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Salinity-induced expression of *HKT* may be crucial for Na^+ exclusion in the leaf blade of huckleberry (*Solanum scabrum* Mill.), but not of eggplant (*Solanum melongena* L.)

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Reduced Na^+ accumulation in the leaf blade is an important aspect of salinity tolerance and high affinity K^+ transporters (HKTs) are known to play a significant role in the process. Huckleberry and eggplant have previously been shown to display 'excluder' and 'includer' characteristics, respectively, under salt stress, but the underlying mechanisms have not been investigated. Here, we isolated the cDNA of the HKT homologs, *Solanum scabrum* HKT (*SsHKT*) from huckleberry and *Solanum melongena* HKT (*SmHKT*) from eggplant, and analyzed their expressions in different tissues under salt stress. *SsHKT* expression was markedly induced in the root (28-fold) and stem (7-fold), with a corresponding increase in Na^+ accumulation of 52% and 29%, respectively. Conversely, eggplant accumulated 60% total Na^+ in the leaf blade, with a lower *SmHKT* expression level in the root (3-fold). Huckleberry also maintained a higher K^+/Na^+ ratio in the leaf blade compared to eggplant, due to the reduction of its Na^+ concentration and unaltered K^+ concentration. Functional analysis demonstrated that SsHKT-mediated Na^+ influx inhibited yeast growth under Na^+ stress, and that SsHKT did not complement the growth of the K^+ uptake-deficient CY162 strain under K^+-limiting conditions. These results suggest that the Na^+ accumulation characteristics of both plants are caused by the differential expression of *HKT* genes, with *SsHKT* exerting a greater control over the ability of Na^+ to reach the leaf blade in huckleberry, than *SmHKT* does in eggplant.

1. Introduction

The ability to restrict the transport of Na^+ to photosynthetic tissues is an important adaptation to salinity stress [1]. Generally, Na^+ uptake at the level of the root proceeds passively or through voltage-dependent channels, such as non-selective cation channels (NSCC). Then, the Na^+ is moved via the activity of some transporters, such as the salt overly sensitive 1 (SOS1), to the stele or vascular bundle, where it is loaded into the xylem and transported to the shoot via the transpiration stream [2,3]. If xylem loading and transport of Na^+ to the shoot is not regulated, toxic levels of Na^+ will accumulate in the leaf blade, leading to the impairment of metabolic functions [4].

Class I HKT transporters exhibit high levels of specificity to Na^+, because of the occurrence of the SGGG motif at the filter position in the pore domains (P_A–P_D), where serine (S) residue restricts the passage of K^+ [5,6]. Conversely, in class II HKTs all four residues are glycines (G) and transporters mediate both Na^+ and K^+ transport [5]. Since Class I HKTs mediate Na^+ exclusion from leaf blade, and hence are involved in maintaining an optimal Na^+/K^+ balance under salt stress, there is a preponderance of research across many species focusing on these transporters. For example, in rice, HKT1;4 and HKT1;5, localized in the xylem parenchyma cells of leaf sheath and root, respectively, mediate Na^+ unloading from the xylem, and thus protect the leaf blade from excess Na^+ accumulation [6]. In addition, the *Arabidopsis thaliana* HKT1 (AtHKT1), which is a functional orthologue of the HKT1;5 in rice, has been shown to participate in Na^+ retrieval from the xylem in both the leaf and root [7,8]. It has also been suggested that AtHKT1 may play a role in Na^+ recirculation from the leaf to the root, via the phloem [7].

