Downregulation of high-affinity potassium and sodium symporter gene, *EcHKT1;1,* in *Eucalyptus* roots enhances salt tolerance

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Abstract

Engineering for restricted root Na^+ uptake could potentially enhance salt tolerance in Eucalyptus. High-affinity K^+ transporters (HKTs) have been implicated in Na^+ uptake from the external medium as in the case of TaHKT2;1 or in the unloading of Na^+ from xylem like in AtHKT1;1. To rapidly determine the *in planta* role of *EcHKT1:1*, composite transgenics in which *EcHKT1:1* was specifically downregulated via RNAi in the roots were generated. Compared to the controls that failed to survive at 350 mM NaCl, 33 % of the composite transgenic plantlets generated using the *EcHKT1;1* silencing construct were able to tolerate up to 400 mM NaCl. In these composite transgenics, *EcHKT1;1* downregulation ranged from 37 % to 74 %. The average shoot to root ratio of sodium was 4.9 folds lower than the controls indicating restricted translocation of Na^+ to the shoots. Relative expression analysis in the leaves of two non-transgenic genotypes contrasting for their salt tolerance also showed downregulated

EcHKT1;1 expression in the tolerant clone. The study thus determined that *EcHKT1;1* is a major gene determining Na^+ transport from the roots to shoots. This study also demonstrated the utility of the composite transgenic approach for screening genes conferring salt tolerance in tree species.

KEYWORDS

gene silencing, RNAi, Na^+ transporters, salt tolerance, composite transgenics

1. INTRODUCTION

Eucalyptus is the second most widely planted tree species. Its wide adaptability, fast growth rate, and its utility in paper and pulping industries make it a preferred plantation tree in arid areas. *Eucalyptus camaldulensis* and *E. tereticornis* have been recommended for growing in arid regions with saline water irrigation, and have been shown to tolerate salinity levels up to 10 dS m-1 (Ansar*i et* al., 2007). However, salinity affects the productivity of Eucalyptus (Chauha*n et* al., 2014). In a trial of 31 trees under saline irrigation, *E. tereticornis* was shown to accumulate a high percent (3.37 %) of Na^+ compared to the mean of 1.04 % in other tree species (Toma*r et* al., 2003). Targeting Na^+ transporters to limit Na^+ uptake to the shoots could hence be a potential strategy for enhancing salt

tolerance in Eucalyptus. Determining the major genes that determine Na^+ uptake in Eucalyptus is essential.

HKT transporters are plasma membrane proteins that mediate Na^+-uniport or Na^+/K^+-symport and Mg^{2+}/Ca^{2+} permeation to maintain K^+/ Na^+ homeostasis (S*u et al*., 2015). Based on the cation selectivity, the HKT family of proteins is classified into two major subfamilies. Subfamily 1 members contain a serine residue in the first MPM domain and are generally Na^+ selective. Subfamily 2 members have the serine replaced by glycine (except for OsHKT2;1) and can catalyse the transport of K^+ and high-affinity Na^+ influx (Mäse*r et al*., 2002; Platte*n et al*., 2006; Hori*e et al*., 2007). *HKT* genes from dicots like *A. thaliana* (*AtHKT1;1*) fall within subfamily 1 while those from monocots are divided between subfamily 1 (*OsHKT1;1, 1;2, 1;3, 1;4, 1;5*) and subfamily 2 (*TaHKT2;1, OsHKT 2;1, 2;2, 2;3, 2;4*) (Platte*n et al*., 2006). Both the Eucalyptus *HKT1* homologs, *EcHKT1;1* and *EcHKT1;2*, belong to subfamily 1, and have serine in the first MpM domain. However, they were shown to mediate K^+ and Na^+ symport when heterologously expressed in *Xenopus oocytes* (Fairbair*n et al*., 2000; Li*u et al*., 2001). The transport selectivity of *EcHKT1*;*1* needs to be confirmed *in planta* or other expression systems (Water*s et al*., 2013).

In the case of *AtHKT1;1* gene, loss of function mutations led to an accumulation of Na^+ in Arabidopsis shoots, rendering the plant Na^+

hypersensitive (Mäser *et al*., 2002; Berthomieu *et al*., 2003). *AtHKT1;1* was identified to play a role in retrieving Na^+ from the root xylem before it reaches the shoot (Sunarpi *et al*., 2005; Davenport *et al*., 2005). Contrastingly, transgenic wheat plants expressing a *TaHKT2;1* antisense construct showed reduced Na^+:K^+ ratios and improved Na^+ tolerance under saline conditions (Laurie *et al*., 2002). These results have suggested that *TaHKT2;1* plays a role in Na^+ uptake from the external medium.

In planta studies in Eucalyptus are required to evaluate if *EcHKT1;1* is involved in unloading of Na^+ from xylem or uptake of Na^+ from the external medium. This will help to determine if the gene needs to be downregulated or upregulated for enhancing salt tolerance in Eucalyptus. In tree species like *Eucalyptus*, low transformation efficiency ranging from 0.003 to 7.4 % using *Agrobacterium tumefaciens* (Oliveira *et al*., 2011; Matsunaga *et al*., 2012), and the time taken for a plant to regenerate from transformed tissues limit rapid gene function analysis. Transformation of *E. camaldulensis* is considered challenging (Chauhan *et al*., 2014). Heterologous gene expression studies in model plants like *Arabidopsis* or *Nicotiana tabacum* may not provide the genomic and physiological context of Eucalyptus for deciphering the exact functional contribution of genes to the trait. Composite plants with transgenic hairy roots derived via *A. rhizogenes* mediated transformation are thus increasingly being used in tree species with such limitations to rapidly study root development and root-biotic interactions (Gherbi *et al*., 2008;

Svistoonoff *et al*., 2013). The composite plant strategy with GFP roots was generated for the first time in the difficult to transform tree, *Eucalyptus camaldulensis* (Balasubramanian *et al*., 2011). The technique has been successfully applied for validating the function of the lignin biosynthesis gene *EgCCR1* in *E. grandis* (Plasencia *et al*., 2016; Dai *et al*., 2020). Roots being the portal of Na^+ entry into plants, composite transgenic approaches may provide a suitable *in planta* system for functional analysis of genes involved in Na^+ uptake from the roots.

