direct uptake of HS into plant tissues results in diverse biochemical outcomes (Arancon et al., 2006; Nardi et al., 2002; Selim et al., 2009; Tan, 2003). Humic acid (HA) improves plant development by regulating metabolic and signaling pathways by acting directly on certain targets in diverse physiological processes (Quaggiotti et al., 2004; Trevisan et al., 2010). The application of HA to plants increases cell membrane permeability, oxygen uptake, respiration, photosynthesis, phosphate uptake, and root elongation (Cacco and Dell Agnolla, 1984; Russo and Berlyn, 1990; Vaughan, 1974). 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 (Wang and Mulligan, 2009). 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-*gl*), *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℃.

Salt tolerance assay

Five-day-old WT Arabidopsis seedlings were transferred to

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., 2004; Meier 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 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 systhesis 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'-TCTTCTTGGAGTGACGGTGC-3'; Rev: 5-AACGATCCAACCAACTTCTC-3') and *AT5G12240* as an internal control (For: 5'-AGCGGCTGCTGAGAAGAAAGT-3'; Rev: 5'-TCTCGAAAGCCTTGCAAAATCT-3') by TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROS) kit (Enzynomics) 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 $GdCl_3$ (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_2O:H_2SO_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.

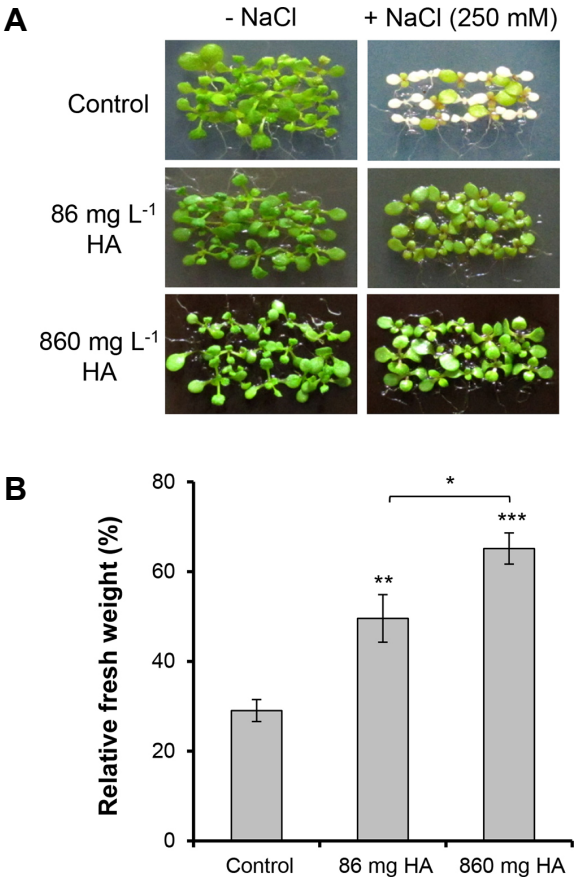

Fig. 1. HA confers salt stress tolerance in a dose-dependent manner. Five-day-old WT seedlings were transferred to 1/2 MS with or without 250 mM NaCl in the absence or presence of HA (86 or 860 mg L^{-1}) and grown for another 7 days. (A) Pictures taken at 7 days after treatments. (B) Fresh weight of 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 in 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*, ×1.56 vs. ×0.94; in *HKT1-OX*, ×2.3 vs. ×1.5) (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 (Møller 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.

