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Cloning and characterization of a novel vacuolar Na^+/H^+ antiporter gene (*VuNHX1*) from drought hardy legume, cowpea for salt tolerance

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Abstract Soil salinity is a major abiotic stress that seriously affects crop productivity worldwide. One of the mechanisms that allow plants to withstand salt stress is vacuolar sequestration of Na^+, through a Na^+/H^+ antiporter. We isolated a new vacuolar Na^+/H^+ antiporter gene (*VuNHX1*) from a drought hardy grain legume, cowpea (*Vigna unguiculata* L.). The cDNA is 1,981 bp, with an open reading frame of 1,629 bp encoding a predicted protein of 542 amino acids with a deduced molecular mass of 59.6 kDa. *VuNHX1* displays a conserved amiloride binding domain ([84]LFFIYLLPPI[93]) in third transmembrane (TM3) region. Phylogenetic and bioinformatic analysis indicated *VuNHX1* belonging to Class-I clade of plant NHX exchangers with high similarity with legume Na^+/H^+ antiporters. To assess its role in Na^+ exchange, we performed complementation studies using the salt sensitive yeast mutant strain AXT3. The results showed that VuNHX1 complemented for the loss of yeast NHX1 under NaCl, KCl and LiCl stress in the salt sensitive phenotype of the yeast strain AXT3. The expression profiles revealed significant induction of *VuNHX1* in cowpea seedlings under salt, cold and dehydration stress. Both expression analysis and ion estimation under salt stress indicated the *VuNHX1* expression preferentially in roots than in leaves. Overexpression of *VuNHX1* in transgenic Arabidopsis conferred enhanced salt tolerance in transgenic *Arabidopsis* lines while the wild type plants exhibited growth retardation. This study shows that *VuNHX1* is a potential gene for salt tolerance, and can be used in future for developing cisgenic salt tolerant cowpea and transgenic salt tolerant crops.

Keywords Na^+/H^+ antiporter gene · Cowpea · RACE · Transgenic Arabidopsis · Salt tolerance

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Introduction

Soil salinity is a major abiotic stress that limits crop growth and productivity worldwide (Kronzucker and Britto 2011). In saline soil, Na^+ is the most predominantly toxic ion. The excess accumulation of Na^+ in the cytosol leads to disruption of ion homeostasis, hyper-osmotic stress, inhibition of cytoplasmic enzyme activities, production of reactive oxygen species, and causes adverse effect on photosynthesis and cellular metabolism (Hasegawa et al. 2000; Zhu 2001). Therefore, salt toxicity is mainly attributed to Na^+ specific damage to the plant cytoplasm (Zhao et al. 2007).

In order to avoid Na^+ toxicity, the plant cell may either exclude the ions from the cell by the plasmamembrane Na^+/H^+ antiporter, or store them inside the vacuole by pumping the Na^+ to the vacuole via vacuolar Na^+/H^+ antiporters (Zhu 2003). These $Na^+(K^+)/H^+$ exchangers (also known as NHX-type cation/proton antiporters) function cooperatively to prevent Na^+ uptake, increase efflux of Na^+ from cell and compartmentalize Na^+ into vacuole (Reguera et al. 2014). Pumping sodium ions into vacuole via vacuolar Na^+/H^+ antiporters facilitates the reduction in the toxic levels of sodium in the cytosol, and the increase in the vacuolar osmotic potential with the concomitant increase in negative water potential which favors enhanced cellular water uptake and tissue water retention under high soil salinity (He et al. 2005). In addition to the known role of Na^+/H^+ antiporters in ion homeostasis, recent findings indicated their involvement in other key cellular processes, not necessarily limited to salinity stress response, such as calcium signaling, sulfur metabolism, cell structure and cell growth, as well as intracellular vesicular trafficking and protein targeting (Apse et al. 2003; Sottosanto et al. 2004), Berry ripening (Hanana et al. 2007), and recessive mutation in vacuolar Na^+/H^+ antiporter gene leading to purple flower of Japanese morning glory (Yamaguchi et al. 2001). Current evidence suggests that halophytes and glycophytes share many fundamental transport and osmotic solute biosynthetic mechanisms for ion compartmentalization and osmotic adjustment (Hasegawa 2013). Vacuolar $Na^{+/}H^+$ antiporter gene of Arabidopsis (AtNHX1) was the first plant homologue of yeast Na^+/H^+ exchanger to be cloned (Apse et al. 1999) and the transcript level of At-NHX1 was up-regulated by NaCl and abscisic acid (Shi and Zhu 2002). Vacuolar NHX1 gene was identified in several glycophytes including rice (Fukuda et al. 1999), calico flower (Zhang et al. 2008), rape seed (Wang et al. 2003), cotton (Wu et al. 2004), hybrid tea rose (Kagami and Suzuki 2005), maize (Zorb et al. 2005), wheat (Brini et al. 2005), and halophytes including ice plant (Chauhan et al. 2000), gmelin's saltbush (Hamada et al. 2001), beetroot (Xia et al. 2002), seepweed (Ma et al. 2004) and umari keerai (Jha et al. 2011). Over-expression of vacuolar antiporter genes resulting in enhanced salt tolerance in different plant species (Apse et al. 1999; Chen et al. 2008; He et al. 2005; Ohta et al. 2002; Wu et al. 2005; Xue et al. 2004; Zhang and Blumwald 2001; Zhang et al. 2001) suggests their important role in salt tolerance and ion homeostasis.