The present work therefore attempts for the first time to study the feasibility of functional evaluation of sodium transporter gene *EcHKT1;1* in *E. camaldulensis* by combining gene silencing via RNAi with the ease of development of composite plants having transgenic hairy roots. Furthermore, sequence and expression variation of *EcHKT1:1* in contrasting genotypes of non-transgenic *E. camaldulensis / E. tereticornis* were also analyzed to gain additional insights on the role of this gene.

2 MATERIALS AND METHODS

2.1 *HKT1;1* sequence variation in salt-tolerant and susceptible *Eucalyptus* clones

Two clones, *Eucalyptus camaldulensis* clone-7 (EC-7) and *Eucalyptus tereticornis* clone-88 (ET-88), were analyzed in this study. These clones had been reported to be contrasting for their salt tolerance (Yasodha *et al.,* 2011). Total RNA was isolated from leaves of salt-tolerant EC-7 and susceptible ET-88 using RNeasy Plant Mini Kit (Qiagen, USA) according to manufacturers instruction with additional use of 2 % polyvinyl polypyrrolidone (PVPP) and TURBO DNase (Thermo Fisher Scientific, USA) treatment. cDNA was synthesized using SMARTScribe reverse transcriptase (TaKaRa, Japan) according to the manufacturer's instructions. Complete coding sequences of *HKT1;1* gene from EC-7 and ET-88 cDNA were PCR (polymerase chain reaction) amplified independently using the primers designed based on *EcHKT1;1* sequence available at NCBI GenBank (Accession Number: AF176035.1), sequenced and analyzed. A phylogenetic tree was constructed for the

publicly available HKT1 proteins (205), EC-7 *HKT1;1* and ET-88 *HKT1;1* translated protein sequences using MEGA 7.0.14.

2.2 *HKT1;1* expression variation in salt-tolerant and susceptible *Eucalyptus* clones

Vegetative propagules of the clones EC-7 and ET-88 were multiplied in the nursery of IFGTB, Coimbatore. The three months old ramets were transferred to pots containing Hoagland's solution (Hoagland & Arnon 1950) and allowed to acclimatize for two months under greenhouse conditions with regular medium replacement in subsequent weeks. The clones were subjected to 150 mM NaCl treatment, and leaves were collected to study *HKT1;1* gene expression prior to salt application (0 min), and 15 min and 24 h post salt treatments. Total RNA was isolated from frozen shoots of salt-stressed (150 mM NaCl: 0 h, 15 min, and 24 h) EC-7 and ET-88, and cDNAs were synthesised.

Determination of relative expression of *HKT1* transcripts

The primer pairs were synthesized for the genes *Actin* (*Act2*) (Boav*a et al*., 2010), *NADP isocitrate dehydrogenase* (*IDH*), and *SAND* (de Almeid*a et al*., 2010). These reference genes had been used in differential expression

studies in different tissues of *E. tereticornis* (Sundari & Dasgupta 2012). The qRT-PCR reaction components included 9.5 µl nucleasefree water, 150 ng of cDNA, 10 µl SYBR pre-mix Ex-Taq (TaKaRa, Japan), ROX reference dye (50X) (TaKaRa, Japan), and 0.25 µl of 100 nM forward and reverse primer. A no-template control was also included. All reactions were conducted in three independent biological replicates in Step-One Plus Real-Time PCR system (Applied Biosystems, USA) using the following program: one cycle of 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. The expression level and stability of three endogenous reference genes in different salt stress conditions were evaluated using GeNorm (Vandesompel*e et al*., 2002), Normfinder (Anders*en et* al., 2004), and BestKeeper (Pfaff*l et al*., 2004).

The relative expression profiles of *HKT1* transcripts of EC-7 and ET-88 were analyzed for two different time points post salt stress at 150 mM NaCl. All samples were tested in three biological replicates, including three technical replicates. The relative expression analysis was obtained through the $2^{-\Delta\Delta CT}$ method using *SAND* transcripts as endogenous control. The C_T values for both the target and internal reference genes were used to quantify transcripts by comparative C_T method normalization. The expression of the target gene was calculated using the formula: $2^{-\Delta\Delta CT}$

where $\Delta\Delta C_T = \Delta C_T$ *HKT1*-ΔC_T *SAND* (Livak and Schmittgen, 2001) and $\Delta\Delta C_T$ values reflect the relative expression of the target gene.