Huckleberry is an important leafy vegetable in many parts of sub-Saharan Africa, and is rich in vitamin A, Ca, Fe, methionine, antioxidant phytochemicals and anti-inflammatory agents [9,10]. Although its growth is known to be adversely affected by water stress [11], very little information exists on its adaptation to salinity. In the first study of its response to salinity, the Na^+ content of its

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leaf (i.e. leaf blade and petiole combined), stem and root was compared with that of eggplant, both belonging to the genus *Solanum* [12]. The study showed that huckleberry and eggplant have different Na^+ accumulation characteristics under salt stress. While, eggplant accumulates more Na^+ in the leaf than in the stem and root ('includer'), huckleberry accumulates less in the leaf and more in the stem and root ('excluder'). This observation suggests the existence of a Na^+ transport regulatory mechanism, controlling the accumulation of Na^+ in the leaf of huckleberry. Although this control mechanism was hypothesized to include a Na^+ transporter, it has not been investigated. Such a regulatory mechanism has widely been believed to be absent in eggplant [13–15], but there is no supporting physiological evidence. Besides, no study on Na^+ transporters has been carried out in huckleberry and eggplant, to date, making both plants good models for the comparative evaluation of Na^+ transport activity. Thus, confirming the expression of a Na^+ transporter in these plants may offer insights into their adaptation to salinity, and open new avenues for engineering salinity tolerance.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, huckleberry and eggplant seeds were sown in seed beds. Two weeks after germination, seedlings were transferred to 65-L hydroponic tubs, containing Hoagland solution (1 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 4.8 mM KNO_3, 5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 4.5 μM $MnCl_2 \cdot 4H_2O$, 0.4 μM $CuSO_4 \cdot 5H_2O$, 29 μM H_3BO_3, 0.1 μM MoO_3, 0.96 μM $ZnSO_4 \cdot 7H_2O$, and 50 μM Fe-EDTA) diluted to one-fourth strength for conditioning. After 10 days, the concentration of the Hoagland solution was raised to half strength, supplemented with 0 mM NaCl (control) and 50 mM NaCl (salt stress). The NaCl was added in 25 mM increments every 3 days, up to a final concentration of 50 mM, after which treatment was considered to have started. The experiment was set up in the green house under natural sunlight conditions, day/night temperature of 18–28 °C/10–15 °C and humidity of 60–70%. The pH of the solution was daily adjusted to 6.5–7.0.

2.2. Measurement of growth, and Na^+ and K^+ concentrations

Sampling was done 10 days after the start of treatment. The plants were harvested, separated into leaf blade, petiole, stem and root, and weighed to get the fresh weight (FW). The samples were then partitioned in two sets, flash-frozen in liquid nitrogen and stored at −80 °C. One set was freeze-dried, weighed to obtain the dry weights (DW), powdered and used for elemental analysis. The second set was maintained fresh and used for gene expression analysis. Na^+ and K^+ concentrations were measured using dry samples as previously described [12].

2.3. HKT cloning

Total RNA was isolated from frozen root samples of huckleberry and eggplant, using TRIzol (Invitrogen, CA, USA). The RNA extracts were then reverse-transcribed into first strand cDNA using oligo dT primers and ReverTra Ace-reverse transcriptase (TOYOBO, Osaka, Japan). The single stranded cDNAs were further converted to double strand cDNA, using the SsHKT and SsEF1 degenerate primers (Table S1). The cDNAs were then ligated into a pGEM-T Easy vector (Promega, Wisconsin, USA), cloned, and sequenced using the M13 primer and the BigDye terminator (Applied Biosystems, Texas, USA).

The full sequence of the huckleberry *SsHKT* gene was obtained by 3′ and 5′ RACE using the 3′/5′ RACE kit (Roche Diagnostics, Mannheim, Germany), Poly A^+ mRNA, and the following primers: 3′SP1, 5′SP1, 5′SP2, and 5′SP3 primers (Table S1). The deduced amino acid sequence of the full *SsHKT* cDNA was then compared with those of AtHKT1;1 (AF237672), TsHKT1;2 (ABK30935) and TaHKT1;5 (AAA52749). For eggplant, only the upstream fragment of the HKT was cloned from cDNA derived from stressed root samples using the 5′SP1, 5′SP3, and SsHKT-F primers (Table S1). The nucleotide sequence of *SsHKT* has been deposited at the DDBJ/EMBL/GenBank data library under the accession number LC028893.

