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Molecular characterization of a novel Na^+/H^+ antiporter cDNA from *Eucalyptus globulus*

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ABSTRACT

Environmental stress factors such as salt, drought and heat are known to affect plant productivity. However, high salinity is spreading throughout the world, currently affecting more than 45 million ha. One of the mechanisms that allow plants to withstand salt stress consists on vacuolar sequestration of Na^+, through a Na^+/H^+ antiporter. We isolated a new vacuolar Na^+/H^+ antiporter from *Eucalyptus globulus* from a cDNA library. The cDNA had a 1626 bp open reading frame encoding a predicted protein of 542 amino acids with a deduced molecular weight of 59.1 KDa. Phylogenetic and bioinformatic analyses indicated that EgNHX1 localized in the vacuole. To assess its role in Na^+ exchange, we performed complementation studies using the Na^+ sensitive yeast mutant strain Δ*nhx1*. The results showed that EgNHX1 partially restored the salt sensitive phenotype of the yeast Δ*nhx1* strain. However, its overexpression in transgenic *Arabidopsis* confers tolerance in the presence of increasing NaCl concentrations while the wild type plants exhibited growth retardation. Expression profiles of *Eucalyptus* seedlings subjected to salt, drought, heat and ABA treatment were established. The results revealed that *Egnhx1* was induced significantly only by drought. Together, these results suggest that the product of *Egnhx1* from *E. globulus* is a functional vacuolar Na^+/H^+ antiporter.

1. Introduction

Plants as sessile organisms have developed several mechanisms that allow them to tolerate unfavorable environmental conditions such as high temperature, drought and high salinity. Even though salt accumulation occurs as a natural process in the environment, the presence of high concentrations severely affect agricultural productivity and biodiversity. Therefore, the maintenance of ion homeostasis by the plants is essential to survive in these conditions.

In order to avoid Na^+ toxicity, the plant cell may either transport the ions outside the cell or store them inside the vacuole, processes mediated by specialized proteins. Some of them belong to the family of Na^+/H^+ exchangers, which can be located in the plasma membrane or vacuole [1]. Na^+/H^+ antiporters have been identified in several mammals, bacteria and plants [2]. These transporters play roles in pumping out Na^+ from the cytoplasm by exchanging it for H^+ at the expense of the proton gradient generated by specialized pumps in the cell and vacuolar membrane [3]. This mechanism allows plant cells to accumulate Na^+ in the vacuole and therefore maintain the appropriate ion concentration in the cytoplasm. In plants, these Na^+/H^+ antiporters consist on a family of proteins known as NHX, with eight members (NHX1-8) being NHX7/SOS1 located in the plasma membrane and the other seven in intracellular membranes [4,5]. Furthermore, transporters found in intracellular membranes are classified as Class I or Class II proteins, depending on their location. Class I transporters are located in the vacuolar membrane (NHX1-4) while class II members are located in the endosome (NHX5, 6 and 8) [4].

One of the most studied vacuolar transporters is NHX1, which carries Na^+ to the inside of the vacuole and exchanges it for H^+, preventing sodium toxicity in the cytoplasm [6,7]. Overexpression of *Atnhx1* in *Arabidopsis thaliana* and other species such as *Lycopersicum esculentum* confers high salinity tolerance [8,9]. Moreover, mutant *Atnhx1 Arabidopsis* plants have shown an increased sensitivity to salt stress compared to wild type *Arabidopsis* [8]. Additionally, an ortholog of *Atnhx1* in *Oryza sativa* (*Osnhx1*) was found to confer salt stress tolerance when it was overexpressed in the same species [10].

Eucalyptus globulus is considered the most important temperate hardwood plantation species in the world due to its wood properties that make it suitable for the pulp and paper industry [11]. Moreover, this tree species has fast growth rates and ability to adapt to a broad range of geographic locations. Most importantly, *Eucalyptus* has been listed as one of the candidate biomass energy crops by the U.S. Department of Energy [12]. However, little infor-

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mation is available on *E. globulus* molecular mechanisms and response to high salt concentration.