Cowpea (Vigna unguiculata L. Walp.) is an important grain legume that serves both as food and fodder for resource poor populace in semi-arid tropics (Singh 2005; Timko et al. 2007). It is cultivated in different parts of the world, viz. Africa, India, USA and Australia, under a wide range of climatic conditions such as semiarid to sub-humid

and is well adapted to heat and drought (Hall 2012). In plants, there exists extensive overlap and crosstalk between salinity and drought stress-responsive signaling pathways. The distinctive character of cowpea to withstand drought makes it a valuable system for mining abiotic stress responsive genes and understanding the molecular mechanisms of abiotic stress tolerance. However, there has been no attempt to clone and understand the function of NHX1 in cowpea. Moreover, salinity and limited water availability impose severe constraints on cowpea production. Consequently, development of cowpea cultivars endowed with enhanced tolerance to salinity and dehydration stress possibly through overexpression of native NHX1 by cis-genic approach offers both environment and consumer acceptable way for sustainable production on hostile soil.

In this study, we report cloning and molecular characterization of a vacuolar Na^+/H^+ antiporter gene from cowpea (VuNHX1), a drought tolerant grain legume. We investigated the VuNHX1 expression patterns under salinity and other abiotic stresses, functional validated in salt sensitive yeast AXT3 mutant and over-expressed in Arabidopsis with intention to reveal the possible role of VuNHX1 in salt tolerance.

Materials and methods

Plant material and stress treatment

Cowpea plants (Vigna unguiculata L. cv. Pusa Komal) were grown in a growth chamber in hydroponic system consisting of Hoagland's solution under conditions of 16 h photoperiod at 25 °C and 70 % relative humidity. Ten days old seedlings were subjected to different abiotic stress treatments such as salt, dehydration and cold for different time periods (0, 6, 12, and 24 h). Salt stress was applied by adding NaCl to the hydroponic medium to a final concentration of 200 mM. For dehydration stress, the seedlings were drawn out of the hydroponic system and their roots were exposed to air in a room at 27 °C. The cold stress treatment consisted of incubating the seedlings at 4 °C.

Isolation of VuNHX1 cDNA

A pair of degenerate PCR primers, FP: 5′-GGKTTTCARG TRAAAAAGAAGCA-3′ and RP: 5′-GTRCTSGTGATCA TRAYTGCATT-3′ were designed for amplification of partial cDNAs of Na^+/H^+ antiporter analogue gene from cowpea based the conserved regions of vacuolar Na^+/H^+ antiporter from legume species. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands). The first strand cDNA synthesis and RT-PCR were performed using Revert Aid™ First Strand cDNA Synthesis Kit (Thermo

