2021 S.T. Yau High School Science Award (Asia)

Research Report

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Title of Research Report

Heterologous Expression of a Mangrove K^+ Transporter, *AoHKTI* increases salt tolerance of *Arabidopsis thaliana*

Date

3rd August 2021

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Abstract

Saline soil limits arable land and reduces agricultural productivity. Mangrove species including *Avicennia officinalis* have adapted to thrive in high salt environments, as they possess membrane transporters that maintain cellular ion homeostasis. In this study, we identified and characterized a High-Affinity Potassium Transporter (HKT) homolog *AoHKT1* through heterologous expression in *Arabidopsis thaliana*. Germination and root length assays in response to salt stress were conducted on *Arabidopsis* seedlings, and the leaf surface areas and FW/DW ratios of 1-month old transgenic *Arabidopsis* plants with *AoHKT1* were assessed compared to the *athkt1;1* mutant. We found that under high NaCl stress conditions, heterologous overexpression of AoHKT1 in particular was able to improve *A. thaliana* root length and leaf surface area significantly, while *AoHKT1* expression in *athkt1;1* mutant background was able to improve germination rate of *A. thaliana* seedlings. Confocal images from the green fluorescence of *GFP-AoHKT1* in tobacco showed that *AoHKT1* was localized to the Golgi Apparatus. Yeast mutants without functional TRK proteins (K^+ influx system) complemented with *AoHKT1* showed enhanced growth under low potassium conditions. Similar to the functional adaptations found in other halophytes such as *Thellungiella salsuginea*, our work suggests that AoHKT1 is a membrane potassium transporter with a role in reducing the cytosolic Na^+/K^+ ratio, making this gene a potential candidate for developing salt-tolerant crops.

Keywords: Salt-Tolerance, Crop Yield, Na^+/K^+ ratio, AtHKT1, AoHKT1, *Arabidopsis*, High-Affinity Potassium Transporter, Leaf Surface Area Assay, Halophyte, *Avicennia Officinalis*, Mangrove, Yeast Complementation Assay, Subcellular Localization, Root Length Assay, Germination Assay.

Acknowledgement

We would like to thank our research mentor Dr. Pannaga Krishnamurthy for her invaluable advice and support throughout this research project, and we would like to thank Dr. Huang Sinong for her timely check-ins and feedback on the research manuscript.

Commitments on Academic Honesty and Integrity

We hereby declare that we

1. are fully committed to the principle of honesty, integrity and fair play throughout the competition.
2. actually perform the research work ourselves and thus truly understand the content of the work.
3. observe the common standard of academic integrity adopted by most journals and degree theses.
4. have declared all the assistance and contribution we have received from any personnel, agency, institution, etc. for the research work.
5. undertake to avoid getting in touch with assessment panel members in a way that may lead to direct or indirect conflict of interest.
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7. observe the safety regulations of the laboratory(ies) where we conduct the experiment(s), if applicable.
8. observe all rules and regulations of the competition.
9. agree that the decision of YHSA(Asia) is final in all matters related to the competition.

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INTRODUCTION

Soil salinity is prominent abiotic stress that majorly reduces crop yield (Wu, et al. 2017). There have been continuous efforts to identify and characterize the genetic variation driving salinity tolerance in plants, especially in *A. thaliana* and *O. sativa*. In saline soils, toxicity primarily arises from the high concentrations of Na^+, which disturbs the osmotic and ion homeostasis of the plant. Specifically, it is difficult to increase the uptake of K^+, an essential macronutrient. Na^+ influx into the cell induces the efflux of K^+ and in some plants, induces the efflux of Ca^{2+} as well leading to an imbalance in the cytosolic Na^+/K^+ ratio. This ultimately leads to reduced cellular metabolism and inhibited growth (Hasegawa, et al. 2001).

It is widely recognized that the three major adaptations against salinity stress are; exclusion and ultrafiltration of Na^+ at the roots, vacuolar compartmentalization of Na^+, and regulation of Na^+ flux across the plasma membrane to reduce Na^+ accumulation in younger leaves and increased recirculation of Na^+ towards the roots via phloem loading (Rogriguez-Navarro et al. 2005). These adaptations are more pronounced in halophytes like *Thellungiella salsuginea* (Yun, et al. 2013) and the mangrove *Avicennia officinalis* compared to glycophytes like *A. thaliana*. Specialized epidermal bladder cells in *Avicennia* regulate the secretion of Na^+ from leaf tissue to maintain K^+/Na^+ ratio homeostasis. *HKT*s are a diverse group of genes encoding ion transporters whose major function in higher plants is to regulate intracellular K^+ and Na^+ homeostasis (Zhang, et al 2018) by transporting these ions across the plasma membrane. HKTs share their function with fungal TRK high-affinity potassium transporters. They are characterized by different species such as *AtHKT1* in model organism *A. thaliana* and *OsHKT1* in rice, *O. Sativa*, a crop plant. They are usually expressed in the xylem parenchyma cells in various organs (An, et al. 2017) and primarily function in unloading Na^+ from the leaves and xylem in the roots.

HKT transporters are classified into two distinct groups based on amino acid polymorphisms. Subclass 1 contains a serine in the first p-loop of the four-pore domains and selectively transports Na^+ (for example, *Arabidopsis* AtHKT1). A notable exception is *T. salsuginea* (Yun, et al. 2013), which contains TsHKT1;2 which acts as an efficient K^+ transporter and is upregulated dramatically upon salt-stress to import K^+. Subclass 2, mostly occupied by monocot crop plants such as wheat and rice have a glycine in place of the serine and usually acts as a symporter for Na^+ and K^+. These proteins can function as Na^+ importers (Na^+ can substitute some functions of K^+ in low potassium environments) under low potassium and a study has reported that downregulation can confer salt resistance to wheat (Laurie, et al. 2002). Recent evidence (Lan, et al. 2010) suggests that Subclass 2 proteins may play a role in Ca^{2+} homeostasis as well, and generally exhibit much less Na^+ selectivity.