2.4. SsHKT and SmHKT expression analysis

To conduct the expression analysis, first-strand cDNA of *SsHKT* and *SmHKT* were synthesized from 1 μg of total RNA using the ReverTra Ace® qPCR RT Kit (TOYOBO, Osaka, Japan). Then, quantitative real-time PCR (qRT-PCR) was performed on cDNA derived from the leaf blade, petiole, stem and root of both huckleberry and eggplant using the Thunderbird SYBR qPCR mix (TOYOBO, Osaka, Japan), the qSsHKT and qSsEF1 primers (Table S1), and cDNA. The reaction was performed as described previously [16], and the fold change of the gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [17].

2.5. Yeast transformation and complementation

In this study, the Na^+-sensitive yeast strain AB11c (*MATa, ena1Δ::HIS3::ena4Δ, nha1Δ::LEU2, nhx1Δ::TRP*) [18] was used in the growth inhibition tests, while the K^+ uptake-deficient strain CY162 (*MATα, ura3-52, his3Δ200, his4-15, trk1Δ, trk2Δ1::pCK64*) was used in the K^+ uptake complementation. SsHKT cDNA was cloned to the plasmid pYES2 (Invitrogen, CA, USA) and used to transform the strains. For AB11c, Ura^+ transformants were selected on Ura-selective medium containing 0.67% yeast nitrogen base, 2% glucose and 2% agar. For CY162, selection of Ura^+ transformants was performed according to the method in Ref. [19]. Growth inhibition and K^+ uptake analyses were performed in liquid AP medium containing 2% galactose, 0.6% sucrose, and the indicated concentrations of KCl and NaCl.

3. Results

3.1. Effect of salt stress on growth of huckleberry and eggplant

Salt stress did not alter the total FW of huckleberry, but decreased that of eggplant by 28% (Fig. S1). With respect to different tissues, salinity did not affect root FW, but markedly reduced the FW of petiole (40%), stem (57%), and leaf bade (27%) of eggplant. Conversely, in huckleberry salinity only significantly reduced the FW of the stem (16%).

3.2. Effect of salt stress on Na^+ and K^+ concentrations, Na^+ accumulation, and K^+/Na^+ ratio

Under salt stress, the Na^+ concentration of the leaf blade of huckleberry plants was about one sixth that in eggplant, whereas the stem Na^+ levels in huckleberry were about 2-fold that of eggplant (Fig. 1A). In huckleberry, the K^+ concentration was not significantly altered by salt stress, but was significantly reduced in eggplant in all the tissues, especially in the leaf blade (27% reduction) (Fig. 1B). In huckleberry, the root had a higher total Na^+ content (52%) than the shoot (48%), whereas in eggplant the shoot Na^+ content was higher (72%) than that of the root (28%) under salt stress (Fig. 1C). Of the total Na^+ accumulated in the shoot, 31%

