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ORIGINAL PAPER

Analysis of *Arabidopsis thaliana HKT1* and *Eutrema salsugineum/botschantzevii HKT1;2* Promoters in Response to Salt Stress in *Athkt1:1* Mutant

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Abstract
Soil salinity imposes a serious threat to the productivity of agricultural crops. Among several other transporters, high-affinity K^+ transporter (HKT)'s play an important role in reducing the phytotoxicity of Na^+. Expression of *Eutrema salsugineum* (a halophyte) *HKT1;2* is induced upon salt exposure. To elucidate the role of its promoter, we compared the sequences of *HKT1;2* promoters from *E. salsugineum* (1822 bp) and *E. botschantzevii* (1811 bp) with *Arabidopsis thaliana HKT1;1* (846 bp) promoter. In silico analysis predicted several *cis*-acting regulatory elements (GT-1 elements, core motifs of DRE/CRT, MYC/MYB-recognition sites and ACGT elements). Activities of the three promoters were analyzed by measuring *HKT1;1* and/or *HKT1;2* transcript level in the *Athkt1;1* mutant plants. NaCl tolerance of the transgenics was also assessed. Our results depicted that expressing either *AtHKT1;1* or *EsHKT1;2* coding regions under the control of *AtHKT1;1* promoter, almost reversed the hypersensitivity of the mutant for salt, on contrarily, when *AtHKT1;1* coding sequence expressed under either *Es* or *EbHKT1;2* promoters did not. Changes in shoot Na^+/K^+ concentrations under salt exposure is significantly consistent with the complementation ability of the mutant. The transcript concentration for genes under the control of either of *Eutrema* promoters, at control level was very less. This may suggest that either an important upstream response motif is missed or that *A. thaliana* misses a transcriptional regulator that is essential for salt-inducible *HKT1* expression in Eutrema.

Keywords *HKT1;2* · Promoter swapping · RT-qPCR · In silico

Introduction

The detrimental effects of high salinity levels on plants are the consequence of osmotic and ionic stresses [1]. Plants with the ability to successfully complete the life cycle under

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continuous exposure to higher salinity stress (> 200 mM NaCl) are categorized as halophytes, however, rest plants are known as glycophytes (salt-sensitive) [2]. The mechanisms underlying high-level salt tolerance among many halophytes are not properly understood, but the trait is often, more or less implicitly, supposed to depend on enhanced capacities of cellular Na^+ and K^+ compartmentalization and homeostasis, or compatible organic osmolyte synthesis, through alterations of the expression patterns of genes encoding Na^+/K^+ transporters or genes involved in the synthesis or breakdown of compatible solutes [2]. The Na^+ transporters, SOS1, NHX1 and HKT1 have often been considered to play key roles in salt tolerance in halophytes [3], however, comparisons between their expression patterns in halophytes and glycophytes are, with few exceptions [4–7], not available to date.

The HKT is Na^+/K^+ transporter and gene family is increasingly studied for its role in Na^+ detoxification within the plant body [8–10]. Plett et al. [11] showed that overexpression of *AtHKT1;1* in the mature root cortex improved the shoot Na^+ exclusion, and thus increased salinity tolerance in transgenics. In a recent study, Nawaz et al. [7] compared *HKT1* expression among halophytic and glycophytic species of Cochlearia, and found much higher expression levels in the halophytic species than in the glycophytic, supporting the hypothesis that enhanced *HKT1* expression may be crucial for high-level salt tolerance, indeed. *Eutrema salsugineum* (formerly known as *Thellungiella halophila*), thought to be a suitable halophytic model plant because of its high DNA identity with *A. thaliana* [12], has at least three *HKT1* genes, *HKT1;1*, *HKT1;2* and *HKT1;3* [13]. EsHKT1;2, is a K^+ transporter, whereas EsHKT1;1 and EsHKT1;3 are Na^+ transporters, like AtHKT1;1 [13–15].

There are many reports suggesting the presence of an efficient transcriptional regulation of the salt-responsive genes in halophytic species. Zhang et al. [16] analyzed the expression pattern of *Suaeda liaotungensis* gene *BADH* (*SlBADH*) promoter. They used 300 bp fragment (upstream from ATG) and showed almost six fold higher expression of gene under 400 mM NaCl stress as compared to control. Sun et al. [17] compared the activities of two promoters from *A. thaliana* and *T. halophila* gene (*Vacuolar H^+-Pyrophosphatase-VP*) and concluded that 130 bp of the *ThVP1* promoter contains *cis*-acting regulatory elements (two enhancers) which are involved in strong induction of the gene under salt stress. Li et al. [18] analyzed the role of *PEAMT* gene promoter from *Suaeda liaotungensis* (a halophyte) and predicted the presence of many salt-responsive *cis*-element (897 bp upstream from start codon). They reported the enhanced GUS activity (18-fold) under the control of *SlPEAMT* promoter at 200 mM of NaCl stress, in the transgenic tobacco. These studies and many others provided a notion that even a small fragment of promoter can contain salt stress-inducible, *cis*-acting motifs to regulate the expression of different genes, upon salt stress.