Fisher Scientific, Waltham, MA, USA) with the pair of degenerate primers. The amplified product was cloned into TA cloning vector, pTZR/T (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced. The cDNA synthesized using gene specific primer (GSP: 5′-GCACCCAAAGTTA TGACAGCAC-3′) and superscript[TM] II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was purified using a SNAP column for efficient removal of unincorporated dNTPs and remaining GSP. Terminal transferase (TdT) enzyme generated a homopolymeric tail by incorporating dCTPs to 3′ end of the purified cDNA. The dC-tailed cDNA was amplified using abridged anchor (AAP: 5′-GGCCACGCGTCGACTAG TACGGGIIGGGIIGGGIIG-3′) and gene specific (GSP1: 5′-TGTACCAATAGCACCAAACAACATGATG-3′) primers. A nested PCR was performed with abridged universal anchor (AUAP: 5′-GGCCACGCGTCGACTAGTAC-3′) and gene specific (GSP2: 5′-GTCATGAAGTTAACAAAAAC TGC-3′) primers using the first PCR product as template. Similarly, the 3′ untranslated sequence was obtained by using "Rapid Amplification of cDNA Ends Kit" (Version E, Life Technologies/Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using a dT-adapter primer (AP: 5′-GGCCAC GCGTCGACTAGTAC(T)$_{17}$-3′). The 3′-RACE PCR was performed with abridged universal anchor (AUAP: 5′-GGCCACGCGTCGACTAGTAC-3′) and gene specific (GSP3:5′-GCTGTATATTGGAAGGCACTCT-3′) primers. The full length of VuNHX1 was amplified using forward (FLP: 5′-ATGGTCTTTGAAATCAGTTCTGTTGTTTC-3′) and reverse (RLP: 5′-TCAACGCCATTGATGACCATTACG TTC-3′) primers, cloned into TA cloning vector pTZR/T (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced (GenBank accession no.: JN641304.2).

In-silico analysis

Multiple sequence alignment and phylogenetic analysis were performed using Clustal W (Thompson et al. 1997). Based on the neighbor-joining (NJ) algorithm, an unrooted phylogenetic tree was constructed using MEGA4: Tree Explorer software (Tamura et al. 2007). The transmembrane prediction was performed with TMpred software (Hofmann and Stoffel 1993) and secondary structure prediction was made using SOPMA (Self-Optimized Prediction Method with Alignment) server (Geourjon and Deleage 1995). Post-translational modification of VuNHX1 was predicted by searching for conserved motifs of N- and O-glucosylation and N-myristoylation sites using Scan-Prosite (Gattiker et al. 2002).

Yeast strain and vector construction

Saccharomyces cerevisiae strains used in tolerance assays were wild-type W303-1B (MATα ade2-1 can1-100 his3-11, 15 leu2-3,112 trp1-1 ura3-1) and mutant strain AXT3 (Δ ena1-4::HIS3 Δnha1::LEU2 Δnhx1::TRP1, ura3-1). Yeast strains were grown in YPD (1 % Yeast extract, 2 % peptone and 2 % glucose), YPGal (1 % Yeast extract, 2 % peptone and 2 % galactose), SC (0.67 % Yeast nitrogen base and 2 % glucose) and APGal synthetic minimal media (10 mM arginine, 8 mM phosphoric acid, 2 mM MgSO$_4$, 1 mM KCl, 0.2 mM CaCl$_2$, 2 % galactose, trace vitamins, and minerals; pH 4.0) supplemented with appropriate amino acids as indicated.

The VuNHX1 ORF was digested from the pTZR/T-Vu-NHX1 vector by KpnI/BamHI and sub-cloned into the KpnI/BamHI site of the yeast expression vector pYES2.0 (Invitrogen, Carlsbad, CA, USA).

Functional assays using the yeast mutant

The yeast mutant AXT3 was transformed with pYES2.0-VuNHX1 and pYES2.0 as a control using Lithium acetate method (Gietz et al. 1992) and selected on SC ura$^-$ medium. All yeast strains were grown at 30 °C for 16–18 h to reach OD$_{600}$-1.0 in liquid YPGlu (Glu/Ura$^-$) media. Cultures were normalized to an OD$_{600}$-0.006 and inoculated into liquid APGal (Gal/Ura$^-$) media (Rodriguez-Navarro and Ramos 1984) supplemented with different concentrations of NaCl (0, 50, 75, and 100 mM), KCl (0, 0.5, 1.0, and 1.5 M), and LiCl (0, 15, 20 and 25 mM). Growth status was compared after culturing strains at 30 °C for 2 days. Similarly, 10, 100 and 1,000 fold serial dilutions of yeast cultures (OD$_{600}$-1.0) were spotted on solid APGal media supplemented with or without 75 mM NaCl, 0.5 M KCl, and 50 mM LiCl and further, on solid YPGal media supplemented with or without 50 μg/ml hygromycin-B antibiotic. Growth status was compared after culturing the strains at 30 °C for 3 days.