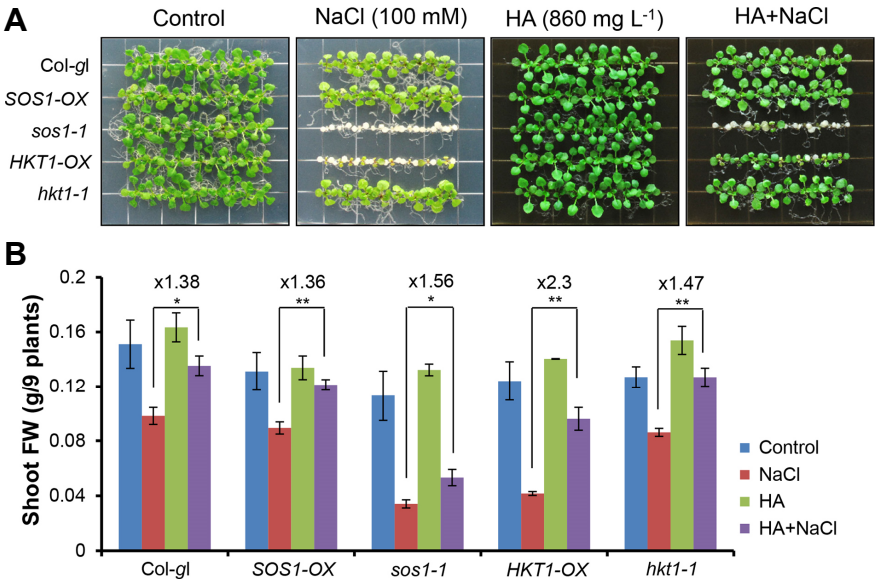

Fig. 2. HA reduces salt-sensitivity in shoots. (A) Seeds of WT, *SOS1-OX*, *sos1-1*, *HKT1-OX*, and *hkt1-1* were stratified on 1/2 MS agar plates. Five-day-old seedlings were transferred to MS media supplemented with 100 mM NaCl or 860 mg L^{-1} HA, respectively. Photographs were taken at 8 days after treatments on the media. (B) Fresh weight of shoots. The fresh weight (FW) of shoots was measured at 8 days after treatments. Three biological replicates were averaged and statistically significant differences between the NaCl treatment and HA plus NaCl treatment are indicated by asterisks (Student's *t*-test, $*P < 0.05$; $**P < 0.01$). The numbers above the bars indicate the ratio of FW by NaCl and HA plus NaCl (NaCl/HA+NaCl).