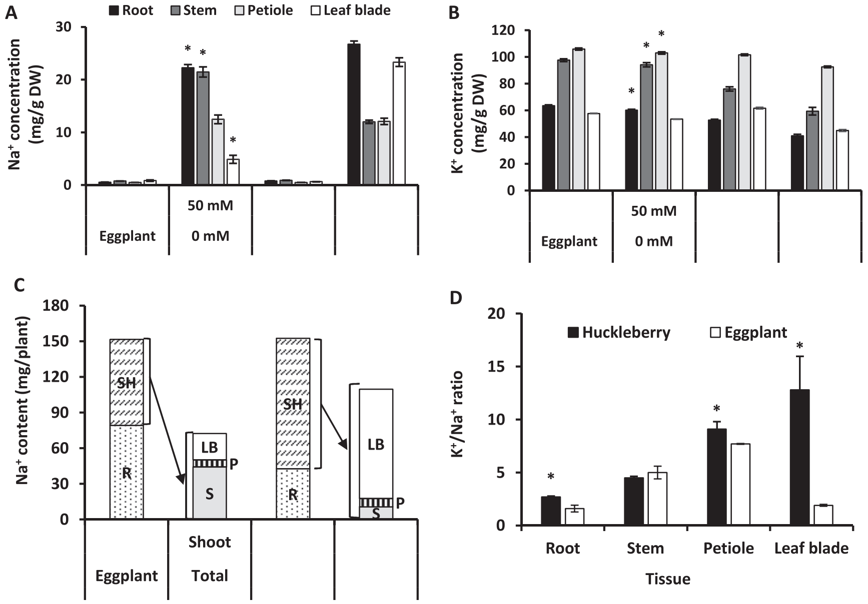

Fig. 1. Huckleberry is able to maintain lower Na^+ content in leaf blade compared to eggplant under salt stress. (A) and (B) Na^+ and K^+ concentrations in different tissues of huckleberry and eggplant under control and salt stress conditions. (C) Na^+ distribution in different tissues of huckleberry and eggplant under salt stress. (D) K^+/Na^+ ratio in different tissues of huckleberry and eggplant under salt stress. SH = shoot, R = root, LB = leaf blade, S = stem, P = petiole. Data represent means (n = 4) and bars, standard error. Asterisks show significant difference from eggplant $P < 0.05$.

accumulated in the leaf blade of huckleberry, as opposed to 83% in eggplant (Fig. 1C). The total Na^+ accumulated under salt stress in huckleberry was distributed as follows: leaf blade contained 15%, petiole 4%, stem 29%, and root 52%; while in eggplant the distribution was as follows: leaf blade contained 60%, petiole 5%, stem 7%, and root 28%. The K^+/Na^+ ratio was more elevated under salt stress in all huckleberry tissues than in those of eggplant. Stem tissue K^+/Na^+ ratio was the exception as this ratio was slightly higher in eggplant (Fig. 1D). The difference in K^+/Na^+ ratio between the two species was most pronounced in the leaf blade; approximately 7-fold reduction was observed in the leaf blade of eggplant compared to huckleberry.

3.3. SsHKT and SmHKT are both class I HKT proteins

The full cDNA of the *SsHKT* gene in huckleberry possessed a nucleotide sequence containing an ORF of 1503 bp encoding 500 amino acid residues (Fig. S2). Comparative analysis of the deduced amino acid of SsHKT with that of other class I HKT transporters revealed that SsHKT contained the S residue at the S70 position of the first pore domain (P_A) responsible for substrate filtration. In addition, the G residues at the filter position in the other 3 pore domains (P_B, P_C, and P_D) are also conserved (Fig. S3), indicating that the SsHKT transporter is a class I HKT transporter. The deduced amino acid sequence of SmHKT, an HKT homolog found in eggplant, also possessed the S residue at the S70 position (data not shown), suggesting that both SsHKT and SmHKT belong to the same group of class I HKT transporters, which facilitates Na^+ transport into cells.

3.4. HKT is induced by salinity in all huckleberry tissues, but only in the root of eggplant

To quantify the expression of the *SsHKT* and *SmHKT* genes, qRT-PCR was performed using cDNAs obtained from the root, stem, petiole and leaf blade of huckleberry and eggplant (Fig. 2). *SsHKT* expression was found to be induced by salt stress in all examined huckleberry tissues, and particularly in the root and stem, where there was 28-fold and 7-fold increase in the expression levels, respectively. In eggplant, on the contrary, although *SmHKT* expression was induced in the root (3-fold), this expression was about 11% that of *SsHKT* in huckleberry root. In all other eggplant tissues, *SmHKT* was repressed.