Surprisingly, very little work has been done to explore the role of the *HKT1* promoter in the transcriptional regulation of the gene. Asins et al. [19] has observed the altered expression of *SlHKT1;1* gene in roots and leaves of NILs due to the difference in their promoter sequences, which affects the transport activity of the gene, as well. The *cis*-regulatory allelic variations among various Arabidopsis ecotypes has also been documented for the *AtHKT1;1*, which lead to differential salinity tolerance of the ecotypes [20, 21]. However, there is not even a single study to compare the promoter activities of a halophyte (like *Eutrema salsugineum/botschantzevii*) and a glycophyte (like *A. thaliana*). In this study, we performed an in silico analysis of three HKT1 promoter sequences, i.e., *A. thaliana* ($Pro_{AtHKT1;1}$, 1822 bp), *Eutrema salsugineum (=halophila)*, ecotype Shandong ($Pro_{EsHKT1;2}$, 1822 bp), and *Eutrema botschantzevii* (formerly known as *Thellungiella botschantzevii*), ecotype Saratov ($Pro_{EbHKT1;2}$, 1822 bp). We compared the activities of four promoter sequences, i.e., $Pro_{AtHKT1;1}$ (846 bp), $Pro_{EsHKT1;2}$ (1822 bp), $Pro_{EbHKT1;2}$ (1811 bp), and the CaMV 35S promoter, through examining *AtHKT1;1* and *EsHKT1;2* gene expression in the *A. thaliana hkt1;1* mutant background. We also compared the potential of the constructs to reverse the Na^+ hypersensitivity phenotype of the *Athkt1;1* mutant.

Materials and Methods

Plant Materials and Experimental Conditions

Eutrema salsugineum and *E. botschantzevii* seeds, originating from a coastal area near Shandong, China, and a solonchak soil in Saratov, Russia, were sown on garden soil (Jongkind B.V., Number 6, Aalsmeer, The Netherlands). When seedlings were three-weeks old, they were transfered to hydroponics culture [22]. Mature plants were harvested which were snap-frozen. For subsequent DNA/RNA extraction, samples were stored at − 80 °C [23].

Surface sterilization of different types of seeds [*Arabidopsis thaliana* (Col) wild type, *Athkt1;1* mutants and transgenic lines] were done using 96% ethanol and 10% bleach, followed by three times washing in distilled water. Surface-sterilized seeds were mixed in agarose (0.1%) and spread on square petri plate containing gelrite (0.8% w/v) with Murashige and Skoog (MS—0.5%) salt, whose pH was adjusted around 5.7–5.9. For transgenic lines, hygromycin (25 µg/ml) and for the *Athkt1;1* mutants, 25 µg/ml kanamycin was used as selective marker. Photoperiod for seeds germination was 10 h light and 14 h dark, at 22 °C temperature.

When seedlings developed their true leaves, they were transplanted to hydroponics 1-l pots, having half-strength modified Hoagland's nutrient solution [22] in climate room. Light intensity in climate room was set 220 µmol/m/s at the plant level, along with 20 °C (day), 15 °C (night) temperature and 75% relative humidity. Hydroponics solutions were renewed two times a week. When plants get adapted to the new environment (almost after two weeks), ten plants from each type were exposed to NaCl (50 mM) using control as a reference. Salt stress was increased gradually (at first 25 mM stress was given then increased to 50 mM). Plants were harvested after two weeks of the final concentration. Fresh weight of the roots and shoots in gram was taken.

RNA/DNA Extraction and First-Strand cDNA Synthesis

Frozen shoot tissues were used for RNA extraction via Trizol™ (Invitrogen) method, as described in Jack et al. [23]. For the synthesis of single-stranded cDNA, total RNA (2.5 µg) which was boiled for 1 min, was used. Reverse transcription was accomplished with M-MLV Reverse Transcriptase (100 Units), 100 mM DTT, 2 mM dNTPs, RT buffer (10×) and 10 µM of oligo dT primer (Invitrogen). For DNA isolation, we used the protocol as described in Rivera et al. [24].