Triplicate samples of leaves were collected at each time point, *viz*., 0 min, 15 min, and 24 h. Real-time PCR was performed on the corresponding cDNA synthesized from each sample. The C_T values were imported into Microsoft Excel. The change in expression of the *HKT1;1* gene was normalized to *SAND* and analyzed for the three-time points *viz.,* 0 min, 15 min, and 24 h. The fold change in the target gene normalized to *SAND* and relative to the expression at time zero was calculated for each sample using the $2^{-\Delta\Delta CT}$ method, where $\Delta\Delta C_T = (C_{T,\ HKT1} - C_{T,\ SAND})_{\text{Time x}} - (C_{T,\ HKT1} - C_{T,\ SAND})_{\text{Time 0}}$. For the untreated control sample at time 0 min, $\Delta\Delta C_T$ equals zero, and therefore, the fold change in gene expression relative to the untreated control equals one. For the treated samples at 15 min and 24 h, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression relative to the untreated control at time 0 min. Similarly, the relative expression of *HKT1* in the salt-tolerant clone, EC-7, was analyzed using the susceptible clone, ET-88, as the calibrator sample.

2.3 Variation in Na^+ levels in salt-tolerant and susceptible *Eucalyptus* clones

Samples were collected to study the variation in Na^+ accumulation in roots and shoots of three ramets each of the salt-tolerant EC-7 and salt-sensitive ET-88 clones that were subjected to salt stress at 150 mM NaCl (at 0 min,

15 min, and 24 h). Sodium levels were estimated using Flame photometer 128 (Systronics, India) (Sadasivam & Manickam 1997).

2.4 Generation of *Eucalyptus* composite transgenic plants for functional analysis of *EcHKT1;1* gene

Plant material, bacterial strain, and transformation vector

Aseptic seeds of *E. camaldulensis* (Clone-1) were inoculated in bottles containing half-strength MS media (Murashige & Skoog 1962) and maintained at 24°C in a transgenic culture room providing a 16 h photoperiod. The axenic seedlings sprouted after two weeks of inoculation. The *Agrobacterium rhizogenes* A4RS strain (Jouanin *et al.,* 1986) was used in this study for hairy root induction. A4RS strain transformed with pHKN29 plasmid harboring a *35SCaMV::GFP* fusion (Kumagai & Kouchi 2003) was used as control. Plasmid pHKN29 is a derivative of pCAMBIA1300 with the hygromycin resistance *(Hyg)* gene replaced with the *sGFP(65T)* gene (Niw*a et al*., 1999) (Figure 1 Supporting Information).

Development of *EcHKT1;1* silencing construct for functional analysis

The full-length sequence of *EcHKT1;1* gene (Accession Number: AF176035.1) was retrieved from NCBI GenBank database. A unique region in this sequence was identified as an RNAi target region by BLASTN analysis of different segments of the *EcHKT1;1* gene in Kazusa and NCBI database. To avoid off-target effects in insect pollinators of

Eucalyptus, the target region was also analyzed for uniqueness using sequences available in FlyBase (http://flybase.org/) database. For PCR amplification of this RNAi target region, the forward primer was designed with an additional 4-nucleotide GGGG at the 5' end, followed by 25 bp of *att*B1 and 22 bp *EcHKT1;1* sequence. For the reverse primer, the four guanine residues were followed by 25 bp *att*B2 and 23 bp of *EcHKT1;1* sequence (Table 1 in Supporting Information). The PCR reaction was performed using 100 ng of genomic DNA isolated from *E. camaldulensis* clone-7 in a 100 µl reaction mixture containing 10 µl of 10X PCR buffer, 1 mM of dNTPs, 25 pM each of primers, and 10 units of Taq DNA polymerase (Invitrogen, USA). Amplification was performed in the Verti PCR system (Applied Biosystems, USA). The temperature profile used for amplification included initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 53°C for 40 sec and synthesis at 68°C for 40 sec, followed by a final extension at 68°C for 10 min. The obtained *EcHKT1;1 attB1/attB2*-flanked PCR product was electrophoresed on a 1.5 % (w/v) agarose gel. The desired 197 bp amplicon was eluted and purified using the GeneJet gel extraction kit (Thermo Fisher Scientific, USA). The amplicon was cloned into a high-throughput binary Gateway® destination vector, pHELLSGATE12 (CSIRO, Australia), to obtain a construct having the target sequence in opposite orientation at both sides of *Pyruvate decarboxylase kinase* (*Pdk) Catalase* intron. Cloning into pHELLSGATE12 containing *attR1* and

attR2 sites was performed via an intermediate entry vector pDONR207 with *attP* sites in a two-step process by Gateway cloning (Invitrogen). Plasmid DNA was isolated from PCR positive *EcHKT1;1* Gateway LR clones and subjected to restriction digestion with *Xba*I and *Xho*I to confirm the presence of the *PDK* intron and the correct orientation of the inserts. The constructed hpRNA cassette was excised, ligated to a green fluorescent protein-based binary vector pHKN29 at *Spe*I and *Sac*I restriction sites to develop pHKN29 *EcHKT1;1*hpRNAi construct. A4RS strain was transformed with the pHKN29 *EcHKT1;1*hpRNAi by electroporation and the integrity was confirmed by PCR, restriction digestion, and back transformation (Sambrook & Russell 2001).