3.5. SsHKT mediates Na^+ uptake in yeast

To assess whether the retention of Na^+ in the root and stem of huckleberry could be under the control of the SsHKT transporter, functional analysis of the SsHKT transporter was performed in two yeast mutants: AB11c, which lacks Na^+-pumps and Na^+/H^+ antiporters, and CY162, which lacks two K^+ transporters. The expression of *SsHKT* rendered the Na^+-sensitive AB11c more sensitive to NaCl, as its growth was much more reduced than that of AB11c

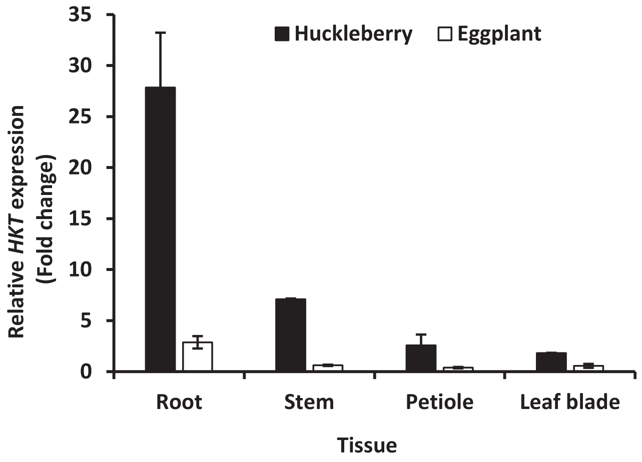

Fig. 2. SsHKT and SmHKT are induced primarily in the root under salt stress. *SsHKT* and *SmHKT* expression in different tissues of huckleberry and eggplant, respectively. The data are the means (n = 3) and bars represent standard errors.

transformed with pYES2 (vector control) (Fig. 3A and B). To determine whether this growth reduction was due to increased Na^+ uptake via SsHKT, the Na^+ concentration within the yeast cells was measured at the end of each growth experiment. The data showed that while the Na^+ concentration of the vector control was unaltered, AB11c cells transformed with *SsHKT* demonstrated a marked increase in Na^+ concentration (Fig. 3C).

3.6. *SsHKT does not complement yeast growth under K^+-limiting conditions*

To determine whether SsHKT could rescue the growth of K^+ uptake-deficient yeast, CY162 transformed with *SsHKT* and pYES2 (empty vector control) was grown under control conditions (100 mM KCl), low KCl (10 and 1 mM), and low KCl supplemented with 100 mM NaCl conditions. Under control conditions, CY162 transformed with either *SsHKT* or the empty vector grew equally well (Fig. 4A). However, the growth of both declined to the same degree, with decreasing concentrations of KCl (Fig. 4B and C). However, upon the addition of NaCl, while the vector control tended to grow, CY162 transformed with *SsHKT* declined (data not shown). Since the growth reduction of CY162 transformed with *SsHKT* was more marked when treated with 10 mM KCl supplemented with 100 mM NaCl (Fig. 4D), we proceeded to determine whether this reduction was due to an increased uptake of Na^+ by the yeast cells. To this end, the Na^+ and K^+ concentrations of cells at the end of each growth experiment was measured. The data showed that CY162 cells transformed with *SsHKT* contained much higher levels of Na^+ than vector control cells (Fig. 4E). Under 10 mM KCl treatment, both transformants had the same K^+ concentrations (data not shown).