While halophytic adaptations to salinity stress have been well-characterized, there is much research to be done to elucidate its genetic basis, including the role of HKT transporters in halophytes. This work is crucial because heterologous expression of these halophyte HKT transporters has potential to improve salt tolerance in crop plants, including *O. sativa* (Hamamoto, et al. 2014). In the present study, we have identified and characterized a HKT gene homolog, *AoHKT1* from the mangrove *Avicennia* through heterologous expression in *A. thaliana*. The subcellular localization and phenotypic response to varying concentrations of NaCl and KCl treatment was studied. Furthermore, we found that *AoHKT1* can complement yeast mutants with the deletion of genes encoding TRK1 and TRK2 (membrane K^+ transporters). To our knowledge, this is the first functional characterization of a mangrove HKT protein channel, and our study suggests that *AoHKT1* is a membrane K^+ importer with a key role in cytosolic homeostasis of the Na^+/K^+ ratio, suggesting that *AoHKT1* is a promising candidate gene for the generation of salt-tolerant crops for improved productivity in saline soils.

RESULTS

Sequence similarity and phylogenetic tree of HKT homologs compared to *Avicennia AoHKT1*

Prior to functional characterization of the *AoHKT1* cation transporter, the similarity of its deduced amino acid sequence compared to homologs in other species was analyzed. A phylogenetic tree (**Fig. 1a)** was constructed based on standard protein-protein BLAST search in the GenBank database. Included in the phylogenetic tree is the model plant *Arabidopsis thaliana*, the crop plant *Triticum aestivum* (wheat), and the halophyte *Thellungiella salsuginea*. *TsHKT1* from *T. salsuginea* reported the highest similarity to *A. thaliana*, which is unsurprising since *T. salsuginea* is a close relative of *A. thaliana* (Yun, et al. 2013). Functional homologies are not well-represented in the phylogenetic tree, as the halophytes

Avicennia, Thellungiella, and *Saueda* are not close relatives of each other.

AoHKT1 expression in shoots was induced by salt stress

To examine the expression profile of *AoHKT1* under salt stress in *Avicennia* seedlings, RTq-PCR analysis was conducted at varying time intervals within 48 hours after salt stress of 0.5M NaCl. In the leaves, an increase in the expression of *AoHKT1* was observed after 4h and the increase was maintained up to 24 hours after salt treatment, with the highest increase observed after 12 hours of salt treatment (**Fig. 1b**). In the roots, there was no significant increase in the expression of the *AoHKT1* gene until 24 hours of salt treatment, where a slight increase in expression was observed. Overall, the results suggest that *AoHKT1* might be playing a prominent role in the shoot to increase salt-tolerance under saline conditions. The results used for the expression profile of *AoHKT1* was obtained from unpublished data from the lab.

AoHKT1 was localized to the Golgi Apparatus under normal conditions

HKT1 channels have been consistently reported to be localized to the cell membrane (Wang, et al. 2018). We studied the subcellular localization of AoHKT1 channels in tobacco plants, *Nicotiana benthamiana*. The gene coding for GFP-AoKHT1 fusion protein was introduced into *Agrobacterium Tumefaciens* via electroporation, and another strain carrying a plasma membrane (PM) marker fused to Red Fluorescent Protein (RFP) was also introduced. Tobacco plants were infiltrated with both strains of bacteria and allowed to grow for 3 days under normal conditions. Interestingly, confocal images **(Fig. 2a)** showed very few regions of co-localization of GFP and RFP, suggesting that the AoHKT1 channels were not found at the cell membrane, and further analysis of the confocal images showed the localization of AoHKT1 possibly to the Golgi apparatus, scattered across the cytoplasm. This result was previously unreported in literature and might be due to post-translational modification prior to intracellular translocation to plasma membrane.

AoHKT1 functionally complements K^+ sensitivity of *Saccharomyces cerevisiae* mutant strains lacking the TRK1-2 genes

For further functional characterization of AoHKT1, we introduced this gene into multiple *Saccharomyces cerevisiae* deletion mutants as summarized in **Table 1**, which was compared to WT.

Yeast strains were first transformed with the *AoHKT1* gene, and were selected by culturing in SD-ura medium. Additional 100mM K^+ was added to the 12X yeast mutants lacking TRK1-2 membrane K^+ transporter to ensure their survival. All yeast strains grew normally in SD-ura medium. Subsequently, each strain was plated on YPD agar with varying Na^+ and K^+ concentrations.

BYT458 strains did not grow under all levels of NaCl stress (data not shown), likely due to its inability to maintain vacuolar K^+ storage. *AoHKT1* gene was unable to rescue the growth of transgenic BYT458 strains, suggesting AoHKT1 does not functionally complement vacuolar K^+ channels. The growth of non-recombinant control and recombinant strains BYT456, BYT459, BYT128 and BYT1289 were comparable across concentrations of NaCl ranging from 0M, 0.5M, 0.7M to 1M.