In this study, we present the isolation and characterization of a novel vacuolar Na^+/H^+ antiporter gene from *E. globulus* (*Egnhx1*). We used *Saccharomyces cerevisiae* wild type and mutant strains as a heterologous model to functionally validate the role of EgNHX1 in Na^+ transport. In addition, we analyzed by quantitative real-time PCR the expression pattern of *Egnhx1* transcripts from *E. globulus* plants subjected to salt, drought, heat and ABA treatments. Overexpression of *Egnhx1* was only observed in plants subjected to drought stress peaking at 120 min of treatment, suggesting that *Egnhx1* participates in response to this adverse condition. Furthermore, *Arabidopsis* plants overexpressing *Egnhx1* subjected to increasing NaCl concentration showed a higher number of flowers, height and rosette diameter. Taken together, these findings suggest that oveexpression of *Egnhx1* provides a way to improve salt tolerance on plants.

2. Materials and methods

2.1. Isolation of Egnhx1 gene and Bioinformatic analyses

Egnhx1 was isolated from a cDNA library built from *E. globulus* plants subjected to 4 °C for 30 min [13]. The *Egnhx1* gene was sequenced using the universal M13 primers and the gene specific primers NHX1F2 (5' aggaaaatcaactgcactcac 3'), NHX1F3 (5' agttgtaaatgccaaattcagat 3'), NHXF2 (5' gaggtgtggttttgctggtt 3'), NHXR2 (5' taggctcgggagatcagaga 3'), NHXR3 (5' tgccccgctgcttttccat 3') and NHXF3 (5' gtttggggaaggagttgtga 3').

Subsequently, the predicted peptide sequence was established using Translate (ExPASy tools) and the molecular weight of the predicted protein established using ProtParam [14].

The multiple sequence alignment was conducted using ClustalX [15] and the construction of the phylogenetic tree using Mega 4.0 with the Neighbour-Joining algorithm with 5000 replicates. The following sequences were extracted from GenBank: **PeNHX5** (ACU01856), **InNHX2** (BAD91200), **OsNHX1** (BAA83337), **GhNHX1** (AAM54141), **RhNHX** (BAD93487), **AgNHX1** (BAB11940), **HvNHX1** (BAC56698), **PeNHX2** (ACU01853), **PeNHX3** (ACU01854), **ZmNHX2** (ACG43655), **HvNHX2** (AAO91943), **ZmNHX4** (NP_001105943), **GmNHX1** (AAY43006), **MdNHX1** (ADB92598), **AthNHX6** (AAM08407), **AthNHX5** (AAM08406), **AthNHX4** (AAM08405), **AthNHX2** (AAM08403), **AthNHX1** (Q68KI4), **AthNHX3** (Q84WG1), **AthNHX7** (Q9LKW9), **AthNHX8** (AAZ76246).

2.2. Plant material and stress treatments

E. globulus seedlings were grown in soil for one month in a growth chamber (16 h photoperiod, 21 °C) prior to being transferred to a hydroponic system that consisted of a solution of 0.4 g/L of Phostrogen (PBI Home & Garden Ltd) under the same growth conditions.

Two-month old *E. globulus* seedlings were subjected to different treatments for various time periods. The heat stress treatment consisted on incubating the seedlings at 37 °C. For drought stress, the seedlings were drawn out of the hydroponic system and their roots were exposed to air in a room at 21 °C [16,17]. For the Abscisic acid treatment, ABA was added to the hydroponic medium to a final concentration of 100 μM. Salt stress was applied by adding NaCl to the hydroponic medium to a final concentration of 100 mM.

2.3. RNA extractions

Total RNA was extracted from the leaves of two-month old *E. globulus* seedlings using the method described by Chang et al. [18]. The leaves were ground in a mortar using liquid nitrogen prior the RNA extraction.