Es/EbHKT1;2 Promoters Sequencing, Constructs Making and the Transformation of the *Athkt1;1* Mutant

The sequence of *HKT1;2* promoters from *E. salsugineum* and *E. botschantzevii* were obtained by chromosome walk, using Universal Genome Walker™ kit by Clontech. Gene specific reverse primers were used for chromosome walk on DNA (genomic). 1822 bp from *E. salsugineum*, 1811 bp from *E. botschantzevii* and 846 bp from *A. thaliana* [25] upstream from start codon (ATG) of *HKT1,* were used as promoters. Following constructs were prepared: Pro_{35S}*::EsHKT1;2*, $Pro_{AtHKT1;1}$*::EsHKT1;2*, $Pro_{AtHKT1;1}$*::AtHKT1;1*, $Pro_{EsHKT1;2}$*::AtHKT1;1* and $Pro_{EbHKT1;2}$*::AtHKT1;1*. PCR's were performed using "Phusion® DNA Polymerase" by Finnzymes, on gDNA for amplification of the promoter region and on cDNA for amplification of *HKT* coding sequences. Forward primers of $Pro_{AtHKT1;1}$, $Pro_{EbHKT1;2}$, $Pro_{EsHKT1;2}$ and $Pro_{EsHKT1;2}$*::AtHKT1;1* contained "CACC" 5′ overhang which is necessary for directional cloning in pENTR/D Topo, while primers for $Pro_{AtHKT1;1}$*::AtHKT1;1*, $Pro_{AtHKT1;1}$*::EsHKT1;2* and $Pro_{EbHKT1;2}$*::AtHKT1;1* contained attB1/2 sites (Table S1: Supplementary Information). BP reaction was performed using BP Clonase® II enzyme mix between vector containing attP-site and DNA fragment having attB-flanked to generate an entry clone. This entry clone was subsequently utilized for LR recombination reaction, using LR Clonase® II, with Gateway® destination vector for the development of an expression vector. Following expression vectors; pH7WG2, pH7WG2 (-Pro_{35S}) {constructed from pH7WG2} and pHGWFS7 [26], were used. pHGWFS7 was used for the promoters analysis, however, pH7WG2 (-Pro_{35S}) was used for the constructs (having a promoter). *Agrobacterium tumefaciens* strain C58 (pMP90) containing specific construct was used to transform *A. thaliana hkt1;1* mutant.

Screening of Transgenic Lines

Athkt1;1 mutants (Col) seeds, obtained from NASC stock center (N6531), and *A. thalaina* wild type seeds were sown on the garden soil (Jongkind B.V., Number 6, Aalsmeer, The Netherlands). Plants of *Athkt1;1* mutants, at silique forming stage, were transformed using 'flower dipping' protocol as described by Clough and Bent [27]. The matured seeds were harvested from T_0 plants and were sowed on square Petri plates containing MS media, after surface sterilization. The transformed plants were easily distinguished from non-transformed ones, after 2 weeks depending on root growth. Transformed plants were transplanted to hydroponics culture [22]. After 2 weeks, leaves and root samples were taken for RNA extraction, from selected T_1 progeny. After selection, null segregants were never found, and selected plants were supposed to be a mixture of heterozygotes and homozygotes. Transcript levels of the genes (*Actin-2* as reference gene) were measured by Real-Time quantitative PCR. Primers for RT-qPCR were designed with a G/C ratio ranging between 50 and 60% with T_m (melting temperature) range between 58 and 60 °C. Gene-specific primers for *Eutrema salsugineum* (*EsHKT1;2*), *Eutrema botschantzevii* (*EbHKT1;2*), *A. thaliana* (*AtHKT1;1*) and *Act-2* (as an internal control for expression analysis) were designed separately (Table S2: Supplementary Information). RT-qPCR data were analyzed as described by Livak and Schmittgen [28]. The gene expression was normalized to the highest expression, which was assigned a value of 1.

Tolerance Index and Water Content

At the time of harvest, fresh weight of root and shoot samples were taken. Samples were air dried at 65 °C for 72 h. Tolerance index was calculated over ten biological replicates of each transgenic line using the formula:

$$\text{Tolerance Index (TI)} = \frac{\text{Average fresh weight at 50 mM}}{\text{Average fresh weight at the control}}$$

The percentage of water was calculated using the following formula:

$$\%\ \text{age of water} = \left(\frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}}\right) \times 100$$

Measurement of Na^+ and K^+

After taking fresh weight, the roots of two plants were pooled together while shoots were dried and stored separately. After measuring shoot dry weight two shoots were pooled together like roots. Na^+ and K^+ concentrations, in roots and shoots were determined (two plants pooled, to make five samples per genotype, per treatment level) by extracting 20 mg of dry material in 2 ml eppendorfs. Two milli liters of water was added in each eppendorf then boiled it for 1 h at 90 °C in a water bath. After cooling, samples were filtered and dilutions were made, if needed. After proper dilution, Na^+ and K^+ analysis on Atomic Absorption Spectrophotometer (AAS100) was done.