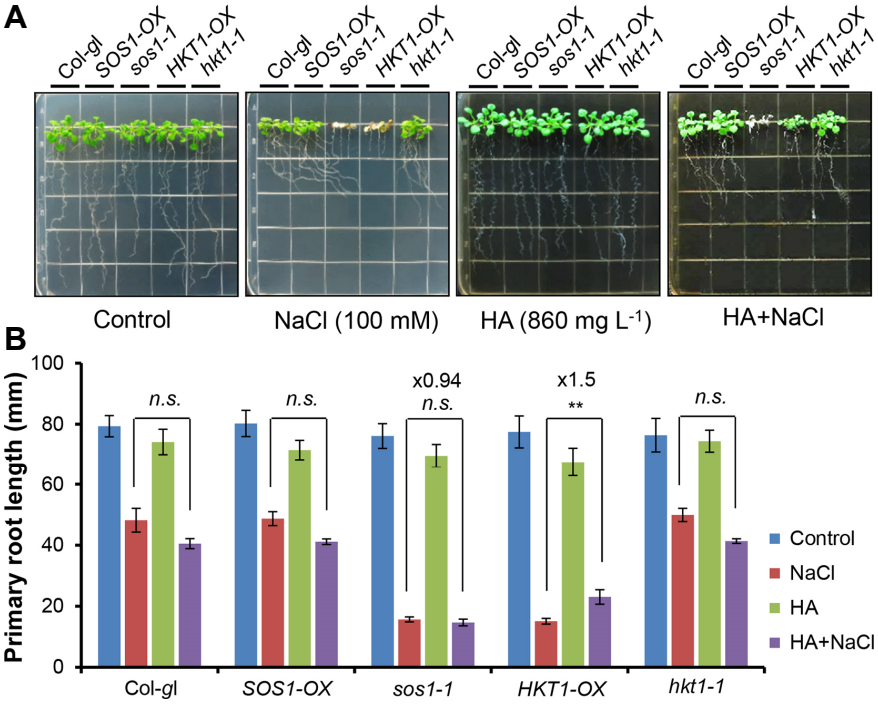

Fig. 3. HA rescues NaCl-induced inhibition of root growth in *HKT1-OX* plants. Seedlings were prepared and treated as mentioned in Fig. 2. (A) The plants were grown vertically on subjected media for 9 days after treatments. (B) Primary root length was measured at 9 days after treatments. Three biological replicates were averaged and statistically significant differences between the NaCl treatment and HA plus NaCl treatment are indicated by asterisks (Student's *t*-test, $**P < 0.01$; *n.s.*, no significant differences). The numbers above the bars indicate the ratio of primary root length by NaCl and HA plus NaCl (NaCl/HA+NaCl).

HA increases the efficiency of Na^+ reabsorption from xylem vessel to xylem parenchyma cells in the root stele

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

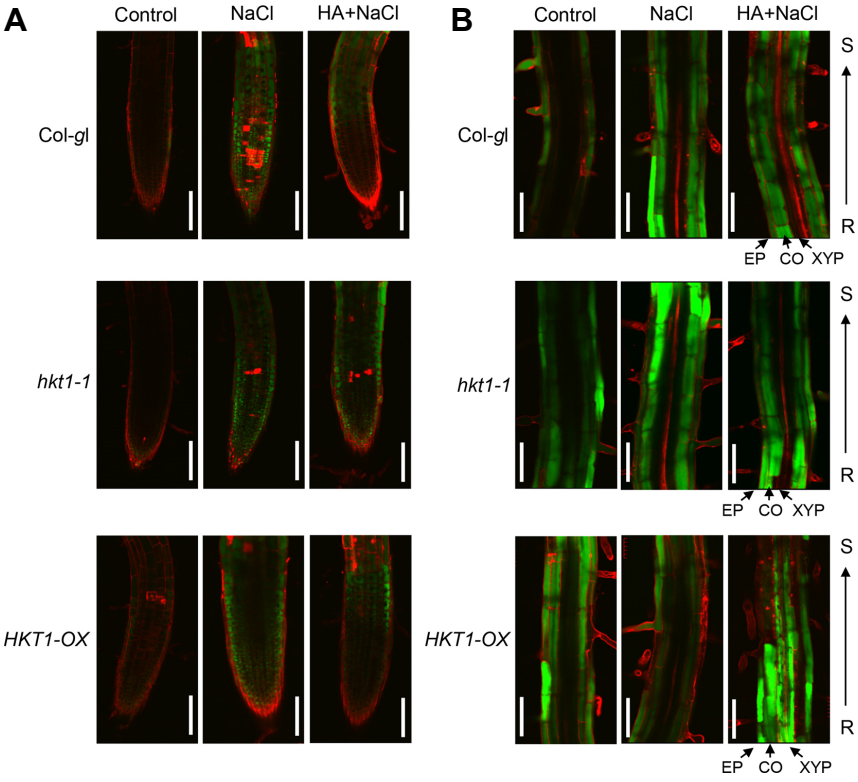

Fig. 4. HA increases the Na^+ reabsorption into xylem parenchyma cells by HKT1 under salinity stress. Five-day-old WT, *HKT1-OX*, and *hkt1-1* seedlings were treated with 100 mM NaCl with or without 860 mg L^{-1} HA for 14 h. Seedling roots were stained with 5 μM CoroNa-Green AM (green) and 1 mg ml^{-1} 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 florescence 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