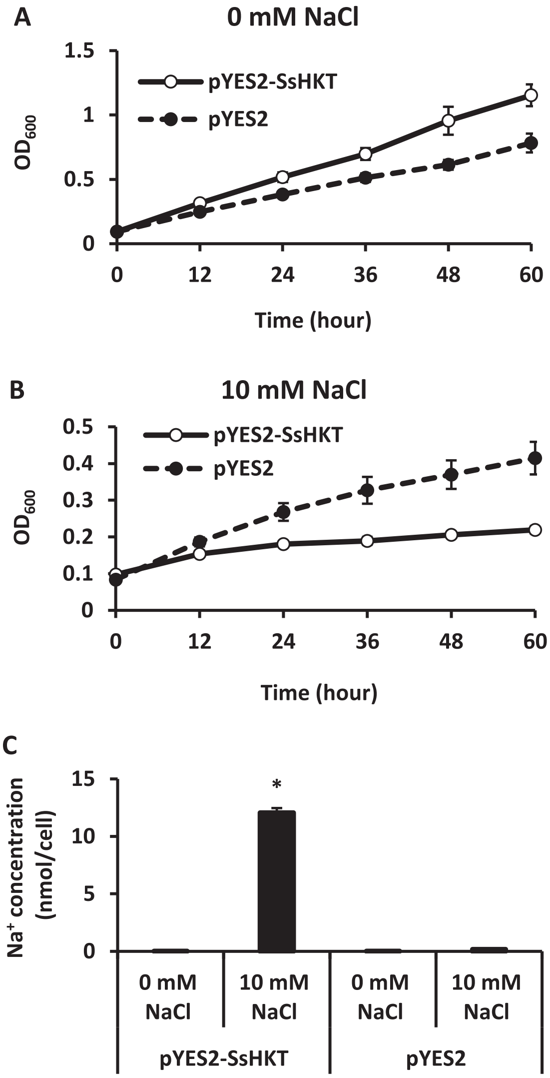

Fig. 3. SsHKT renders the Na^+-sensitive yeast, AB11c, more sensitive to Na^+ in liquid AP medium. Growth of AB11c in the absence of NaCl (A) and in the presence of 10 mM NaCl (B). (C) Na^+ concentration of AB11c cells, transformed with pYES2-SsHKT and pYES2, under control and NaCl stress. The data are means of 3 independent experiments, using 3 different colonies and bars represent standard errors. Asterisks show significant difference from control $P < 0.05$.

4. Discussion

Class I HKT transporters are specific Na^+ transporters located mainly in the membranes of xylem parenchyma cells, and are responsible for unloading Na^+ from the xylem, thereby preventing the accumulation of toxic levels of Na^+ in the leaf blade [2]. In this study, we investigated the mechanism underlying the reduction in Na^+ accumulation in leaf blade of huckleberry by isolating the cDNA of a novel HKT-like transporter, SsHKT, and analyzing its expression and function in huckleberry and yeast, respectively. We have shown that SsHKT is a Na^+-specific transporter, whose induction under salt stress may mediate the retention of high Na^+ levels in root and stem of huckleberry. The data further show that the widely reported susceptibility of eggplant to salt stress, due to the uncontrolled delivery of Na^+ to its leaf blade, could be related to the HKT activity.

The primary characteristic feature of class I HKTs lies in the configuration of the amino acid residues at the entrance of the selectivity filter of their pore-domains (P_A–P_D), where in the first pore domain (P_A), a conserved G residue is substituted by S residue, giving it the SGGG configuration, as opposed to the GGGG motif of class II HKTs [5]. The GGGG selectivity pore-forming motif is conserved in all class II transporters except OsHKT2;1, and confers high K^+ permeability, especially under conditions of low Na^+ concentration [5,20]. The S residue in the P_A loop has been shown to be responsible for the inhibition of K^+ permeability in class I HKTs, due to steric hindrance, in favor of Na^+ [21,5,6]. This makes class I transporters specific Na^+ transporters, and hence their role in controlling the transport of Na^+ in the tissues in which they are localized [7]. However, there exist exceptions regarding this function. Despite the occurrence of the S residue at the filter position of some class I HKT transporters, variations in other residues elsewhere along the polypeptide can alter the transport properties of the proteins, rendering some K^+-selective [22] and others weakly permeable to Na^+ [23,6,24]. In this study, analysis of the amino acid sequence of the SsHKT transporter showed that it is a class I HKT and possesses no amino acid variations that could affect the selectivity properties of the transporter [22,24,25] (Fig. S3).