Intracellular measurement of Na$^+$ and K$^+$ distribution in yeast mutant

Yeast strains were grown in liquid APGal media, pH 4.0 supplemented with or without 70 mM NaCl, and harvested at an OD$_{600}$ of 0.3–0.4. Cells were centrifuged at 3,000g/3 min, washed twice in ice-cold 10 mM MgCl$_2$, 10 mM CaCl$_2$ and 1 mM HEPES buffer and resuspended in the same buffer. The relationship between cell density (Absorbance at OD$_{600}$) and yeast dry weight was determined. Total intracellular ion was determined by addition of HCl to a final concentration of 0.4 % and incubated at 95 °C for 20 min. After removal of cell debris the supernatant was measured for presence of total Na$^+$ and K$^+$. To differentiate Na$^+$ and K$^+$ concentration in cytoplasmic and vacuolar region, cells were grown and washed as above and resuspended in 2 % cytochrome c, 18 μg/ml antimycin,

1 mM HEPES, 10 mM $MgSO_4$, 10 mM $CaCl_2$, and 5 mM 2-Deoxy D-Glucose solution. Cytochrome c is known to selectively permeabilize the plasma membrane of cells. After 20 min incubation at room temperature, cells were washed thrice with the same solution without cytochrome c. Cytoplasmic ion content was determined by pooling the supernatants. The remaining vacuolar ions were extracted with addition of HCl in a final concentration of 0.4 % and incubated at 95 °C for 20 min (Venema et al. 2003). The Na^+ and K^+ distribution in the cytoplasmic and vacuolar fractions were measured in Flame Photometer (Systronics, Bhopal, MP, India).

Gene copy determination by Southern analysis

For gene copy number analysis in cowpea, genomic DNA (20 μg) of cowpea was digested separately with restriction endonucleases EcoRI, HindIII and PstI. Digested DNA was electrophoretically fractionated on a 1 % agarose gel and blotted onto Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA, USA). The blot was hybridized with DIG-labeled 0.9 kb PCR product, corresponding to the coding region of *VuNHX1* using the primers, forward (SF: 5′-GCTGTA-TATTGGAAGGCACTCT-3′) and reverse (RF: 5′-TCA ACGCCATTGATGACCATTACGTTCAG-3′). Southern hybridization was carried out using hybridization solution containing 50 % formamide, 5 X SSC, 5 X Denhardt's solution, 0.05 M sodium phosphate pH 6.5, 0.1 % SDS, 10 % Dextran sulfate, 0.1 mg/ml sheared denatured salmon-sperm DNA and 20 ng/ml probe at 42 °C for 18 h. Washing and detection was performed according to instructions of the DIG Labeling and Detection system (Roche Diagnostics, Mannheim, Germany).

Expression analysis by semi-quantitative PCR and determination of Na^+ and K^+ content

Cowpea seedlings subjected to different treatments were used for expression analysis by semi-quantitative PCR. Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands). The first strand cDNA synthesis was performed using Revert Aid^{TM} First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the products were subsequently used for PCR using gene specific primers (RFP: 5′-GCTGTATATTGGAAGGCACTCT-3′ and RRP: 5′-CA-ATGTCCAAGGCATCCATACC-3′). The PCR conditions were as follows: 95 °C for 10 min followed by 28 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and finally 72 °C for 10 min. Housekeeping *VuNSR9* primers (FN: 5′-GCACAGTTTGGGTATATTG-3′ and RN: 5′-GAG-TAAAACTGGCAAAAATTAG-3′) were used as an internal control. The PCR conditions for housekeeping

gene were as follows: 95 °C for 10 min followed by 28 cycles at 95 °C for 30 s, 49 °C for 30 s, 72 °C for 30 s and finally 72 °C for 10 min. Entire experiments were performed in triplicate. Leaves and roots of untreated and salt-treated cowpea seedlings were harvested at different time intervals (0, 6, 12, 18, 24, and 48 h). The samples were dried, digested with concentrated HNO_3 at 90 °C for 30 min and centrifuged at 12,000 rpm for 10 min. The suspension was diluted with sterile water and analyzed for Na^+ and K^+ content in Flame photometer (Systronics, Bhopal, MP, India).