Generation of *Eucalyptus* composite transgenics using *EcHKT1;1* silencing construct

A4RS strain harboring pHKN29 *EcHKT1;1*hpRNAi transformation construct was grown on Yeast Extract Peptone (YEP) broth, pH 7.2, with respective antibiotics and incubated overnight at 28ºC. The overnight-grown culture was re-inoculated in YEP broth, pH 5.3, and incubated at 28ºC until an OD_{600nm} 0.8 was obtained. The cells were then centrifuged at 4000 rpm for 2 min at 4°C, and the supernatant was discarded. The pellet was resuspended in 100 mM modified MS media. Roots of *E. camaldulensis* plantlets were removed aseptically, and the shoots were placed in sterile disposable Petri plates. The plantlets were pricked

manually 4-6 times using a sterile syringe needle at the hypocotyl region, immersed in the A4RS suspension culture, and incubated for 20 min. The excess bacterial inoculum was removed off by blotting the plantlets on a sterile filter paper. All the explants were co-cultivated on modified MS medium, pH 5.3, supplemented with 100 µM acetosyringone, casamino acid 300 mg L^{-1} (BD Bioscience, France), 20 g L^{-1} D-glucose, and 0.7 % bactoagar (BD Bioscience, France) for 7 days at 16.5ºC. Co-cultivated plantlets were subcultured in modified MS media with cefotaxime 500 mg L^{-1} and kept at 24°C in the dark. Plantlets were subcultured every two weeks onto modified MS media containing decreasing concentrations of cefotaxime (500, 250, 100 mg L^{-1}) to remove A4RS agrobacteria from the explants completely. The control plantlets that were transformed using A4RS strain with the pHKN29 control vector and without silencing insert were also generated. The expression of the GFP gene in hairy roots emerging from the infected sites of *Eucalyptus* was examined under a stereo fluorescence microscope SMZ-1000 (Nikon, Japan) following excitation by a blue light source (λex = 460-500 nm excitation filter; λem = 510 nm Long pass barrier filter). The transformation efficiency was calculated as a percentage of GFP expressing roots over total explants having hairy roots. The non-transformed roots were dehisced off using sterile blades and transferred to Hoagland hydroponic solution, and

hardened in the transgenic greenhouse. Composite plantlets were subjected to weekly incremental concentration of 50 mM NaCl in hydroponic solution. Total RNA was isolated from roots of salt treated (150 mM NaCl: 15 min) *Eucalyptus EcHKT1;1*hpRNAi and pHKN29 control plantlets, cDNA synthesized. Relative quantitative expression analysis of *EcHKT1;1* gene was performed using *SAND* protein as endogenous reference gene. Sodium concentrations in roots and shoots of untreated and salt-stressed (350 mM) *Eucalyptus* composite plantlets generated using A4RS pHKN29 and A4RS pHKN29*EcHKT1;1*hpRNAi were estimated.

3. RESULTS

3.1 *HKT1;1* sequence variation in salt-tolerant and susceptible *Eucalyptus* clones

PCR amplification of the coding region of *HKT1;1* homologues from cDNA of EC-7 and ET-88 using gene-specific primers resulted in amplification of 1653 bp of *HKT1* gene. The *HKT1;1* coding region of EC-7 and ET-88 were found to have high sequence homology (>99 % and >97 % respectively) with the already reported *EcHKT1;1* gene sequences (Accession No. AF176035.1). Fifty-one SNPs were observed between EC-

7 and ET-88 *HKT1;1* coding regions (Table 2 in supplementary data). ExPASy tool was used to obtain the translated amino acid sequence. The *HKT1;1* translated sequences of EC-7 and ET-88 comprised 550 and 521 amino acid sequences, respectively. The *HKT1* amino acid sequences of EC-7 and ET-88 showed > 84.5 % homology with the already reported *EcHKT1;1* protein sequences (AAF97728.1). Eighty-four amino acid sequence variations were identified between *HKT1* amino acid sequences of EC-7 and ET-88 (Table 3 in Supporting Information). The *HKT1;1* amino acid sequences of EC-7 and ET-88, along with 203 other plant *HKT1* protein sequences (205) that are available in public databases, were used to reconstruct a phylogenetic tree. The resultant cladogram was divided into three subfamilies. Subfamily 1 had 5 subclusters with *EcHKT1;1* and *EtHKT1;1* grouping in the fifth subcluster (Figure 2 in Supporting Information). The multiple sequence alignment of these 205 proteins was used to determine the four putative P-loops of the HKT1;1 protein of EC-7 and ET-88. Both these proteins had the signature S-G-G-G in the four MPM domains, with the MP_cM motif showing a relatively higher number of variations in amino acid sequences.

3.2 *HKT1;1* expression variation in salt-tolerant and susceptible *Eucalyptus* clones

Using Normfinder, GeNorm, and BestKeeper, *SAND* protein was identified as the most stable reference gene that could be used for data normalization in *HKT1;1* expression experiments (Table 1).

The relative expression analysis of *HKT1;1* was obtained through the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) using *SAND* transcripts as an endogenous control in salt-stressed (150 mM NaCl: 0, 15 min, and 24 h) shoots of salt-tolerant EC-7 and susceptible ET-88 *Eucalyptus* clones. Relative quantification was carried out both using 0 min and ET-88 as the calibrator. Time-course salt stress experiments revealed a 10 fold reduction in *HKT1;1* gene expression in the salt-tolerant clone EC-7. The susceptible clone showed no such changes (Figure 1).

3.3 Variation in Na^+ and K^+ levels in salt-tolerant and susceptible *Eucalyptus* clones

Na^+ levels in roots and shoots of salt-stressed (150 mM NaCl: 0, 15 min, and 24 h) salt-tolerant EC-7 and salt-sensitive ET-88 were estimated using Flame photometer (Figure 2). At 0 min, 15 min, and 24 h post salt treatments, the roots of EC-7 had respectively 4.51, 4.17, and 2.75 fold higher Na^+ concentrations when compared to ET-88. In the case of shoots, the Na^+ concentrations were 1.52, 2.51, and 6.84 folds higher in ET-88 when compared to EC-7 at 0 min, 15 min, and 24 h (Figure 3). Under

untreated and treated conditions, the salt-tolerant clone, EC-7, showed a higher accumulation of Na^+ in roots when compared to the salt-sensitive clone ET-88. Under salt treated conditions, there was a higher accumulation of Na^+ in shoots of the salt-sensitive clone, ET-88, compared to the salt-tolerant clone EC-7. Analyses of the shoot to root ratio of sodium clearly showed that the tolerant clone, EC-7, could retain sodium in the roots while accumulating lesser sodium in shoots.