Results

In Silico Analysis of *EsHKT1;2, EbHKT1;2* Promoters in Comparison with the *AtHKT1;1* Promoter

The sequences of the *EsHKT1;2*, *EbHKT1;2* and *AtHKT1;1* promoters (each was 1822 bp in length) were used for in silico analysis via web-based tools (PLACE/Signal Scan database-http://www.dna.affrc.go.jp/) [29]. The promoters from *EsHKT1;2* and *EbHKT1;2* have 95% identity among themselves, while both promoters showed 38% identity with the *AtHKT1;1* promoter, on nucleotide basis (sequence alignment S1: Supplementary Information).

We identified several putative *cis*-acting regulatory biotic/abiotic stress-responsive elements which include the core motif for DRE/CRT (dehydration-responsive element/C-repeat) and putative GT-1 *cis*-elements (2 motifs in *EsHKT1;2* and *EbHKT1;2* and 3 in the *AtHKT1;1* promoter sequences; Table S3: Supplementary Information). A common *cis*-acting element, CAAT-box, which is mostly present in promoters and enhancer regions, was identified at several places in three promoter sequences. The ACGT motif was found 3, 4 and 5 times in *AtHKT1;1*, *EbHKT1;2* and *EsHKT1;*2 promoter sequences, respectively. Potential MYB-, MYC- and MYBCORE recognition sites have been identified at several places in the analyzed sequences (Table S3: Supplementary Information).

Selection and Molecular Analysis of T_0/T_1 Transgenic Plants

The amplified *E. salsugineum* cDNA sequence appeared to be *EsHKT1;2*. It shared 83% identity, on a nucleotide basis, with *A. thaliana HKT1;1* (sequence alignment S2: Supplementary Information). On a protein basis, EsHKT1;2 and AtHKT1;1 were 79% identical (sequence alignment S3: Supplementary Information). The *EbHKT1;2* (1811 bp) and *EsHKT1;2* (1822 bp) promoters were cloned for this experiment. We successively performed two experiments with two sets of independent transgenic lines.

PCR and Real-Time PCR analyses were performed on all the T_0 plants and 3–4 randomly selected plants from the T_1 progeny (the first generation of transgenic plants) to determine the expression levels (Fig. 1) of the transgenes, under control condition. Only those transgenic lines were selected which showed the similar transcript expression level of *AtHKT1;1* and/or *EsHKT1;2*, for the subsequent experiments (at least two independent lines per genotype). *EsHKT1;2* was most strongly expressed from $Pro_{AtHKT1;1}$, in comparison with any other promoter (Fig. 1). Also *AtHKT1;1* was well expressed, approximately at the level of wild type *A. thaliana*, when expressed under its native promoter. The $Pro_{EbHKT1;2}$*::AtHKT1;1* and $Pro_{EsHKT1;2}$*::AtHKT1;1* constructs were not detectably expressed, under un-stressed condition.

Tolerance Index

There were evident differences in salt tolerance among various transgenic lines. *A. thaliana* wild type and transgenic under the control of $Pro_{AtHKT1;1}$ and Pro_{35S} looked green and remained healthy. There were no apparent foliar chlorosis/necrosis, however, they showed senescence of the cotyledons and the oldest leaves (~ 10 to 20%). These transgenic lines did not exhibit any mortality upon exposure to 50 mM NaCl. Transgenic lines under the control of $Pro_{EsHKT1;2}$ or $Pro_{EbHKT1;2}$ proved to be sensitive with much smaller leaves. These lines exhibited chlorosis/necrosis with enhanced senescence of the older leaves (~ 40%), as well.

Under exposure to 50 mM NaCl both of the constructs with the *A. thaliana* promoter, $Pro_{AtHKT1;1}$*::AtHKT1;1* and $Pro_{AtHKT1;1}$*::EsHKT1;2*, complemented the *Athkt1;1* mutant, approximately to wild type level (Fig. 2). As expected, the constructs with extremely low expression, did not complement the *Athkt1;1* mutant to any significant extent.

The Pro_{35S}*::EsHKT1;2* constructs complemented the mutant in one of the experiments, but not in the other, in spite of the fact that in both experiments the *HKT* expression level was at least one order of magnitude higher under the CaMV 35S promoter than under any of the others. Overall, the results of the two experiments were consistent (Fig. 2).