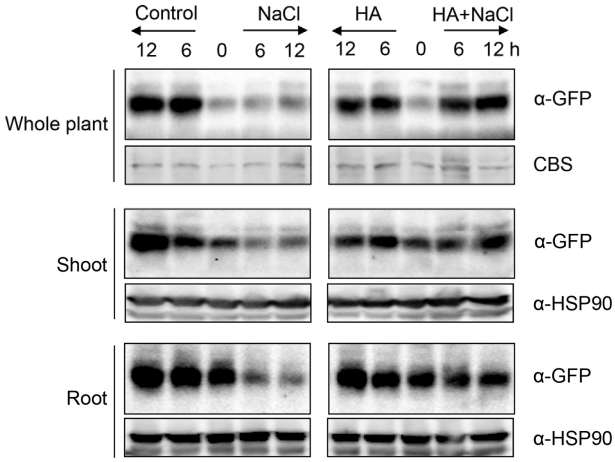

Fig. 5. HA positively regulates the stability of HKT1 protein under salinity stress. Nine-day-old *HKT1-OX* (GFP fused) plants were treated with 100 mM NaCl and/or 860 mg L^{-1} HA. Whole plant, shoot and root parts were harvested separately at 0, 6 and 12 h after treatments. HKT1 protein from total 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.

and trough during the night (Supplementary Fig. S2). HKT1

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 to confirm the effect of HA under salt stress. 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.

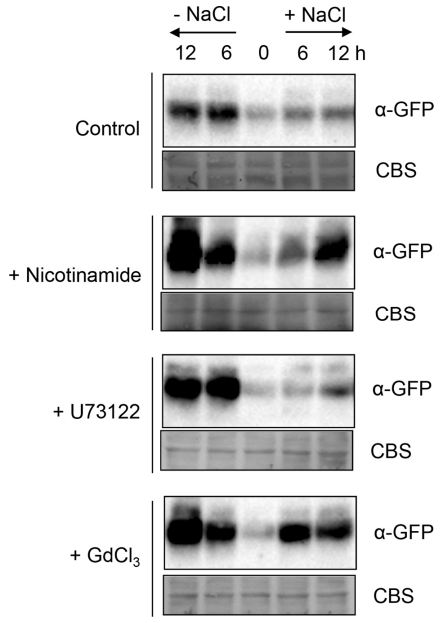

Fig. 6. NaCl-induced cytosolic calcium enrichments destabilize HKT1 protein. Nine-day-old seedlings were subjected to 50 mM nicotinamide (an inhibitor for calcium release by cADPR), 1 mM $GdCl_3$ (a calcium influx inhibitor), or 5 μM U73122 (an inhibitor for calcium released from vacuolar) with or without 100 mM NaCl for 6 or 12 h after treatments. Immunoblot analysis was carried using anti-GFP antibody to detect GFP-fused HKT1 protein. The CBB-stained membrane is shown as a loading control (lower panel). Two or more independent experiments showed similar results.

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 polyelectrolytic compounds. HS contains a large number of complex humate molecules. Humate can bind to positive metal cations such as Iron (Fe^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}), calcium (Ca^{2+}), manganese (Mg^{2+}), and magnesium (Mg^{2+}) (Tunstall, 2005). Salinity (NaCl) stress induces Ca^{2+} influx; the elevated levels of cytosolic free Ca^{2+} serve as a second messenger (Tracy et al., 2008). To investigate the positive effects of HA on HKT1 stability/activity due to the Ca^{2+} chelating effect of humate on plants under salt stress conditions, we used various pharmacological agents to inhibit Ca^{2+} release and flux (Fig. 6). Nicotinamide inhibits cyclic ADP-ribose (cADPR), a potent Ca^{2+}-releasing agent, while $GdCl_3$ blocks stretch-activated cation channels, thereby functioning as a Ca^{2+} influx inhibitor, and U73122 inhibits phospholipase C, thus acting as an inhibitor of Ca^{2+} efflux (Dodd et al., 2007; Tracy et al., 2008). In the absence of salt, HKT1 protein abundance increased by nicotinamide, U73122, or $GdCl_3$ treatment compared to the control condition (Fig. 6). In the presence of 50 mM nicotinamide or 1 mM $GdCl_3$, 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 Ca^{2+} 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; Mäser 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 Fawy, 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^{2+} reverses this effect. Ca^{2+} 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; Guminski, 1968). These findings are consistent with our hypothesis that influx of the secondary messenger Ca^{2+} 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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