2.4. Quantitative RT-PCR

cDNA prepared from *E. globulus* seedlings subjected to different treatments was used for quantitative RT-PCR assays. Total RNA concentration was estimated by gel electrophoresis and reverse transcription was performed using MMLV-RT Reverse Transcriptase (Promega) and oligo dT. PCR was carried out using a Mx3000P Quantitative PCR System (Stratagene), with gene specific primers RTNHXF3 (5' gtgattgctctttgcgtgtt 3') and RTNHXR3 (5' actcgaactctccccttta 3') and Brilliant® SYBR® Green QPCR Master mix (Stratagene). The thermal cycling conditions were as follows: 95 °C for 10 min, then 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. All samples were done in triplicate, and positive and negative controls were included. Efficiency was estimated using the corrected relative expression method described by Pfaffl [19]. Subsequently, *Egnhx1* expression was normalized by histone H4 expression. For each gene, standard curves were generated using serial dilutions of plasmidial DNA and RNA mix. The efficiencies obtained with the standard curves and the Ct (threshold cycle for target amplification) values were used to estimate the normalized relative expression (ratio) of the target gene. Therefore, *Egnhx1* ratio was plotted as fold change in expression relative to the control sample and normalized by histone expression for each time treatment.

2.5. Yeast strains and plasmid constructions

S. cerevisiae strains used in tolerance assays were wild type BY4743 strain (ΔHis3, ΔLeu2, ΔMet15, ΔLys2, ΔUra3) and mutant (Δ*Scnhx1*) YDR456 W strain (ΔHis3, ΔLeu2, ΔMet15, ΔLys2, ΔUra3) lacking the functional *Scnhx1* gene.

Egnhx1 was cloned in pCR8/GW/TOPO Gateway entry vector (Invitrogen) and Gateway LR reaction was used to shuttle *Egnhx1* into yeast expression vector pYES-dest52. On the destination vector, *Egnhx1* was controlled by the GAL1 inducible promoter. *Egnhx1* was amplified with primers NHX1F3 (5' agttgtaaatgccaaattcagat 3') and NHX1F2 (5' aggaaaatcaactgcactcac 3').

2.6. Yeast transformation and tolerance assays

Yeast transformation was performed according to the lithium acetate method [20] and selected on YMM agar medium with glucose lacking uracil (Glu/-Ura). Liquid YMM media (Raf/-Ura) cultures were grown until they reached log or midlog phase. Cultures were then normalized to OD_{600} 1.5–2.0, and 640 μL of a 12.5% galactose solution was added to a 4 mL culture. Afterwards, they were serially diluted and spotted onto YMM agar medium with 1.5% Raf-Gal(-Ura) supplemented with 1 M NaCl.

2.7. Generation and analyzed of transgenic Arabidopsis plants

A. thaliana Col-0 plants were grown in a culture cabinet with a 16/8 h photoperiod and an average temperature of 21 °C. Genetic transformation was performed using the method described by Clough and Bent [21] using *A. tumefaciens* strain GV3101 previously transformed with the pGWB2 binary vector (Invitrogen) containing the CaMV35S promoter and the *Egnhx1* cDNA (pGWB2/*Egnhx1*). Transgenic seeds were selected by plating on agar supplemented with 50 mg/L kanamycin. Plants were subsequently screened and grown until we obtained a T_2 to identify homozygous transgenic lines. Transgenic and wild type 5–6 weeks old plants were grown in soil in order to evaluate rosette size, plant height and the number of flowers after watering every four days with dif-

ferent NaCl solutions (increasing from 50 mM through 100, 150, 200 and a final concentration of 250 mM). We analyzed six plants for each assay and performed three independents experiments. Data were graphed using the software Graphpad Prism5.

3. Results

3.1. Bioinformatic analyses of EgNHX1

The full-length cDNA of *Egnhx1* from *E. globulus* was 1,917 pb long with a 5' untranslated region of 61 bp, an open reading frame of 1,626 bp and a 3'-untranslated region of 230 bp. The ORF encoded a predicted transmembrane protein containing 542 amino acids with a molecular weight of 59.1 KDa. Multiple alignment of the predicted amino acid sequences showed that the identity between EgNHX1 and other Na^+/H^+ antiporters ranged from 78% (PeNHX5) to 62% (MdNHX1). Moreover, the phylogenetic analysis (Fig. 1) showed that EgNHX1 clustered with other vacuolar exchangers.