Generation of transgenic Arabidopsis plants

The CaMV35S::*VuNHX1*::35ST (2.3 kb) was digested from the pRT101*VuNHX1* (4.9 kb) vector by PstI and subcloned into the *Pst*I site of plant binary vector pCAMBIA2301 (11.6 kb) to result the plant binary construct pCAMBIA2301-35S::*VuNHX1* (13.9 kb). Further, a RD29A promoter region (0.898 kb) of *AtRD29A* (DQ071887.1) was amplified from *A. thaliana* genomic DNA using forward (RD29F: 5′-CTGAA ATTTCTGCAAGAATC-3′) and reverse (RD29R: 5′-TCCA ATAGAAGTAATCAAACC-3′) primers and cloned into TA cloning vector pGEMT easy (3.015 kb) (Promega, Madison, WI, USA). The RD29A promoter was digested as *Eco*RI fragment from pGEMTRD29A (4.2 kb) and cloned into *Eco*RI site of plant binary construct pCAMBIA2301-35S::*VuNHX1* (13.9 kb) by replacing the 0.4 kb CaMV35S promoter in 35SP::*VuNHX1*::35STer cassette resulting pCAMBIA2301-RD29A::*VuNHX1* (14.4 kb). The plant binary constructs, pCAMBIA2301-35S::*VuNHX1* and pCAMBIA2301-RD29A::*VuNHX1* were mobilized to *A. tumefaciens* strain GV3101 by electroporation at 1,250 V with capacitance of 25 mF and resistance of 400 ohm.

Arabidopsis thaliana (ecotype Columbia-0) plants were transformed using floral dipping method (Clough and Bent 1998). The T_1 transgenic plants were screened on ½ MS medium (Duchefa, Haarlem, Netherlands) supplemented with 50 mg/l kanamycin (Duchefa, Haarlem, Netherlands). The transgenic selections were continued until T_4 generation to obtain homozygous transgenic lines (35S::*VuNHX1* or RD29A::*VuNHX1*).

Analysis of transgenic Arabidopsis by real-time PCR

Total RNA extraction from wild-type and T_4 transgenic lines and First-strand cDNA synthesis were carried out as per the procedures described previously. The products were subsequently used as templates for real-time PCR analysis using gene specific forward primer (VrRTF: 5′-TGATCCAATC CATCGTCCAA-3′) and 35S poly-A reverse primer (TerparR: 5′-GCGAAACC CTATAAGAACCCTAATTCC-3′) for amplification of a 0.28 kb fragment of *VuNHX1*::35S poly-

A. The *AtUbiquitin* was used as internal control using UBQ1FP: 5′-AGAGCTG TCAACTGCAGGAAGAA-3′ and UBQ1RP- 5′-ACAAGAAAAACAAACCCTATCAAAGG primers to amplify a 150 bp fragment. Real-time PCR was performed using USB VeriQuest™ SYBR Green qPCR Master Mix (2X) (Affymetrix, Santa Clara, CA, USA) on a 7500 Real-Time PCR System (Applied Biosystem, Foster City, California, USA) according to manufacturer's instructions with a final primers concentration of 200 nM for both genes. The experiment was repeated twice independently with three replicates. The expression values relative to the standard curve was calculated for each sample. The relative expression of *VuNHX1* in wild-type (WT) and transgenic *Arabidopsis* lines was estimated by normalizing expression values of *VuNHX1* with that of housekeeping *AtUBQ1*.

Effect of salinity on transgenic 35S::*VuNHX1* and RD29A::*VuNHX1 Arabidopsis* lines

In order to evaluate the effect of salinity on seed germination, T_4 seeds of wild-type (WT) and homozygous transgenic *Arabidopsis* were surface sterilized and vernalized for 3 days at 4 °C on ½ MS medium (Murashige and Skoog 1962) supplemented with or without 150 mM NaCl and transferred for 7 days to a growth chamber maintained at 22 °C and 60 % relative humidity with a 16 h photoperiod.