3.4 Generation of *Eucalyptus* composite transgenic plants for functional analysis of *EcHKT1;1* gene

Development of *EcHKT1;1* silencing construct for functional analysis

A 197 bp sequence from 1079 to 1275 bp in the first exon of *EcHKT1;1* was selected as the unique RNAi target region. For any continuous stretch of 21-23 nucleotides in this region, BLAST analysis did not show similarities with any other gene sequences. The unique RNAi target region was PCR amplified from EC-7 genomic DNA using sequence-specific primer flanked with *attB* sites to get a 247 bp fragment. This product was cloned into a pDONR207 vector containing the corresponding *attP* signals in BP reaction to generate *EcHKT1;1* entry clone. Recombination was initiated between a flanking pair of *attL* sites

and *attR* sites of the *EcHKT1;1* entry clone and pHELLSGATE12 destination vector, respectively. Restriction digestion of *EcHKT1;1* Gateway LR clones with *Xho*I and *Xba*I enzymes resulted in the release of plasmid fragments of 257 bp and 259 bp, respectively, indicating the correct orientation of the inserts in pHELLSGATE12 (Figure 3 in Supporting Information). The linearized pHKN29 vector and *EcHKT1;1*hpRNAi Gateway cassette using *Spe*I and *Sac*I restriction enzymes were ligated using T4 DNA ligase. The plasmid containing *EcHKT1;1*hpRNAi gene construct (pHKN29 *EcHKT1;1*hpRNAi) was electroporated into *A. rhizogenes* A4RS strains.

Generation and analysis of *Eucalyptus* composite transgenics using *EcHKT1;1* silencing construct

The A4RS strain harboring the *EcHKT1;1* silencing construct (pHKN29 *EcHKT1;1*hpRNAi) was used to generate 108 composite transgenic plants of *E. camaldulensis*. A transformation efficiency of 61.71 % at an average of 1.92 GFP expressing roots per plant was generated (Figure 4). Sixty-five composite transgenic plants were also generated using *A. rhizogenes* strain A4RS pHKN29 with a transformation efficiency of 51.72 % at an average of 1.44 GFP expressing roots per plant (Figure 5). Additionally, 90 composite plants were generated using A4RS strain for use as control

plants that do not express GFP. The expression of GFP in roots of transgenic plants was confirmed, and non-transgenic roots were removed aseptically. *Eucalyptus* composite transgenic plants with *EcHKT1;1* silencing constructs, and controls were transferred to Hoagland's solution and hardened.

Eighty composite transgenics generated using A4RS pHKN29 *EcHKT1;1*hpRNAi construct, 40 composite transgenics generated using A4RS pHKN29, and 40 composite plants generated using A4RS were subjected to weekly incremental salt stress conditions from 50 mM to 400 mM NaCl (Figure 6). Ten composite plants each generated using A4RS, A4RS pHKN29, and A4RS pHKN29 *EcHKT1;1*hpRNAi constructs were kept without salt treatment for use as controls.

qRT-PCR analysis was performed using *SAND* protein as endogenous control on roots of five independent *EcHKT1* silenced composite transgenic plants. Downregulation of *EcHKT1;1* in the *EcHKT1;1*hpRNAi composite transgenics ranged from 37 % to 74 % compared with the *Eucalyptus* control transgenic plant (Figure 7).

After 1 week on 200 mM NaCl stress, 90 % of the control plants showed rapid growth and had larger green leaves, whereas no morphological changes were observed in *EcHKT1;1* downregulated plants (Figure 8).

One week post 300 mM NaCl stress, leaf discoloration and mortality (67.5 % and 57.5 %) were observed in the control group whereas no mortality was observed in *EcHKT1;1* silenced composite transgenic plants. At one week post 350 mM NaCl stress, none of the A4RS derived control plants survived, compared to 60 % survival in the A4RS pHKN29 *EcHKT1;1hpRNAi* derived plants. A survival of 12.5 % of the control plants generated using A4RS pHKN29 was observed at 350 mM NaCl. The survival of these plants could be attributed to genotypic variations in seedling-raised plants. Thirty percent of *Eucalyptus* composite transgenic plants generated using A4RS pHKN29 *EcHKT1;1hpRNAi* were able to survive up to 400 mM NaCl stress when compared to 0 % survival for *Eucalyptus* composite transgenic plants transformed using pHKN29 vector (Figure 9).

Sodium (Na^+) levels in roots and shoots of salt-stressed (350 mM NaCl) *Eucalyptus* composite transgenic plantlets were quantified using Flame photometer (Figure 10). Under salt treated conditions, the Na^+ accumulation in roots of *Eucalyptus* composite transgenic plants generated using the *EcHKT1;1* silencing construct was 2.04 fold higher at 0.51 %, compared to the control plants developed using the pHKN29 vector (0.25 %). Contrastingly, in the shoots of composite plantlets generated using *EcHKT1;1* silencing construct, the percentage of Na^+ was 2.5 fold lower at 0.52 % when compared to the control plants developed using pHKN29

vector (1.30 %) under salt treated conditions. The average shoot-to-root ratio of sodium in the salt treated *EcHKT1;1* silenced composite transgenics was 4.88 folds lower than the controls. In untreated conditions also, the *EcHKT1;1* silenced composite transgenics showed 1.26 folds lower shoot to ratio of sodium (Figure 11). These observations indicated that downregulation of *EcHKT1:1* in roots results in restricted translocation of Na^+ to the shoots resulting in improved salt tolerance in *Eucalyptus EcHKT1;1 hp*RNA*i* composite transgenic plants.