We hypothesized that similar to *T. salsuginea*, AoHKT1 could have a role in potassium transport across the plasma membrane. This prompted us to test the growth of control and recombinant 12X yeast mutants under low K^+ conditions. Deletion mutant BYT12 lacking the membrane K^+ transporters TRK1-2 exhibited sensitivity towards low K^+ concentrations of 10mM and 5mM because they could not take up K^+ into cytoplasm, while the growth of wildtype-BYT4741 was less affected. (**Fig. 2b**) The introduction of *AoHKT1* recombinant vector into this mutant functionally complemented the TRK1-2, and recombinant BYT12 exhibited better growth compared to the non-recombinant control. It is likely that AoHKT1 enables yeast to thrive under low potassium conditions, outgrowing the

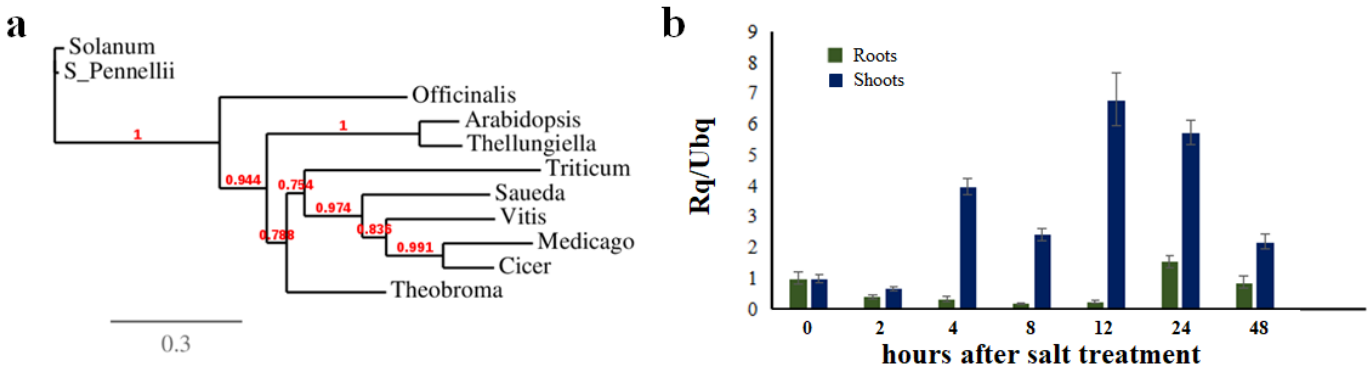

Fig. 1a A phylogenetic tree was constructed using the deduced amino acid sequences of AoHKT1 along with other plant genera: *Avicennia, Arabidopsis, Thellungiella, Triticum, Saueda, Vitis, Medicago, Cicer, Theobroma*. The phylogenetic trees were constructed using Phylogeny.fr (http://www.phylogeny.fr/) by the bootstrap method. The scale bar indicates the branch lengths. **b** Gene expression analyses by RTq-PCR of *AoHKT1* in *A. officinalis* plants. *AoHKT1* expression level in shoots increased significantly after 4 hours of 0.5M NaCl treatment, while there was no significant difference in the root expression levels. Relative expression levels of transcripts with reference to Ubiquitin transcript levels are plotted, RTq-PCR data represent mean ± SD from 3 biological replicates each with 3 technical replicates.