The Water Content of Fresh Leaves

Consistent with their tolerance index, lines harbouring the $Pro_{AtHKT1;1}$*::EsHKT1;2* and $Pro_{AtHKT1;1}$*::AtHKT1;1* constructs maintained wild type-like water percentages in their leaves (> 90%) under salinity stress, whereas the plants with the *Eutrema* promoters desiccated to a degree comparable with that of the *Athkt1;1* mutant (Fig. 3). The

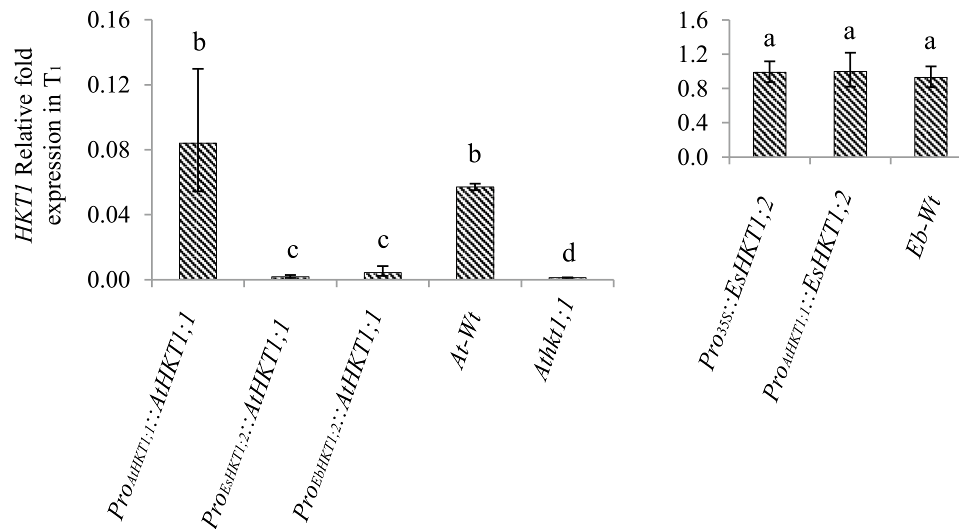

Fig. 1 Expression of *HKT1* in transgenic lines (T_1) harbouring Pro_{35S}::*EsHKT1;2*, $Pro_{AtHKT1;1}$::*EsHKT1;2*, $Pro_{AtHKT1;1}$::*AtHKT1;1*, $Pro_{EsHKT1;2}$::*AtHKT1;1*, $Pro_{EbHKT1;2}$::*AtHKT1;1* construct, using Real-Time quantitative PCR. *A. thaliana* wild type (*At-Wt*) and *Athkt1;1* mutant were used as positive and negative controls, respectively. Transgenic lines with comparable levels of *HKT1* expression were used in subsequent experiments. Each value is an average of 3–4 independent biological replicate ± SE. Significant differences ($p < 0.05$) between means are indicated by different superscripted letters

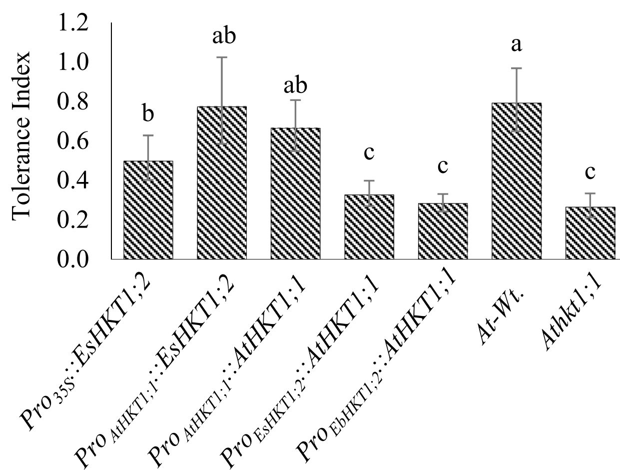

Fig. 2 Tolerance index on fresh weight basis measured in T_1 progeny harbouring Pro_{35S}::*EsHKT1;2*, $Pro_{AtHKT1;1}$::*EsHKT1;2*, $Pro_{AtHKT1;1}$::*AtHKT1;1*, $Pro_{EsHKT1;2}$::*AtHKT1;1*, $Pro_{EbHKT1;2}$::*AtHKT1;1* constructs. Plants were grown hydroponically at 50 mM NaCl stress for 14 days. *A. thaliana* wild type (*At-Wt*) and *Athkt1;1* mutant were used as positive and negative controls, respectively. Ten replicates were used for each genotype and two independent T_1 lines were used in the experiment. Each bar is a mean of 20 plants ± SE. Significant differences ($p < 0.05$) between means are indicated by different superscripted letters