4. DISCUSSION

HKT1;1 sequence variation in salt-tolerant and susceptible *Eucalyptus* clones

The full-length coding regions of *HKT1;1* from EC-7 and ET-88 were of the same length of 1653 bp and had homology of 99 % and 97 %, respectively, with the sequence of *EcHKT1;1* (Acc. No. AF176035.1) reported by Fairbair*n et al*. (2000). The *HKT1;1* homologue of EC-7 and ET-88 were found to have high sequence homology (97 %) with 51 identified SNPs. The translated protein of HKT1;1 from EC-7 encodes a protein of 550 (Acc. No. AAF97728.1) amino acid residues and had 99 % homology and 4 amino acid sequence variations with *EcHKT1:1* (Acc.No. AF176035.1). Interestingly, the HKT1;1 from ET-88 encoded a shorter ORF of 521 amino acids and lacked C- terminus due to a stop codon in the 3rd exon. It had 84.75 % homology with HKT1;1 from EC-7. Eighty-four

amino acid variations between *EcHKT1;1* homologue of EC-7 and ET-88 were identified. ET-88 HKT1;1 protein as visualized through interactive protein feature visualization via Protter analysis (Omasit*s et al*., 2014) depicts the loss of the 8th membrane-spanning region (Figure 4 in supporting Information).

Phylogenetic analyses of 205 publically available HKT1 protein sequences, including the two *Eucalyptus* sequences identified in this study *viz.,* EC-7 HKT1;1 and ET-88 HKT1;1, grouped the HKT1 transporters into three subfamilies. HKT1 proteins of EC-7 and ET-88 along with other *Eucalyptus* HKT1;2 protein (Acc. No. AAD53890.1) formed a separate clade in subfamily 1, which also included salt-tolerant species. Plant HKTs are classified as Ser-Gly-Gly-Gly type and Gly-Gly-Gly-Gly type (Hauser & Horie 2010; S*u et al*., 2015). Members of subfamily 1 HKT transporters, which includes those of monocots and dicots, are all characterized by Serine in the first loop (P_A) (Garciadeblá*s et* al., 2003), whereas subfamily 2 and 3 members have glycine residue (S*u et al*., 2015). HKT transporters of subfamily 3 are related to subfamily 2 with Gly in the first MP_AM motif. Table 2 provides a list of HKTs along with their Na^+ or Na^+/ K^+ transport properties. The two HKT1;1 sequences identified in this study *viz.,* EC-7 HKT1;1 and ET-88 HKT1;1 have serine residue present at the first MP_AM motif that is characteristic of a Na^+

uniporter. Interestingly, EcHKT1;1 and EcHKT1;2, when expressed in *Xenopus* oocytes, showed Na^+ and K^+ influx rather than a Na^+ selective transport property (Fairbair*n et al*., 2000; Li*u et al*., 2001). Unlike the wheat *TaHKT2;1, EcHKT1;1* was reported to show sensitivity to changes in the external osmolarity of the solution, thereby possibly enabling *E. camaldulensis* to maintain K^+ homeostasis under water-logged conditions (Li*u et al*., 2001). Similar Na^+ and K^+ transport activity has also been reported in *OsHKT2;1,* which has Ser-Gly-Gly-Gly selective motif (Jabnoun*e et a*l. 2009). The K^+- Na^+ co-transport observed in EcHKT1;1, despite having Ser-Gly-Gly-Gly selective motif, indicate that additional domains may be involved in aiding K^+ transport.

HKT1;1 **expression variation in salt-tolerant and susceptible** ***Eucalyptus*** **clones**

Three different endogenous genes, *viz., SAND* protein*, IDH,* and *Act2,* were selected based on earlier reports from other *Eucalyptus* species (de Almeid*a et al*., 2010; Moura *et al*., 2012; Sundari & Dasgupta 2012). *SAND* protein was identified as the most suitable reference gene that could be used for gene expression analysis in *E. camaldulensis* and *E. tereticornis* under salt-stressed conditions. In a study by Moura *et a*l. (2012), *SAND, IDH, Act, UBQ,* and *ATub* were identified as suitable

normalizer gene for expression studies in *E. globulus* and *E. urograndis* under different stress conditions like temperature, water, and light.

Fairbair*n et a*l. (2000) reported that *EcHKT1;1* and *EcHKT1;2* transcripts were expressed in leaves, stems, and roots. *EcHKT1;1* transcripts were more abundant than *EcHKT1;2*. *EcHKT1;1* expression was high in the stem when compared to leaves and roots, whereas *EcHKT1;2* expression was higher in stem and leaves than in roots. In the present study, the relative expression profiles of *HKT1;1* transcripts in leaves of salt-tolerant EC-7 and susceptible ET-88 were analyzed under salt stress conditions. The tolerant clone, EC-7 showed a 9.61 fold downregulation of *HKT1;1* expression 24 h post salt stress, while the susceptible clone ET-88 showed no such changes. Under untreated conditions, there was a higher accumulation of Na^+ in roots of the salt-tolerant clone EC-7 than in the salt-sensitive clone ET-88. Under treated conditions, it was observed that the Na^+ concentration in the shoot increased by 6.57 folds in the salt-sensitive clone, ET-88, while in EC-7, Na^+ concentration increase was only by 1.47 folds. These results indicate that the tolerant clone EC-7 is able to restrict Na^+ uptake to the shoot, while the susceptible clone has limited capacity to restrict the transport of Na^+ into the shoot. It was also observed that the susceptible clone ET-88 had a higher shoot to root ratio