The marked induction of the *SsHKT* expression in the root and stem of huckleberry (Fig. 2) correlated with the high accumulation of Na^+ in these tissues, as well as with the marked reduction in Na^+ accumulation in the leaf blade (Fig. 1C). Additionally, the induction level appears to be dependent on the Na^+ concentration of each tissue (Fig. 1A), indicating that Na^+ transport and delivery to the leaf blade at the level of the root, stem, and petiole is strictly regulated. This controlled delivery of Na^+ to the leaf blade of huckleberry, and hence its improved salt tolerance (Fig. S1) could therefore be attributed to the SsHKT transporter. The induced expression of the rice *OsHKT1*;5 was shown to be associated with reduced Na^+ accumulation in the leaf blade of the rice cultivar, Egyptian Yasmine, making it more tolerant than the susceptible cultivar that showed repressed *OsHKT1*;5, and consequently

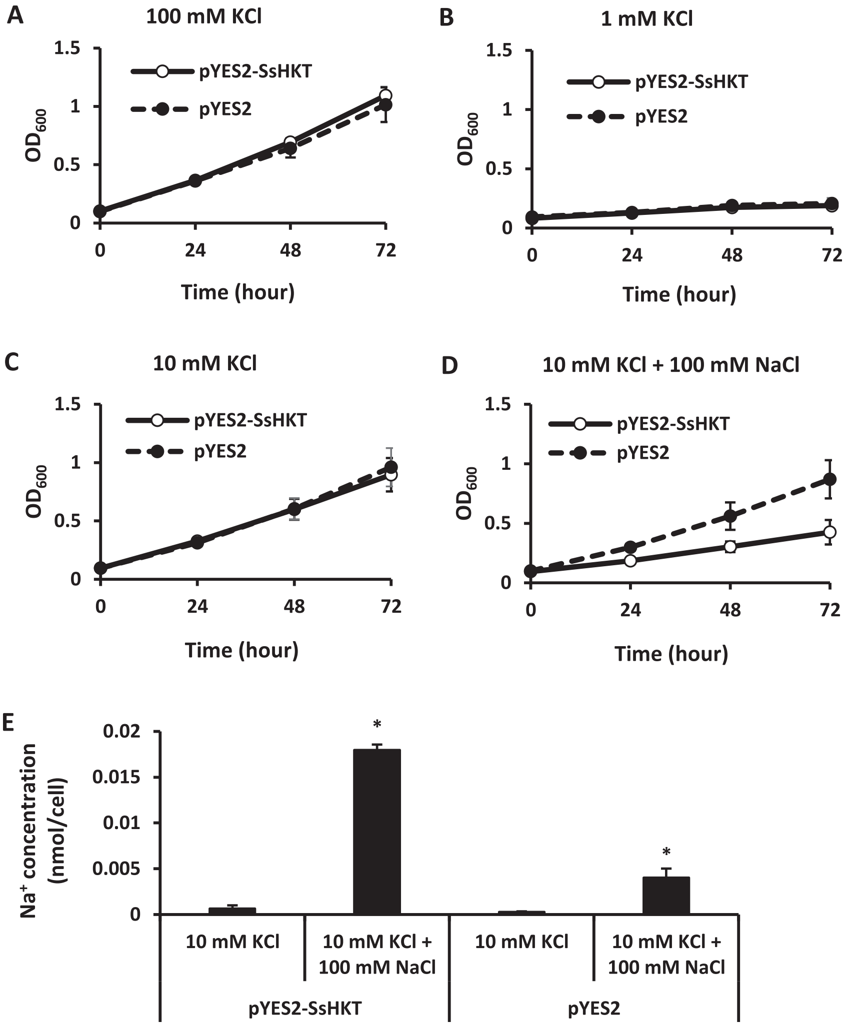

Fig. 4. The SsHKT protein does not complement yeast growth under low KCl condition. (A) Growth of CY162 under control (100 mM KCl). (B) Growth of CY162 under 1 mM KCl. (C) Growth of CY162 under 10 mM KCl. (D) Growth of CY162 under 10 mM KCl, supplemented with 100 mM NaCl. (E) Na^+ concentration of CY162 cells grown in 10 mM KCl and 100 mM NaCl. The data represent the means of 3 independent experiments, using 3 different colonies and bars represent standard error. Asterisks show significant difference at $P < 0.05$.

enhanced Na^+ accumulation in its leaf blade [16]. Similar observations have also been reported of the class I HKT transporters of other plants, such as Arabidopsis [7], tomato [24] and wheat [26]. Based on these observations, it is plausible to conclude that the accumulation of excess Na^+ in eggplant leaf blade would be associated with the weaker induction of *SmHKT* in the root, and its repression in other tissues (Fig. 2).