Analysis of EgNHX1 with InterproScan showed that the predicted protein had four conserved regions of 14, 15, 14 and 12 residues respectively, common in Na^+/H^+ exchangers. Additionally, a 289-residue region that is present near the C-terminal end of NHX1-4 isoforms was also found in EgNHX1. An analysis with TMpred was carried out to establish the number and position of putative transmembrane domains in EgNHX1, showing that the predicted protein contained 11 domains, of which number 6 and 7 are highly conserved among NHX1 proteins. Furthermore, EgNHX1 was compared to NHX1 from *S. cerevisiae*, establishing a 25.1% identity probably due to different lengths (633 versus 542) and a different number of transmembrane domains (8 for ScNHX1 and 11 for EgNHX1).

3.2. EgNHX1 restores the wild-type phenotype of yeast mutant

In order to assess whether EgNHX1 had a role in Na^+/H^+ exchange, we used a *S. cerevisiae* mutant lacking NHX1 that had been previously used in other studies [22,23]. Yeast cells transformed with the empty vector pYES-dest52 and the construct pYES-dest52/*Egnhx1* were compared for their ability to grow under salt stress. As shown in Fig. 2, yeast cells transformed with either construct displayed normal growth on YMM plates. However, when the transformed yeast cells were plated on media with an additional 1 M NaCl, growth was restricted. Wild type yeast cells transformed with the construct pYES-dest52/*Egnhx1* displayed slightly higher growth than the one transformed with the empty vector. As for the yeast Δ*nhx1* mutant, transformation with the empty vector was not capable of rescuing the Na^+ sensitive growth phenotype. Nevertheless, the *Egnhx1* cDNA was capable of suppressing the salt sensitivity of the yeast mutant strain at the same level of the wild type strain transformed with the empty vector.

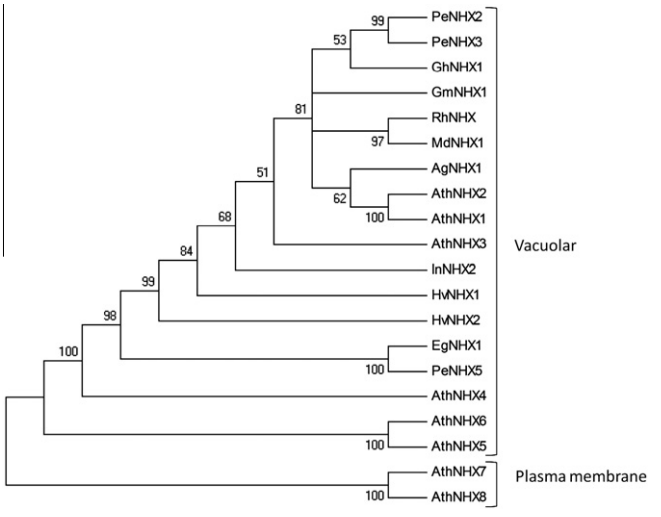

Fig. 1. Phylogenetic tree of different NHX proteins. Bootstrap values are indicated for each branch divergence. The accession numbers for the proteins are specified in Section 2.

3.3. Egnhx1 expression patterns by quantitative Real-time PCR

To understand the potential role of *Egnhx1* in abiotic stress, the expression of this gene was assessed under different stress conditions. *E. globulus* seedlings were subjected to salt, drought, heat or ABA treatment. Leaves of the treated and control plants were collected for total RNA extraction, which was used for quantitative RT-PCR assays. As Fig. 3 shows, the level of *Egnhx1* transcripts was not significantly affected by salt, heat or ABA treatment. However, the expression of *Egnhx1* in seedlings subjected to drought stress was significantly upregulated showing a peak at 120 min.

3.4. Growth of transgenic Arabidopsis plants subjected to salt stress

To investigate whether *Arabidopsis* transgenic plants were more tolerant to salt stress, we randomly chose ten independent lines for analysis. As shown in Fig. 4, the transgenic plants seemed to cope better to increasing concentrations of NaCl in comparison to wild type plants. Transgenic plants height and rosette diameter were significantly higher than that of wild type plants (Fig. 4). Additionally, the total number of flowers formed at the end of the treatment was more than two-fold on transgenic plants in comparison to their control.