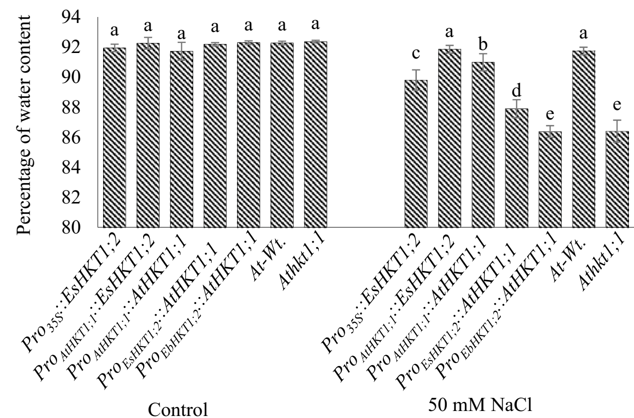

Fig. 3 Water content (percentage of shoot fresh weight) in T_1 progeny harbouring Pro_{35S}::*EsHKT1;2*, $Pro_{AtHKT1;1}$::*EsHKT1;2*, $Pro_{AtHKT1;1}$::*AtHKT1;1*, $Pro_{EsHKT1;2}$::*AtHKT1;1*, $Pro_{EbHKT1;2}$::*AtHKT1;1* constructs. Plants were grown hydroponically at control and 50 mM NaCl stress for 14 days. *A. thaliana* wild type (*At-Wt*) and *Athkt1;1* mutant were used as positive and negative controls, respectively. Ten replicates were used for each genotype and two independent T_1 lines were used in the experiment. Each bar is a mean of 20 plants ± SE. Significant differences ($p < 0.05$) between means are indicated by different superscripted letters

construct with the CaMV 35S-promoter completely complemented the mutant in the first experiment, but only incompletely in the second one. Overall, the results of the two experiments were consistent.

Na^+ and K^+ Analysis in Transgenic T_1 Plants

We compared Na^+ and K^+ accumulation in shoots and roots of wild type and transgenic lines, in two sets of experiment. Average values of Na^+ contents from both experiment were used to depict the results (Fig. 4). In both experiments the

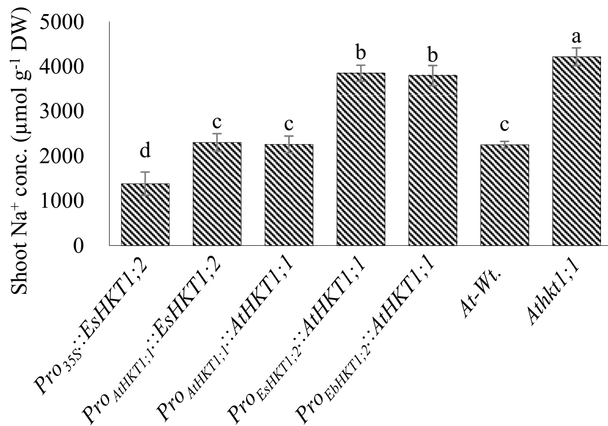

Fig. 4 Na^+ concentrations in shoots of T_1 progeny from *Pro*$_{35S}$*::EsHKT1;2*, *Pro*$_{AtHKT1;1}$*::EsHKT1;2*, *Pro*$_{AtHKT1;1}$*::AtHKT1;1*, *Pro*$_{EsHKT1;2}$*::AtHKT1;1*, *Pro*$_{EbHKT1;2}$*::AtHKT1;1*. Plants were grown hydroponically at control and 50 mM NaCl stress for 14 days. *A. thaliana* wild type (*At-Wt*) and *Athkt1;1* mutant were used as positive and negative controls, respectively. Five replicates (two shoots were pooled together) were used for each genotype and two independent T_1 lines were used in the experiment. Each bar is a mean of 10 plants ± SE. Significant differences ($p < 0.05$) between means are indicated by different superscripted letters