of Na^+ when compared to the tolerant clone. This preferential retention of Na^+ in the roots of the tolerant clone, EC-7, could possibly be due to reduced transport of Na^+ effected through a lower expression of *HKT1;1* gene in EC-7. Under salt treated conditions, salt-sensitive clone, ET-88 accumulates 3.42 % of Na^+ in its shoots. The results agree with the finding of Tomar *et a*l. (2003) in a trial of 31 trees under saline irrigation, wherein *E. tereticornis* was shown to accumulate a high percent (3.37 %) of sodium when compared to the mean of 1.04 % in other tree species. It is, therefore, expected that engineering reduced uptake of sodium by the roots, or partitioning the sodium into vacuoles could increase salt tolerance in *Eucalyptus*.

Generation and analysis of *Eucalyptus* composite transgenic plants for functional characterization of *EcHKT1;1* gene

Fairbairn *et a*l. (2000) and Liu *et a*l. (2001) have identified *EcHKT1;1* and *EcHKT1;2* genes involved in K^+-Na^+ cotransport in *E. camaldulensis*. The *Eucalyptus HKT1* homologs, *EcHKT1;1* and *EcHKT1;2*, showed K^+ and Na^+ currents in oocytes (Fairbairn *et al*., 2000). In wheat, silencing the *HKT1* gene (*TaHKT2:1*) resulted in improved Na^+ tolerance under saline conditions indicating a role in Na^+ translocation to the shoots (Laurie *et al*., 2002). However, in Arabidopsis, loss of function mutants in the

AtHKT1;1 gene rendered the plants Na^+ hypersensitive, indicating a role in the unloading Na^+ from the xylem. The phylogenetic analysis of *HKT* genes in this study and earlier studies by Platte*n et a*l. (2006) and S*u et a*l. (2015) showed that *EcHKT1;1* grouped in subfamily 1, and has more sequence similarity with *Arabidopsis AtHKT1;1* than Wheat *TaHKT2;1*. However, functional diversity of HKT1 has been reported. Unlike *AtHKT1;1*, few HKT proteins of subfamily 1 like *SbHKT1;4* (Wang *et al.,* 2014) have been reported to function in Na^+ translocation to the shoots. Similar interesting functional diversity include *EpHKT1;1, HvHKT1;5, HvHKT2;1* and *OsHKT2;2* (Table 2).

To evaluate *in planta* function of *EcHKT1;1,* we used an RNAi-based approach to downregulate its expression in roots of *Eucalyptus*. Roots, being the major portal of entry of sodium ions into plants, composite transgenic approach in which transgenic roots are generated from non-transgenic shoots offers a rapid system for functional analysis of salt tolerance conferring genes. qRT-PCR was used to determine the levels of *EcHKT1;1* transcript in *A. rhizogenes* transformed roots that shown GFP expression. Downregulation of *EcHKT1;1* ranged from 37 % to 74 % in roots compared with the control composite transgenic plants. Similar differences in downregulation of *EgCCR1* mRNA have been observed in silenced roots of transgenic *E. grandis* (Plasenci*a et al*., 2016).

The tolerance to salt stress can be due to the control of the acquisition and allocation of sodium inside the plant, osmotic readjustment and other physiologic processes (Cheeseman 1988). In our earlier study in the tree species *Casuarina equisetifolia*, the root-to-shoot ratio of Na^+ was identified as a differentiating parameter for salt tolerance, indicating the ability of salt-tolerant clones to restrict Na^+ in roots (Selvakesava*n et al*., 2016). In the present study, composite transgenics of *Eucalyptus* downregulated for *EcHKT1;1*, were analyzed for ascertaining its role in Na^+ transport to the shoots. Under salt treated conditions, Na^+ accumulation in roots of the composite transgenic plants with the *EcHKT1;1* silencing construct was 2.04 fold higher at 0.51 % when compared to the control *Eucalyptus* plants developed using *A. rhizogenes* strains A4RS (0.08 %) and A4RS pHKN29 (0.25 %). The shoot to root ratio of Na^+ in the composite transgenics generated using the *EcHKT1;1* silencing construct was 4.88 folds lower than the controls. The lower shoot to root ratio of sodium indicated that the *EcHKT1;1* downregulated composite transgenics could limit Na^+ transport to the shoot suggesting a function similar to *TaHKT2;1*. The antisense expression of *TaHKT2;1* in wheat resulted in a decrease in Na^+ uptake by the roots and reduced Na^+ translocation to the shoots, but K^+ homeostasis was not affected. Under

saline conditions, *TaHKT2;1* is responsible for substantial Na^+ influx into roots and transport to other parts of the plant (Laurie *et al*., 2002).

The results obtained in this study are interesting as downregulation of HKT transporters of the subfamily 1, generally result in salt susceptibility, and overexpression confers salt tolerance (Table 2). Fairbair*n et a*l. (2000) had suggested that *EcHKT1;1* may play a role in limiting Na^+ transport by excluding Na^+ from transpiration stream. In the same study, it was shown that *EcHKT1;1* expression was lowest in the roots, followed by leaves, and highest in the stems. *EcHKT1;1* downregulation was hence expected to result in salt susceptibility. Contrastingly, downregulation of *EcHKT1;1* specifically in the roots, resulted in enhanced salt tolerance indicating that *EcHKT1;1* may play a role in sodium influx from the roots as in the case of *TaHKT2;1* (Laurie *et al*., 2002). Localization studies of *EcHKT1;1* expression in the root tissues of Eucalyptus and radioactive Na^+ uptake studies may provide further insights. It is interesting to note that salt-tolerant (non-transgenic) clone EC-7 also showed downregulation of *EcHKT1;1* expression 24 h post salt stress, while the susceptible (non-transgenic) clone ET-88 did not. Taken together, these results suggest that reducing expression of *HKT1;1* could be a potential strategy for enhancing salt tolerance in Eucalyptus.