4. Discussion

Plants regularly face abiotic stress throughout their lives. In some cases, the stress inflicted onto the plants is so severe that it affects their growth and physiology, resulting in a reduction of productivity and sometimes death. High salinity, which occurs in more than 45 million ha [24], has been characterized as one of the most severe stresses causing ion imbalance and hyperosmotic stress. Hence, the manipulation of plant vacuolar Na^+/H^+ antiporter to reestablish ion homeostasis may solve this issue.

In the present study an *Egnhx1* cDNA from *E. globulus* was isolated, containing a 1626 bp ORF. Phylogenetic analysis of the predicted EgNHX1 protein revealed a clear separation between the Na^+/H^+ antiporters located in the vacuole from those located at the endosomal or plasma membrane (Fig. 1). Furthermore, the analysis showed that EgNHX1 was evolutionarily closer to a vacuolar N^+/H^+ antiporter from a woody species, *Populus euphratica* [25]. The topological analysis predicted that EgNHX1 contained 11 transmembrane domains, in addition to the putative amiloride binding domain FFLYLLPP located at the third transmembrane domain, common to vacuolar Na^+/H^+ antiporters. The number of spanning transmembrane domains differs slightly from the 12 present in *AtNHX1* [26], HcNHX1 [27], OsNHX1 [10] and SsNHX1 [28] or the 10 domains described for TrNHX1 [23]. Nonetheless, EgNHX1 contains domains 6 and 7 that are known to be involved in ion transport, which are highly conserved among NHX1 [29]. These results suggest that EgNHX1 corresponds to a Na^+/H^+ antiporter localized in the vacuole.

The role of *Egnhx1* was assessed by complementation of *S. cerevisiae* wild type and mutant Δ*nhx1* strains. The expression of *Egnhx1* partially suppressed the Na^+ sensitive phenotype of the

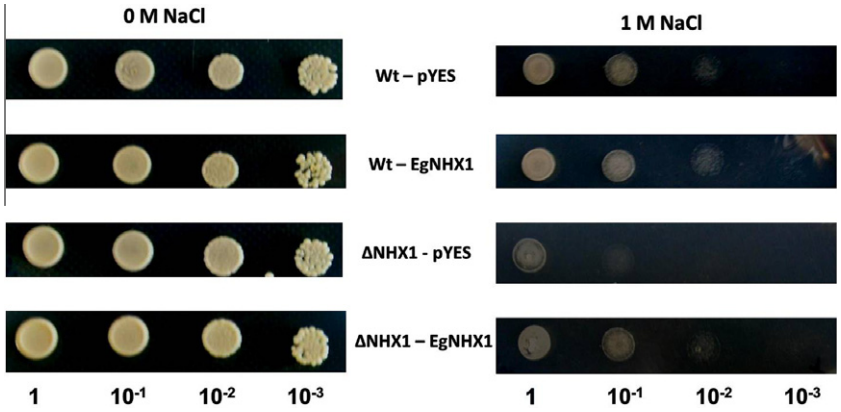

Fig. 2. Expression of *Egnhx1* in wild type and yeast mutant strains. The pYES-dest52 (pYES) vector and pYES-dest52/*Egnhx1* (EgNHX1) were introduced into wild type and mutant yeast. Ten fold serial dilutions of transformed yeast strains were spotted in 0 M and 1 M NaCl YMM medium and incubated at 30 °C for 2 days.