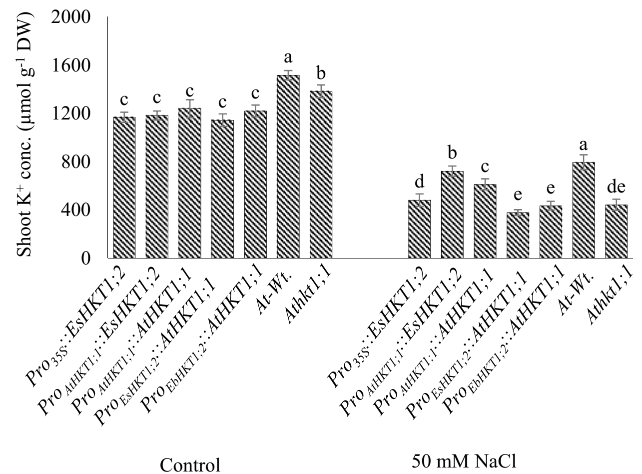

Fig. 5 K^+ concentration in the shoots of T_1 progeny from *Pro*$_{35S}$*::EsHKT1;2*, *Pro*$_{AtHKT1;1}$*::EsHKT1;2*, *Pro*$_{AtHKT1;1}$*::AtHKT1;1*, *Pro*$_{EsHKT1;2}$*::AtHKT1;1*, *Pro*$_{EbHKT1;2}$*::AtHKT1;1*. Plants were grown hydroponically at control and 50 mM NaCl stress for 14 days. *A. thaliana* wild type (*At-Wt*) and *Athkt1;1* mutant were used as positive and negative controls, respectively. Five replicates (two shoots were pooled together) were used for each genotype and two independent T_1 lines were used in the experiment. Each bar is a mean of 20 plants ± SE. Significant differences ($p < 0.05$) between means are indicated by different superscripted letters

genotype with the *Pro*$_{35S}$*::EsHKT1;2* constructs exhibited a lower Na^+ concentration in their shoots than any of the other lines. The foliar Na^+ concentration in the *Pro*$_{AtHKT1;1}$*::AtHKT1;1* and *Pro*$_{AtHKT1;1}$*::EsHKT1;2* lines was significantly higher than in the *Pro*$_{35S}$*::EsHKT1;2* line, but significantly lower than in the lines under either *Es* and/or *Eb* promoters. Although in second experiment, the Na^+ concentration was detected comparatively higher in all genotypes, however, the trend of Na^+ contents remained the same as in both experiments. Overall, results from both experiments exhibited the similar trend (Fig. 4).

The *Athkt1;1* mutant and transgenic lines under either *Es* and/or *Eb* promoters showed similar shoot K^+ concentration, however, wild type showed highest foliar K^+ concentration than all the other lines, under NaCl exposure (Fig. 5). Transgenic line expressing construct *Pro*$_{AtHKT1;1}$*::EsHKT1;2* also maintained significantly higher K^+, upon salt stress, in its shoot as compared to the lines expressing *Pro*$_{AtHKT1;1}$*::AtHKT1;1*.

Na^+/K^+ ratios of shoots and roots of all genotypes were also calculated (Supplementary Figure S1). Transgenic lines under the control of *Es* and/or *Eb* promoter showed Na^+/K^+ ratio in shoot as high as found for *Athkt1;1* mutants and was significantly higher as compared to all other genotypes (Supplementary Figure S1a). Root Na^+/K^+ ratio for lines harbouring constructs *Pro*$_{AtHKT1;1}$*::EsHKT1;2*, *Pro*$_{EsHKT1;2}$*::AtHKT1;1*, *Pro*$_{EbHKT1;2}$*::AtHKT1;1* and *Athkt1;1* mutant, were found significantly low as compared to other genotypes (Supplementary Figure S1b).

Discussion

In this study, we compared the expression pattern and activities of four promoters, i.e., *Pro*$_{AtHKT1;1}$, *Pro*$_{EsHKT1;2}$, *Pro*$_{EbHKT1;2}$ and CaMV 35S promoter, using *AtHKT1;1* and *EsHKT1;2* cDNA. As clearly shown by the tolerance index, only the *Pro*$_{AtHKT1;1}$*::AtHKT1;1* and *Pro*$_{AtHKT1;1}$*::EsHKT1;2* constructs more or less completely complemented the *Athkt1;1* mutant regarding its salt hypersensitivity phenotype. The *Pro*$_{EsHKT1;2}$*::AtHKT1;1* and *Pro*$_{EbHKT1;2}$*::AtHKT1;1* constructs did not yield any detectable complementation at all, while the *Pro*$_{35S}$*::EsHKT1;2* construct only incompletely complemented the mutant. The same conclusion can be drawn on the basis of the foliar water contents in the salt treatment. The complete lack of complementation observed with the *Pro*$_{EsHKT1;2}$*::AtHKT1;1* and *Pro*$_{EbHKT1;2}$*::AtHKT1;1* constructs is doubtlessly owing to low *AtHKT1;1* expression in the transgenic lines. The latter could be due to the absence of essential transcriptional activator/s or, more likely, the lacking an essential response element located upstream of the nucleotides that we used as promoter [30], which are required in *A. thaliana*. The incomplete complementation provided by the *Pro*$_{35S}$*::EsHKT1;2* construct is most probably owing to a non-tissue-specific expression of *EsHKT1;2* [8].