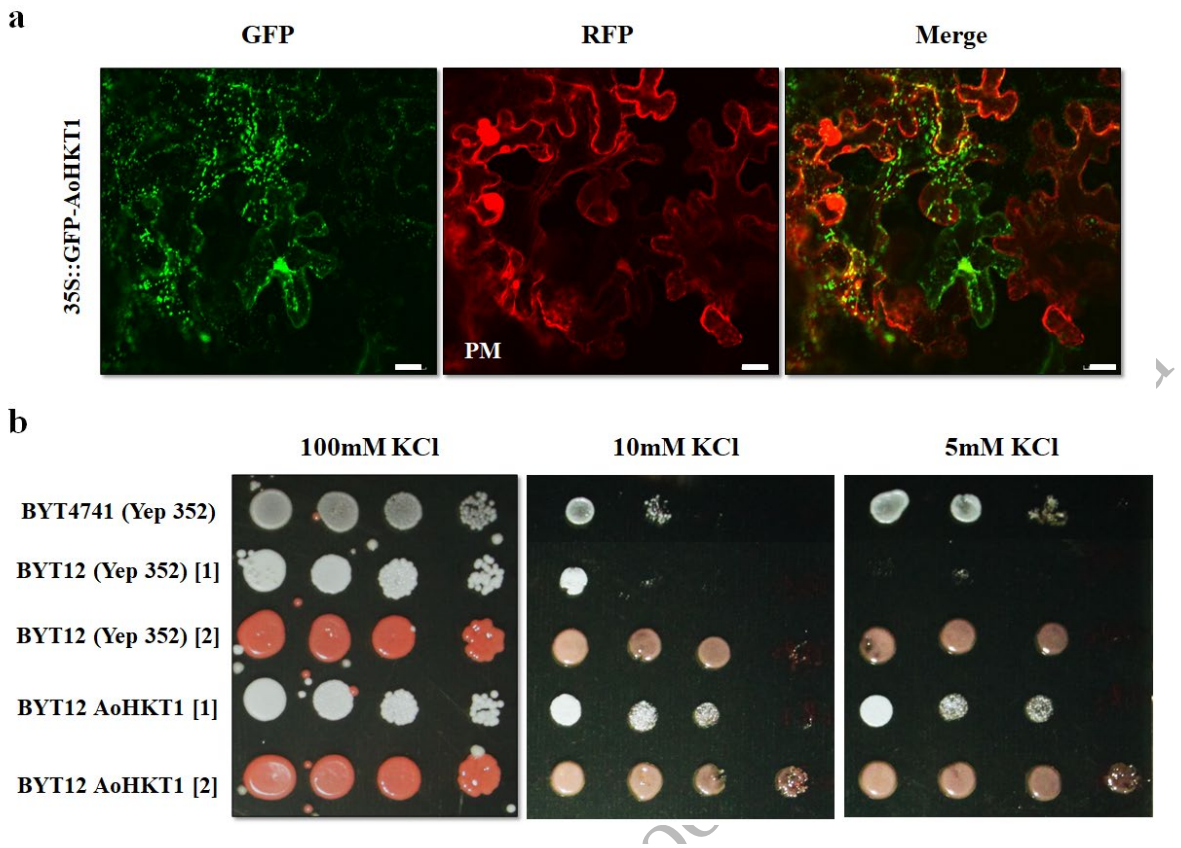

Fig. 2. a Subcellular localization of AoHKT1. Confocal microscopy images of the leaf epidermis of *Nicotiana benthamiana* expressing 35S::GFP-AoHKT1 along with a PM marker. The expression of 35S::GFP-AoHKT1 was visualized with 488nm (green) and the plasma membrane marker was visualized with 561nm (red). Merged images show the co-localization of the fusion protein and the marker, in yellow. Previously unreported, we found that *AoHKT1* was localized to the Golgi regions in the cell. Scale bar = 20µm. **b** AoHKT1 protein functionally complements the K^+ sensitivity of BYT12 mutant strain of *S. cerevisiae* lacking the TRK1-2 membrane K^+transporter genes. Results are shown for low-potassium concentration (10mM, 5mM) assay, for which yeast strains containing recombinant plasmid (BYT12 AoHKT1 [1] and [2]) show better growth compared to the control strains transformed with the empty vector (BYT12 Yep 352).

wildtype strains. (**Fig. 2b**). Hence, AoHKT1 may be a membrane K^+ importer, functionally similar to TRK1-2 in yeast.

Code	Protein	Function
45	ENA1-5	membrane Na^+/K^+ efflux transporter
12	TRK1-2	membrane K^+ influx transporter
6	NHX1	vacuolar Na^+/H^+ antiporter
8	VNX1	vacuolar K^+ transporter
9	VHC1	vacuolar K^+/Cl^- cotransporter

mutant strains	45	12	6	8	9
BYT456	*ena1-5Δ*		*nhx1Δ*		
BYT458	*ena1-5Δ*			*vnx1Δ*	
BYT459	*ena1-5Δ*				*vhc1Δ*
BYT12		*trk1-2Δ*			
BYT128		*trk1-2Δ*	*nhx1Δ*		
BYT1289		*trk1-2Δ*		*vnx1Δ*	*vhc1Δ*

Table 1. (Top) Summary of functions of proteins knocked-out in yeast deletion mutants used in the complementation assay. (Bottom) Summary of yeast deletion mutants of strains 45X (BYT456, BYT458, BYT459) and strains 12X (BYT12, BYT128 and BYT1289), which were compared to WT in the yeast complementation assay.

Heterologous expression of *AoHKT1* increases salt tolerance of *A. thaliana*

We used 4 independent homozygous strains of *A. thaliana,* including Wild Type (**WT**), *AoHKT1* overexpression in WT background (**WTOE**), *athkt1* knockout mutant (***athkt1;1***), and *AoHKT1* transgenic lines in *athkt1* mutant background (**AoHKT1**) to carry out the following assays.

Seed Germination Assay

A. thaliana seed germination assays were conducted over 4 days under 0mM, 50mM and 75mM NaCl concentrations. Although overexpression of *AoHKT1* resulted in a germination rate comparable to WT in 0mM and 50mM, in 2 out of 3 WTOE lines *AoHKT1* overexpression was able to improve the seed germination rate under 75mM high NaCl concentration (**Fig. 3a**). AoHKT1 is also able to partially rescue the mutant phenotype, as the seed germination rate in the transgenic lines is consistently higher than the mutant line in all salt concentrations, most successfully under 50mM NaCl (**Fig. 3a**). These results suggest that heterologous expression of *AoHKT1* in *A. thaliana* is able to improve the germination rate under salt stress compared to the WT. Furthermore, *AoHKT1* overexpression in the WT background is able to produce the highest seed germination rate, suggesting that *AoHKT1* could