To determine whether the enhanced accumulation of Na^+ in the root and stem of huckleberry is caused by the activity of the SsHKT transporter, we analyzed its function in the yeast *Saccharomyces cerevisiae*. In yeast, an HKT Na^+ transporter will normally mediate Na^+ influx, leading to excess Na^+ accumulation, which slows or inhibits the growth of the yeast greatly in comparison to the less affected control yeast [26,27]. In the present study, the SsHKT mediated significant Na^+ uptake in the Na^+-sensitive strain AB11c, rendering it more sensitive to NaCl (Fig. 3A–C), and indicating that SsHKT is a Na^+ transporter. To determine the ion specificity of the transporter, we analyzed its activity in the K^+ uptake-deficient CY162. Generally, K^+ complementation will be achieved if the growth of CY162, transformed with HKT, is better than that of the vector control under both low K^+ and low K^+ plus NaCl conditions [22,25]. In the current study, the growth of CY162 transformed with SsHKT and vector control showed that SsHKT failed to complement the K^+ uptake deficiency of the yeast, and the growth was further reduced by addition of NaCl (Fig. 4A–D). This is due to enhanced uptake of Na^+ by CY162 transformed with *SsHKT* (Fig. 4E). This indicates that SsHKT is a Na^+-specific transporter, and thus is capable of mediating Na^+ retrieval from the xylem and storing it in the root and stem cells of huckleberry plants grown under salt stress.

Although class I HKT transporters are generally considered to be specific Na^+ transporters [20], some are Na^+/K^+ symporters and have been shown to maintain the optimum K^+/Na^+ homeostatic balance needed for the sustenance of metabolic functions under salt stress [25]. However, even Na^+ uniporters, such as the Arabidopsis HKT1;1 (AtHKT1;1) have been shown to maintain an optimum K^+/Na^+ balance under salt stress [7], due to the Na^+ transport activity of the HKT localized in the membrane of xylem parenchyma cells. Although HKT is not involved in root-shoot K^+ transport [28], its activity during Na^+ retrieval from the xylem sap and into the xylem parenchyma has been shown to trigger the release of K^+ from the xylem parenchyma cells and into the xylem sap [29]. This Na^+-K^+ exchange activity is thought to be caused by the depolarization of xylem parenchyma cell membranes following HKT-induced Na^+ influx in to the cells, and the subsequent activation of channels, such as the K^+ outwardly rectifying channel (KORC) in the xylem parenchyma membrane, which mediates K^+ release [7,29,5]. It has been proposed that this exchange activity simultaneously excludes Na^+ from the leaf and raising leaf K^+, resulting in a high leaf K^+/Na^+ ratio and improved tolerance to salt stress [5]. In the present study, the difference in *HKT* induction in huckleberry and eggplant may explain the difference in K^+ concentration (Fig. 1B) between the two species, and hence the marked difference in leaf K^+/Na^+ ratio (Fig. 1D), which is important for the adaptation of huckleberry to salt stress.

In conclusion, the adaptation of huckleberry to salinity is due to its ability to exclude Na^+ from the leaf blade thereby maintaining K^+/Na^+ ratios optimal for enhancing stress tolerance. This restricted transport of Na^+ to the leaf blade has been shown here to be under the control of the SsHKT protein, which as a specific Na^+ transporter is believed to function by unloading Na^+ from the xylem and retaining it primarily in the root and stem. In addition, the susceptibility of eggplant to salinity is due to the weak induction of *SmHKT* in the root and its repression in the other tissues, thus making the stem and petioles 'toll-free highways' for Na^+ delivery to the leaf blade.

Conflict of interest

The authors have no conflicts of interest directly relevant to the content of this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.048.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.048.

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