5. CONCLUSION

The study has unraveled the *in planta* role of *EcHKT1;1* in Na^+ uptake through the roots, much like *TaHKT2;1*. This function is contrasting to the role attributed to the members of HKT1 subfamily 1, such as *AtHKT1;1*, in unloading of Na^+ from xylem. Further studies are required to unravel the reasons for this difference. The enhancement in salt tolerance by at least 50 mM NaCl, indicates the significant role played by the *EcHKT1;1* in determining salt tolerance in *E. camaldulensis*. *EcHKT1;1* could thus be a potential target for enhancing salt tolerance in Eucalyptus by root-specific RNAi mediated downregulation or advanced gene-editing approaches for improved K^+ transport and reduced Na^+ uptake. The study has also demonstrated the feasibility of rapid functional analysis of genes conferring salt tolerance in *Eucalyptus* using the model composite transgenic system. The technique could be applied by combining CRISPR/Cas9 tools for functional analysis of genes conferring salt tolerance in many other non-model tree species that are recalcitrant to tissue culture, and transformation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

Dr. A. Balasubramanian was involved in experimental execution, data collection, analysis, and drafting the article. Ms. Usha Jayachandran, Ms. Shamili Krishnaraj, and Mrs. S. Sudha assisted in experimental execution. R. K. Selvakesavan, M. C. Sandhya and Dr. K. S. Sowmiyarani assisted in gene isolation and sequence analysis. Dr. V. Sivakumar and Dr.V. K. W. Bachpai provided the breeding resources used in this study. Dr. Claudine Franche contributed to the analysis of the results and commented on the manuscript. Dr. Mathish Nambiar Veetil and Dr. Hassen Gherbi were involved in design of experiments, data analysis, interpretation, and wrote the manuscript.

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TABLE 1 Ranking of candidate reference genes to their order of stability calculated by Normfinder, geNorm and BestKeeper

Software	**NormFinder**		**GeNorm**		**BestKeeper**	
Gene ID	Stability value	Ranking order	Stability value M-value	Ranking order	CV±SD	Ranking order
Act2	1.2402	3	1.4359651269	3	2.43±0.65	1
IDH	0.9505	2	1.2594035276	1	6.40±1.55	3
SAND	0.8262	1	1.2594035276	1	3.95±0.96	2

TABLE 2 Reports of overexpression / downregulation of different HKT1 gene and their corresponding phenotypes.

		***AtHKT1;1* type (Na^+ retrieval from xylem)**		***TaHKT2;1* type (Na^+ influx into roots)**	
	S. No.	**Downregulation & salt sensitive**	**Overexpression & salt tolerance**	**Downregulation & salt tolerance**	**Overexpression & salt sensitive**
Dicots	1	*AtHKT1;1* * (Mäser *et al*., 2002; Berthomieu *et a*l. 2003)	*CmHKT1;1** (Gao *et* al., 2020)	***EcHKT1;1*** ** (This report)	***EpHKT1;1*** (Ali *et al*., 2018)
	2	*EsHKT1;1** (Ali *et al*., 2012)	*PeHKT1;1** (Xu *et* al., 2018)		
	3	*ScHKT1;2* & *SlHKT1;2*** (Jaime-Pérez *et al*., 2017)	*EsHKT1;2*** (Ali *et al*., 2012)		
	4		*EpHKT1;2* (Ali *et al*., 2018)		
	1		***HvHKT2;1*** ** (Mian *et al*., 2011)	*TaHKT2;1*** (Laurie *et al*., 2002)	*OsHKT2;1* * (Miyamoto *et al*., 2015)
	2		***OsHKT2;2*** (Oomen *et al*., 2012)		*PutHKT2;1*** (Ardie *et* al., 2009)

	3	*TaHKT1;5D* * (Byr*t et al*., 2014)	*TmHKT1;5-A* * (Munn*s et al*., 2012)	*HvHKT1;5* * (Huang *et al*.. 2020)	
	4		*ZmHKT1;5* * (Jiang *et al*., 2018)		***SbHKT1;4*** ** (Wang *et al*., 2014)
	5		*OsHKT1;5* (Kobayash*i et al*., 2017)		
	6	*HvHKT1;1** (Ha*n et al*., 2018)	*HvHKT1;1** (Ha*n et al*., 2018)		
	7		*ZmHKT1;1a** *ZmHKT1;1b** (Re*n et al*., 2015)		

* Na^+ selective transporter , ** K^+ -Na^+ symporter.

HKTs showing contradictory functional roles are indicated in boldfonts.

At-Arabidopsis thaliana, Cm- Cucumis melo, Ec- Eucalyptus camaldulensis, Es- Eutrema salsuginea, Ep-Eutrema parvula, Hv-Hordeum vulgare, Os- Oryza sativa, Pe- Populus euramericana, Put-Puccinellia tenuiflora, Rt-Reaumuria trigyna, Sb- Sorghum bicolor, Sv-Sporobolus virginicus,, Sl-Solanum lycopersicum, Sc- Solanum cheesmaniae, Ta- Triticum aestivum, Tm- Triticum monococcum, Zm- Zea mays