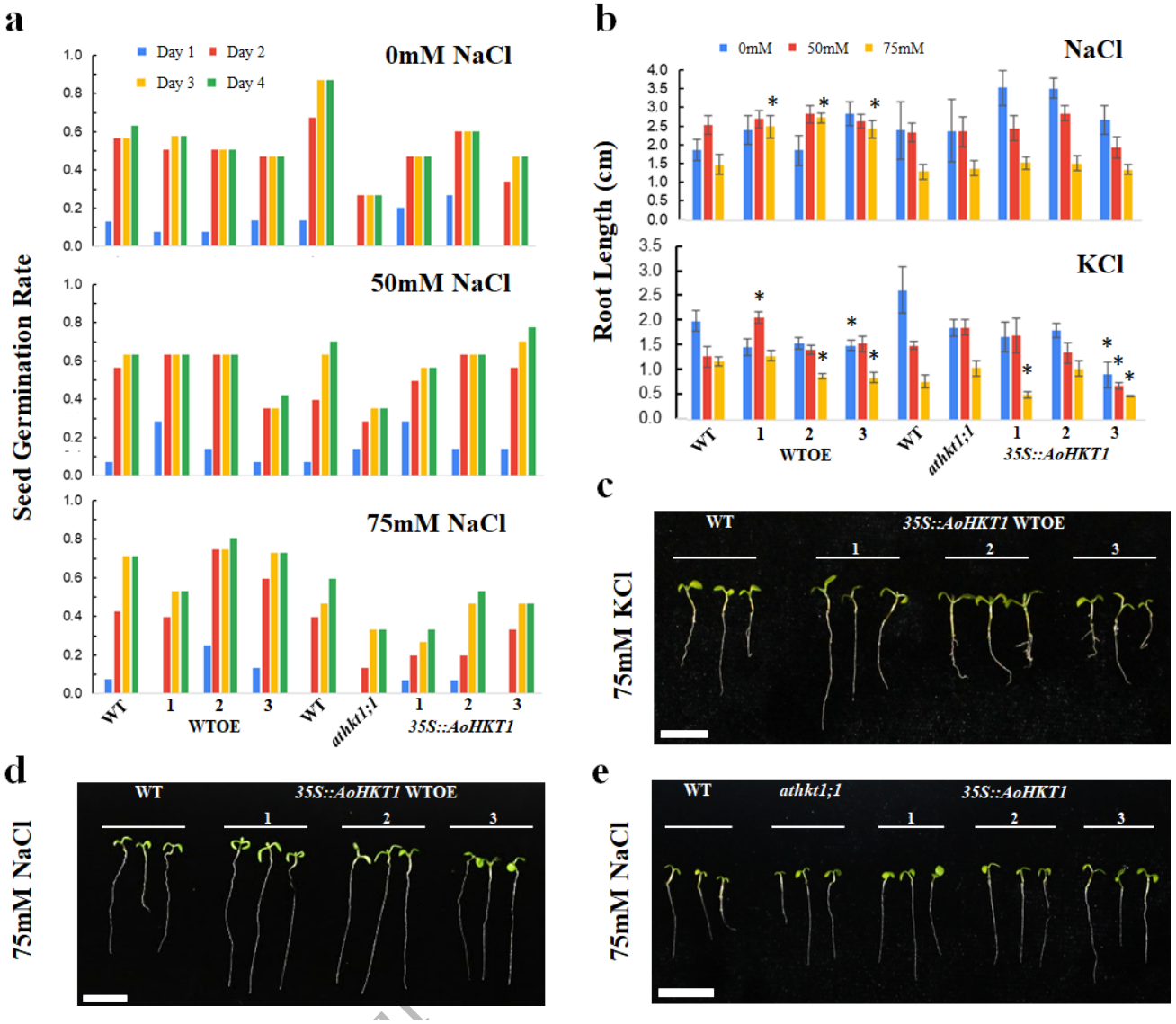

Fig. 3. a Seed germination rate under 0mM, 50mM, and 75mM NaCl treatments. Under both 0mM and 50mM NaCl conditions, WTOE showed comparable results to WT. However, WTOE showed an improved germination rate under 75mM NaCl. AoHKT1 was able to partially rescue the mutant phenotype consistently throughout all concentrations of NaCl. **b** 1-week root length measured under 0mM, 50mM, and 75mM NaCl/KCl treatments. Data in the graphs represent mean ± SE of at least 10 replicates. Asterisks indicate statistically significant differences between WT and mutant as well as between mutant and *AoHKT1* transgenic lines: *P<0.05. **c-e** Comparison of seedling growth among WT and WTOE, *athkt1;1* mutant and *AoHKT1* transgenic lines, under 75mM NaCl or KCl. Scale bar = 10mm. **c** Under 75mM KCl, both WTOE and *AoHKT1* transgenic lines (only WTOE is shown) reported a significant decrease in root length. **d-e** Under 75mM NaCl, WTOE exhibited a significant increase in root length compared to WT, while *AoHKT1* transgenic lines reported no difference.

be to working in concert with *AtHKT1* to further improve salt tolerance of *Arabidopsis*.

Root Length Assay

Root lengths were measured from 1-week-old seedlings under NaCl and KCl concentrations ranging from 0mM, 50mM, to 75mM. The root length of *AoHKT1* transgenic lines did not differ significantly when compared to the *athkt1-1* mutant line under NaCl salt treatment (**Fig. 3b**). However, the roots from WTOE lines were significantly (P<0.05) longer than the roots from WT under high concentration of NaCl (75mM). The results suggest that the overexpression of *AoHKT1* in the WT background is able to produce the most salt tolerant *Arabidopsis* plants, which are able to thrive under higher salt concentrations compared to the control.

Opposite results were obtained when the plants are subjected to KCl salt stress. Under 75mM KCl, the roots from both WTOE and *AoHKT1* transgenic lines were found to be significantly (P<0.05) shorter than the *athkt1-1* mutant background. (**Fig. 3b**). We hypothesize that this may be due to two reasons. First, the expression of *AoHKT1* could have been inhibited by high potassium concentrations, since there is no need to further decrease the cytosolic Na^+/K^+ ratio. Second, the *AoHKT1* could be a high-affinity K^+ transporter, which only operates well under low potassium concentrations.

Leaf Surface Area Assay

A. thaliana seeds from each line were planted in soil for 1 month, followed by 2 weeks of 100mM NaCl salt stress, then 1-week recovery from salt stress. Leaf surface areas were