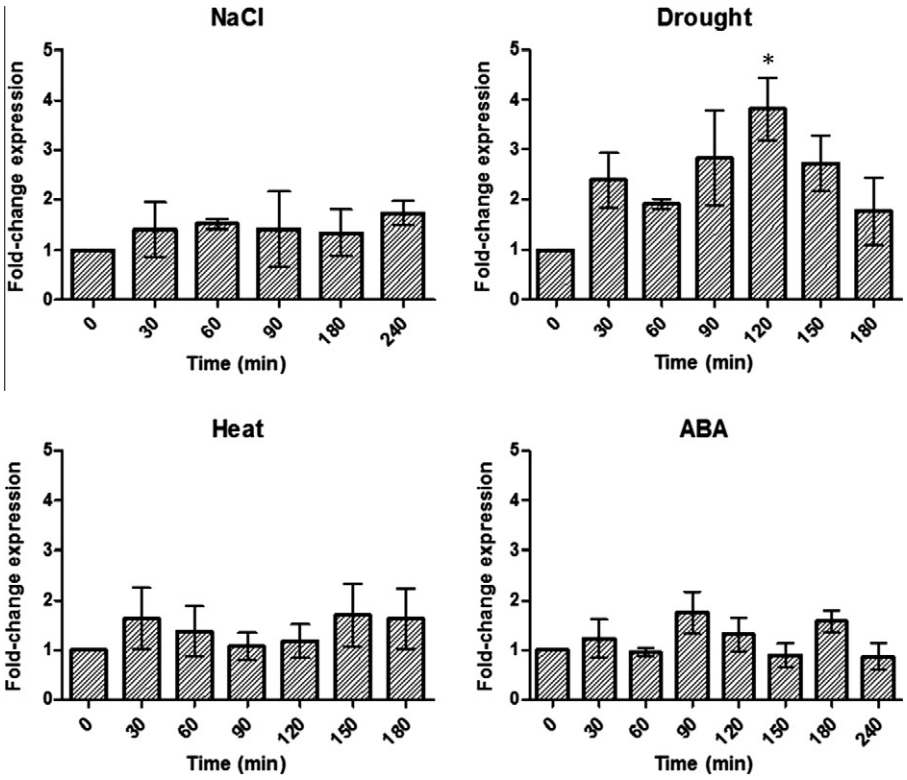

Fig. 3. Quantitative RT-PCR analysis of *Egnhx1* gene in response to NaCl, heat, drought and ABA treatments. *Egnhx1* data is expressed as normalized fold-changes relative to the calibrator sample. H4 is the reference gene. Data represent means and standard errors of three replications. Asterisk represents $P < 0.05$ by Dunnette's multiple comparison test.

yeast mutant strain in the presence of high NaCl concentration (Fig. 2). Partial compensation of the mutant yeast phenotype have been described by other studies that have performed complementation studies using *Atnhx1* [3], *Bvnhx1* [30], *Osnhx1* [10], *Trnhx1* [23] and *Zjnhx1* [31]. Interestingly, a very similar response has been observed on all complementation studies even though different NaCl concentrations (ranging from 25 to 1000 mM) have been used.

Understanding the potential role of NHX proteins in abiotic stress is important to elucidate the biological roles of these Na^+/H^+ antiporters in plants. Since the involvement of vacuolar NHX exchangers in plant salt tolerance has been previously reported [10,27], our interest focused on the *Egnhx1* transcript level in *Eucalyptus* seedlings subjected to 100 mM NaCl. We showed that *Egnhx1* transcript levels did not vary significantly throughout the four-hour treatment (Fig. 3). It is likely that *Egnhx1* transcripts could rise significantly if the measurements were performed for longer periods of time as evidence from other studies has shown a slight rise in *Atnhx1* transcripts at 5 h of treatment and a significant rise at 24 h [22,32]. Similarly, *Osnhx1* transcripts increased two-fold after 5- and 24-h in shoots from rice seedlings subjected to 0.2 M NaCl [10]. The same level of induction was observed for *Innhx2* transcripts in leaves, stems and roots of *Ipomoea nil* [33]. It is possible that *Egnhx1* is overexpressed rapidly under salt conditions in tissues other than leaves, and the time of treatment is not sufficient to observe a significant increase in *Egnhx1* transcript in *Eucalyptus* leaves. Since salinity and drought exert their effect mainly by disrupting the ionic and osmotic equilibrium of the