Our observation that *AtHKT1;1* and *EsHKT1;2* are both able to complement the *Athkt1;1* mutant is not self-evident, since AtHKT1;1 is a Na^+-selective transporter [31], whereas

EsHKT1;2 is a K^+-selective transporter, even in the presence of NaCl [14]. AtHKT1;1 is supposed to provide salinity tolerance through resorbing Na^+ from the xylem, thus preventing its accumulation in the shoot [32, 33], while EsHKT1;2 is supposed to maintain a sufficient K^+ concentration in shoot, under salinity stress (Fig. 5) [14, 15]. Foliar Na^+ concentrations were significantly lower for the wild type *A. thaliana* and lines having gene under *AtHKT1;1* promoter, surprisingly regardless of the origin of the coding region, thus both genes doubtlessly complemented the *Athkt1;1* mutant under our experimental conditions. Which suggested that maintenance of sufficient K^+ concentration in shoot might be crucial for the salinity response of the transgenic line expressing $Pro_{AtHKT1;1}$*::EsHKT1;2* [34, 35]. However, the lines with constructs under *Es* and/or *Eb* promoters showed the foliar Na^+ contents as high as in the *Athkt1;1* mutant plants and failed to revert the Na^+ hypersensitivity of the mutants. Lines having construct under *AtHKT1;1* promoter showed comparatively high K^+ concentrations as compared to the lines with the construct under *Es* and/or *Eb* promoters, however, the difference was not too high between all lines.

Low-cytosolic Na^+/K^+ ratio is a major determinant for the salt stress tolerance of any plant which ultimately play important role in growth and developmental success of the plant. Lower Na^+/K^+ ratio in shoots of the lines harbouring construct Pro_{35S}*::EsHKT1;2*, $Pro_{AtHKT1;1}$*::EsHKT1;2* and $Pro_{AtHKT1;1}$*::AtHKT1;1* as well as *At-Wt*. Positively correlate with salinity tolerance of these genotypes. Low Na^+/K^+ ratio in shoot depicting plants ability to restrict more Na^+ contents in roots, and has been proved as a determinantal factor in *Zea mays* L. salt tolerance [36]. Low Na^+/K^+ ratio in roots of transgenic lines with $Pro_{AtHKT1;1}$*::EsHKT1;2* indicate the higher contents of K^+. Surprisingly, Pro_{35S}*::EsHKT1;2* showed high Na^+/K^+ ratio, which may be attributed to ectopic expression of *EsHKT1;2* under CaMV 35S promoter [8]. Overall, Na^+/K^+ ratio in roots of other genotypes did not correlate positively with their salinity tolerance.

We identified many putative *cis*-acting regulatory abiotic stress-responsive elements. The identified core motif related to dehydration-responsive element/C-repeat (DRE/CRT), has been reported for its role in drought and high salt-stress conditions [37]. Another important *cis*-acting regulatory element, GT-1 was also found which has been predicted to be involved in gene induction (*SCaM-4*) under salt stress, in *Glycine max* [38]. Identified CAAT-box and ACGT *cis*-acting DNA sequences have been reported to be required for induction of various genes such as dehydration-responsive gene (*AtNCED3*) [39, 40]. Prabu and Parsad [41] demonstrated the putative role of *Saccharum officinarum MYB* gene and showed that the promoter activity was increased by 2- to 4-fold under salt, cold and dehydration stress. The indentified putative *cis*-acting elements (ACGT and MYC) were present in higher number in the promoter sequence of *EbHKT1;2*, whereas the GT-1 *cis*-element, CAAT-box and MYBCORE were present at higher numbers in *A. thaliana.* Baek et al. [30] have reported two tandem repeats (R1 and R2) in *AtHKT1;1* promoter region (~ 3.9 kb upstream of the start codon) and suggest that these repeats are involved in *AtHKT1;1* regulation under salt stress. Unfortunately, we could not include longer fragment of *AtHKT1;1* promoter however, expression of *AtHKT1;1* transcripts in transgenic lines harboring *AtHKT1;1* under the control of its native promoter (0.846 kb from ATG), was comparable to wild type expression level (Fig. 1). This indicates that 0.846 kb of *AtHKT1;1* promoter is functionally active and is complementing the *Athkt1;1* mutant up to wild type level (Fig. 2). In any case, the role of above mentioned identified *cis*-acting elements in the Eutrema *HKT1;2* promoters, if any, should be established by a series of promoter-deletion experiments.

Conclusions

Expression under the *E. salsugineum* and *E. botschantzevii* promoters did not yield any significant expression, either because of the lack of an essential upstream response element/s, or lacking an essential *trans*-acting regulator/s, in *A. thaliana*, and thus failed to complement the *Athkt1;1* mutant. When the gene is expressed under *AtHKT1;1* promoter, both *AtHKT1;1* and *EsHKT1;2* fully complemented the mutant, in that they restored a wild type-like salt tolerance level. Several abiotic stress-responsive element have been identified in *EsHKT1;2* and *EbHKT1;2* promoter regions.

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