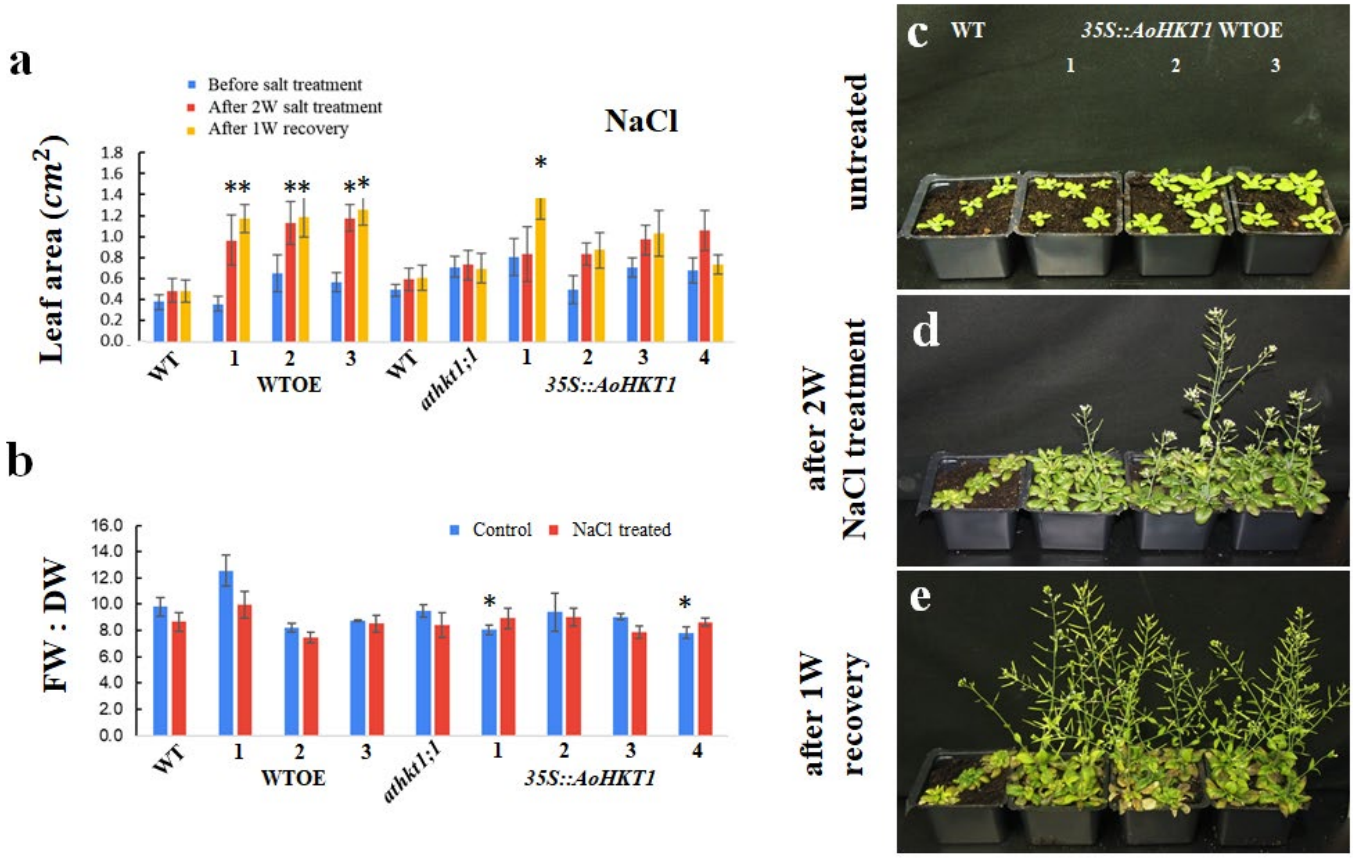

Fig. 4. a Leaf surface area measured before salt, after salt, and after recovery. Under 75mM NaCl, WTOE exhibited a significant increase in leaf surface area compared to WT, while *AoHKT1* transgenic lines showed no difference. **b** FW/DW ratio after 1-week recovery. No significant effects of salt treatment on FW:DW was observed. Data in the graphs represent mean ± SE of at least 3 replicates per experiment. Asterisks indicate statistically significant differences between WT and mutant, and between mutant and *AoHKT1* transgenic lines: *P<0.05. **c-e** Heterologous overexpression of *AoHKT1* increases salt tolerance of *Arabidopsis* seedlings. WTOE showed significantly better growth and larger leaf surface area after salt stress and recovery. **c** Growth of WT and *35S::AoHKT1* WTOE under untreated condition. **d** Growth of WT and *35S::AoHKT1* WTOE after 2 weeks of NaCl treatment. **e** Growth of WT and *35S::AoHKT1* WTOE after 1 week recovery.

measured at the end of each stage. Similar to root length assay, WTOE showed a significantly higher leaf surface area under both 50mM and 75 mM NaCl stress, while *AoHKT1* transgenic lines report no significant difference when compared to the mutant line (**Fig. 4a**). This further affirms our result from the germination and root length assays that AoHKT1 is able to increase salt tolerance of *Arabidopsis* best in the WT background containing *AtHKT1* gene.

Fresh Weight to Dry Weight Ratio (FW: DW)

As an index of cell water content, FW: DW is hypothesized to decrease rapidly when salt sensitive plants are placed in a high osmolarity environment. The FW:DW ratio of each plant was measured after 1-week recovery. However, the FW:DW ratio before and after salt treatment was comparable, with no significant decrease in FW:DW ratio being observed in WT lines (**Fig. 4b**).

DISCUSSION

Plants have evolved several mechanisms to survive under saline conditions and respond to salinity stress (Hamamoto, et al. 2014). Among these adaptations, the export of Na^+ away from the leaves, for example in *AtHKT1* in *A. thaliana* and the import of K^+ into root tissue, for example in *TsHKT1* in the halophyte *T. salsuginea* is crucial to maintain the homeostasis of cytosolic Na^+/K^+ ratio (Wang, et al. 2018). This is to fully engage K^+ in its function as an essential macronutrient. HKT transporters are known to play a crucial role in this homeostasis by transporting cations across the plasma membrane. HKTs are a diverse group of proteins that display different selectivities to Na^+, K^+, or other divalent cations depending on the plant species (Hasegawa, et al. 2001). In this paper, we have reported, to our knowledge, the first functional characterization of a mangrove *HKT* transporter, *AoHKT1* through heterologous expression in *Arabidopsis thaliana*, and a yeast complementation assay.

We have determined the subcellular localization of the channel, reporting for the first time the localization of *AoHKT1* to the Golgi apparatus instead of the plasma membrane under normal conditions. This is a novel finding, and further experimentation needs to be conducted to characterize the subcellular localization of AoHKT1 in detail. The same experiment should be repeated with a Golgi marker, allowing us to confirm the colocalization of AoHKT1 to the Golgi body. A potential function for the localization to the Golgi body could be to dock vesicles containing the AoHKT1 channel to the plasma membrane upon salt stress. To test this hypothesis, an experiment should be conducted where the same plants are allowed to grow under salt treatment. Subsequent confocal

imaging would allow us to determine whether AoHKT1 migrates to the plasma membrane under salt stress.