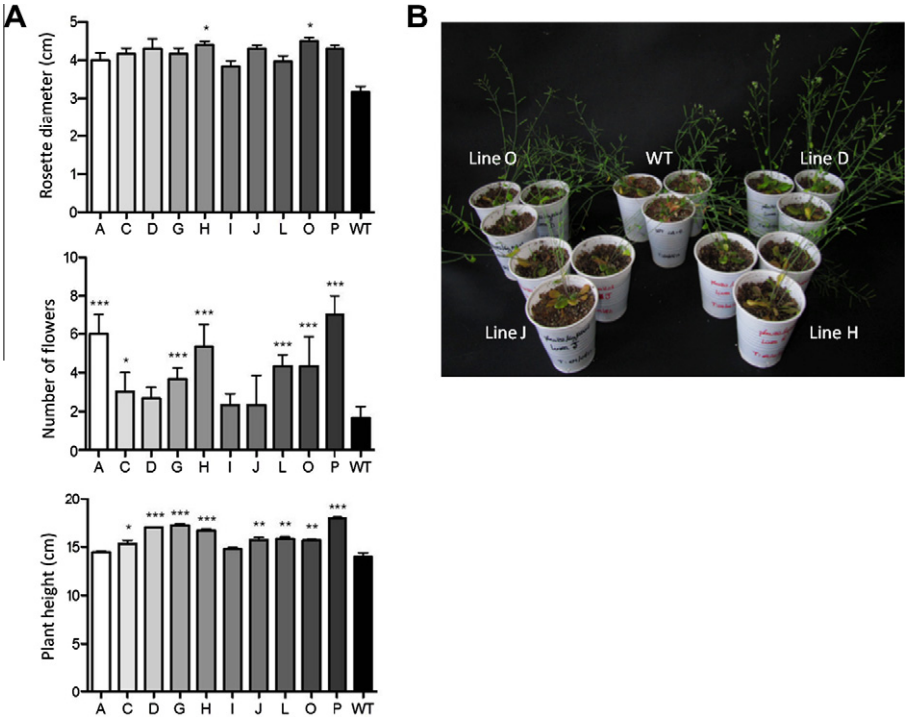

Fig. 4. Wild type and transgenic plants subjected to salt stress. (A) Rosette diameter, plant height and total number of flowers for registered for each transgenic line. Each bar represents the mean of three measurements (n = 3 each). (B) Photograph was taken at the end of the salt treatment. Similar results were observed in the other six transgenic lines.

plant cell, we also studied the expression pattern of *Egnhx1* in seedlings subjected to drought stress. Surprisingly, there was a 3.8-fold increase in expression of the transcripts at 2 h of drought treatment, suggesting that *Egnhx1* might be involved in drought stress response in *Eucalyptus*. Likewise, the *Thnhx1* gene has been described to be upregulated within the first three hours of drought treatment in *Thellungiella halophila* [34]. Concomitantly, *Atnhx1* and *Osnhx1* transcripts have been shown to accumulate in seedlings subjected to osmotic stress [10,22]. The fact that the NHX gene family is made up of multiple members may account for the expression difference established on different tissues and/or developmental stages [30]. Similar to osmotic stress, the plant response to heat is primarily regulated at the transcriptional level [35]. However, only a single study has reported the temporary induction of the cNhx1 gene in grapefruit by a heat treatment [36]. In *E. globulus*, *Egnhx1* gene expression did not vary in response to heat stress. The transgenic lines ovexpressing *Egnhx1* did not exhibit any growth inhibition when watered with a saline solution. Similar results were described by Guan et al. [27] while overexpressing HcNHX in *A. thaliana*. Furthermore, the increased number of flowers in the transgenic plants confirms the results obtained by Bassil et al. [37] that associated NHX1 and NHX2 to cell expansion and flower development.

Abiotic stress pathways are usually studied in isolation in order to examine the expression at one particular stress. However, many of them converge into osmotic stress, inducing a plant response that is regulated by abscisic acid (ABA) [38]. Even though previous studies have reported the overexpression of genes in plants subjected to ectopic ABA treatment [22,32] suggesting an ABA dependent pathway, we showed that *Egnhx1* was not induced by this hormone.

In summary, we have isolated a novel vacuolar Na^+/H^+ antiporter gene from *E. globulus* for the first time, which allowed a yeast mutant strain lacking Sc*nhx1* to suppress its Na^+ sensitive phenotype. Additionally, we showed that *Egnhx1* transcripts increased significantly in response to drought stress, even though most studies associate its role to salt stress. Moreover, *Egnhx1* stable expression in *A. thaliana* plants subjected to high salt concentration improved their performance in comparison to wild type plants. These results suggest that linear metabolic pathways in abiotic stress are not independent; therefore, future studies should consider a wider range of environmental triggers.

Acknowledgments

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