The RTq-PCR measuring gene expression levels shows that *AoHKT1* is upregulated in leaves under salt stress of 0.5M NaCl. The root length assay showed that under high concentrations of KCl (0.5M, 0.75M), the roots from WTOE and *AoHKT1* transgenic lines were significantly shorter compared to the Wild Type, suggesting that *AoHKT1* is either downregulated under high K^+, or that AoHKT1 is a high-affinity K^+ transporter that functions effectively under only low concentrations of K^+. In the yeast complementation assay, *AoHKT1* recombinants for yeast strain BYT12 lacking TRK1-2 membrane potassium transporters showed better growth compared to the mutant strains under low K^+ conditions. Overall, our results suggest that AoHKT1 is involved in K^+ transport, specifically K^+ import to reduce cytosolic Na^+/K^+ ratio for optimal cell growth.

Across root length, leaf surface area, and germination rate assays, WTOE plants with overexpression of *AoHKT1* in a WT background consistently performed better than the WT and *athkt1;1* knockout mutants under salt stress. These results together suggest that the heterologous expression of *AoHKT1* improves the salt tolerance of *A. thaliana*. However, salt tolerance is best improved when *AoHKT1* is overexpressed in the WT background - that is, when AoHKT1 and AtHKT1 act in concert to maintain cytosolic Na^+/K^+ ratio upon salt stress.

It is worthwhile to note that *AoHKT1* in *Avicennia* has many similarities to *TsHKT1* in *Thellungiella salsuginea*. They are functionally homologous - both are Subclass 1 HKT cation channels from eudicots that are unusual because they are K^+ selective, instead of the Na^+ selectivity that most glycophytic Subclass 1 HKT channels exhibit, including AtHKT1. This may suggest a possible mechanism that the enhanced tolerance to salt stress in the transgenic *Arabidopsis* plants is due to the upregulation of *AoHKT1* under salt stress to keep cytosolic K^+, an essential macronutrient at a high level to minimize Na^+ toxicity. It is possible that halophytes, including *Avicennia* evolved this powerful adaptation for K^+ import under salt stress to decrease the cytosolic Na^+/K^+ ratio, despite the increased effect of Na^+ induced K^+ efflux under the salt stress exerted by saline soils.

Therefore, the enhanced physical traits displayed by salt-tolerant *Arabidopsis* plants in this study were due to the reduced Na^+ toxicity and resultant cell damage under high salt concentrations - consequently, increasing plant productivity. This study shows that the heterologous expression of *AoHKT1* in crop plants such as *O. sativa* and *T. aestivum* has potential to increase crop productivity in saline soils.

Conclusion

We report, to our knowledge, the first characterization of a mangrove HKT transporter, AoHKT1 from *Avicennia officinalis*, through its heterologous expression in *Arabidopsis thaliana* and a yeast complementation assay. Our results suggest that AoHKT1 is a membrane K^+ importer that is upregulated in shoots upon salt stress. Our proposed mechanism through which AoHKT1 confers salt tolerance is through the reduction of the cytosolic Na^+/K^+ ratio. For the first time, we found that AoHKT1 could be localized at the Golgi Apparatus under normal conditions and further experiments are required to analyze the intracellular translocation of AoHKT1 under salt stress in detail. Expression of *AoHKT1* improved K^+ uptake in yeast *trk1-2* mutants under low K^+ conditions. Finally, heterologous expression of *AoHKT1* in *Arabidopsis thaliana* rescued essential phenotypes in mutant plants and worked in concert with *AtHKT1* to further improve salt tolerance under salt stress. Our research suggests that *AoHKT1* is a promising candidate gene in developing salt-tolerant crops to enhance crop viability in saline soils.

APPENDIX

Materials and Methods

Transformation of *GFP-AoKHT1*
A single round plate with LB Agar, along with the following three agrobacterium-specific antibiotics: Rifampicin, Gentamicin, Kanamycin was prepared. The electroporation cell was prepared by rinsing and evaporating ethanol inside. 1µl of the plasmid containing *GFP-AoKHT1* was added to the cell, along with *Agrobacterium* competent cells. Electroporation was then conducted. Following this, 1ml of lb broth was added and the *Agrobacterium* cells were incubated for 3 hours at 28 degrees Celsius. The bacteria were then plated onto the previously prepared selection plate.

Transformation of *AoHKT1* into yeast mutants

Different strains of yeast were taken from the respective glycerol stock and inoculated in YPD broth. They were incubated for 2 days at 30 degrees Celsius in an orbital shaker incubator. The yeast strains in YPD broth were centrifuged and resuspended, following which their optical density (OD) was checked. The yeast was centrifuged and resuspended in water, and then in 200ul of Te/LiAc to prepare yeast competent cells. 5µl of plasmid, 5µl of yeast carrier gene, 50µl of competent cells, and 500µl of PEG/LiAc solution were then incubated at 30 degrees Celsius for 30 minutes in the orbital shaker incubator. 20 µl of DMSO was added, and the yeast was then shifted to a 42 degree Celsius heat block for 15 minutes. The yeast was then centrifuged and resuspended in -ura selection broth for recovery, and then suspended in 500ul of 0.9% w/v NaCl solution. The yeast cells were then plated onto YPD agar. The yeast was then incubated for 3-5 days at 30 degrees Celsius.

Yeast Complementation Assay
Following transformation, the yeast cells were inoculated in YPD broth. A 1µl sample was taken from each strain, and the OD was measured. The OD of all the strains was normalised via dilution using water. Following this, 3 further dilutions were prepared for each strain : 10x, 100x and 1000x. There were hence 4 different concentrations of each yeast strain. 5µl of each of the samples were taken and dropped onto the YPD agar.

Germination and Root Length Assays
For the germination assays, seeds were sown onto a circular plate, whereas for root assays they were sown onto square plates. There were a total of 12 plates for both. The plates were of concentrations 0mM NaCl, 50mM NaCl, 75mM NaCl, 0mM KCl, 50mM KCl and 75 mM KCl. For each different concentration, there were two plates. One plate with the WT and WTOE strains, as well as one with WT, mutant and transgenic strains. For each strain, approximately 15 seeds were planted. The number of seeds that had germinated each day was observed and recorded for each strain as part of the germination assay. The root assays were then grown in a Tissue Culture Room for 1 week under the following conditions: 16 hours of light, 8 hours of darkness at 22-23 degrees. Photos of the results were taken, and ImageJ software was used to analyse the root lengths.

Leaf Surface Area
The plants were grown under the following conditions: 16 hours of light, 8 hours of darkness. The leaf surface are was measured using ImageJ software.

Fresh Weight to Dry Weight Ratio (FW/DW)
The fresh weight and dry weight of the infiltrated tobacco plants was measured after subjection to salt stress. Each plant was separated into roots and shoots, and the fresh weight of the plants of each strain was measured. Subsequently, the plants were dried at 60 degrees Celsius. The dry weight of the plants of each strain were then measured.

References

1. Ali A, Cheol Park H, Aman R, Ali Z, Yun DJ. Role of HKT1 in Thellungiella salsuginea, a model extremophile plant. Plant Signal Behav. 2013 Aug;8(8):e25196. doi: 10.4161/psb.25196. Epub 2013 Jun 10. PMID: 23759555; PMCID: PMC3999061.

2. Alonso Rodríguez-Navarro, Francisco Rubio, High-affinity potassium and sodium transport systems in plants, *Journal of Experimental Botany*, Volume 57, Issue 5, March 2006, Pages 1149–1160, https://doi.org/10.1093/jxb/erj068

3. Ana Rus, Shuji Yokoi, Altanbadralt Sharkhuu, Muppala Reddy, Byeong-ha Lee, Tracie K. Matsumoto, Hisashi Koiwa, Jian-Kang Zhu, Ray A. Bressan, Paul M. Hasegawa. AtHKT1 is a salt tolerance determinant that controls Na+ entry into plant roots.
4. Proceedings of the National Academy of Sciences Nov 2001, 98 (24) 14150-14155; DOI: 10.1073/pnas.241501798

5. An D, Chen J-G, Gao Y-Q, Li X, Chao Z-F, Chen Z-R, et al. (2017) *AtHKT1* drives adaptation of *Arabidopsis thaliana* to salinity by reducing floral sodium content. PLoS Genet 13(10): e1007086. https://doi.org/10.1371/journal.pgen.1007086

6. Hamamoto S, Horie T, Hauser F, Deinlein U, Schroeder JI, Uozumi N. HKT transporters mediate salt stress resistance in plants: from structure and function to the field. Curr Opin Biotechnol. 2015 Apr;32:113-120. doi: 10.1016/j.copbio.2014.11.025. Epub 2014 Dec 18. PMID: 25528276.

7. Han Y, Yin S, Huang L, Wu X, Zeng J, Liu X, Qiu L, Munns R, Chen ZH, Zhang G. A Sodium Transporter HvHKT1;1 Confers Salt Tolerance in Barley via Regulating Tissue and Cell Ion Homeostasis. Plant Cell Physiol. 2018 Oct 1;59(10):1976-1989. doi: 10.1093/pcp/pcy116. PMID: 29917153.

8. Honghong Wu, Plant salt tolerance and Na+ sensing and transport, The Crop Journal, Volume 6, Issue 3, 2018, Pages 215-225, ISSN 2214-5141, https://doi.org/10.1016/j.cj.2018.01.003.

9. Krishnamurthy, P., Vishal, B., Khoo, K. *et al.* Expression of *AoNHX1* increases salt tolerance of rice and *Arabidopsis*, and bHLH transcription factors regulate *AtNHX1* and *AtNHX6* in *Arabidopsis*. *Plant Cell Rep* 38, 1299–1315 (2019). https://doi.org/10.1007/s00299-019-02450-w

10. Lan, W. Z., Wang, W., Wang, S. M., Li, L. G., Buchanan, B. B., Lin, H. X., Gao, J. P., & Luan, S. (2010). A rice high-affinity potassium transporter (HKT) conceals a calcium-permeable cation channel. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(15), 7089–7094. https://doi.org/10.1073/pnas.1000698107

11. Laurie S, Feeney KA, Maathuis FJ, Heard PJ, Brown SJ, Leigh RA. A role for HKT1 in sodium uptake by wheat roots. Plant J. 2002 Oct;32(2):139-49. doi: 10.1046/j.1365-313x.2002.01410.x. PMID: 12383080.

12. LXXXII (82) 1998, No. 3. (1998, April 19). Retrieved January 28, 2021, from http://www.ipni.net/publication/bettercrops.nsf/issue/BC-1998-3

13. Su, H., Balderas, E., Vera-Estrella, R. *et al.* Expression of the cation transporter McHKT1 in a halophyte. *Plant Mol Biol* 52, 967–980 (2003). https://doi.org/10.1023/A:1025445612244

14. Wang, L., Liu, Y., Feng, S. et al. AtHKT1 gene regulating K+ state in whole plant improves salt tolerance in transgenic tobacco plants. *Sci Rep* 8, 16585 (2018). https://doi.org/10.1038/s41598-018-34660-9