Wild type (WT) and T_4 homozygous transgenic *Arabidopsis* seeds were grown on ½ MS medium (Murashige and Skoog 1962) for 5 days and thereafter transferred to ½ MS medium supplemented with NaCl (150 and 200 mM). The corresponding root length was measured in WT and transgenic lines (35S:: *VuNHX1* and RD29A::*VuNHX1*) after 7 days of NaCl treatment and the difference was calculated. The emergence of lateral roots in each case was also studied. Mean data was collected from ten replicates (n = 10) for wild-type (WT) and T_4 transgenic *Arabidopsis* lines.

To test the effect of salinity on mature plants, germinated seedlings were grown initially on ½ MS medium for 5 days and subsequently transferred to soilrite for 2 weeks. The WT and T_5 transgenic lines (35S:: *VuNHX1* and RD29A::*VuNHX1*) were subjected to salt stress for 2 weeks by watering them with ½ MS nutrient liquid media supplemented with 200 mM NaCl. The leaves were harvested and the Na^+ and K^+ content was estimated using method described previously.

For measurement of chlorophyll content, leaf samples were homogenized in 95 % ethanol, lysate was centrifuged at 3,000 rpm for 10 min and absorbance was recorded for the extract at wavelength of 648 and 664 nm (Lichtenthaler 1987).

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction. Briefly, 0.2 g of fresh leaf samples were homogenized with 5 ml of 0.25 % TBA containing 10 % TCA (tricloroacetic acid). The homogenate was boiled for 30 min at 95 °C and centrifuged at $10,000g$ for 10 min. Absorbance values were recorded at 532 nm and values corresponding to non-specific absorption at 600 nm were subtracted (Heath and Packer 1968).

For colorimetric estimation of proline, leaf samples (0.5 g) were homogenized with 5.0 ml of sulfosalicylic acid (3 %). 2 ml of homogenate was incubated with 2 ml glacial acetic acid and 2 ml ninhydrin reagent at a ratio of 1:1:1 in boiling water bath at 100 °C for 30 min. After cooling, 4 ml toluene was added to the reaction mixture, mixed vigorously and absorbance was measured at 520 nm (Bates et al. 1973). Mean data was collected from three replicates (n = 3) for wild-type (WT) and T_4 kanamycin selected transgenic *Arabidopsis* lines.

Statistical analysis

Statistical comparison between the variances was determined by ANOVA (Analysis of variance) and significant differences between mean values were determined by Bonferroni analysis. Statistically significant mean values were denoted as different letters ($P \leq 0.05$).

Results

Molecular characterization of *VuNHX1* and sequence analysis

The full-length cDNA of *VuNHX1* was obtained by RT-PCR and RACE method. The 1,981 bp cDNA of *VuNHX1* contained an open reading frame (ORF) of 1,629 bp which encoded a polypeptide of 542 amino acid residues with an estimated molecular mass of 59.60 kDa and isoelectric point of 6.76. The sequence of *VuNHX1* cDNA has been deposited at GenBank under accession number JN641304.2.

Multiple sequence alignment revealed a higher degree of homology between amino acid sequences of *VuNHX1* and vacuolar Na^+/H^+ antiporter of other higher plants (Online Resource 1). The highest identity was found to be 97.42 % with mungbean (*Vigna radiata*), an Asiatic grain legume, 92.44 % with soybean (*Glycine max*), 88.1 % with Peashrub (*Caragana korshinskii*), 87.45 % with narrow-leaved bird's-foot trefoil (*Lotus tenuis*), 87.25 % with alfalfa (*Medicago sativa*), 86.9 % with chickpea (*Cicer arietinum*), 86.51 % with white clover (*Trifolium repens*), and 84.84 % with Goat's rue (*Galega orientalis*) (Online Resource 1).

Recent phylogenetic analysis of the Na^+/H^+ exchanger (NHE) family had revealed two distinct subgroups corresponding to plasma membrane (PM-NHE) and intracellular transporters (IC-NHX) (Brett et al. 2005a). The two subgroups were distinct from one another in ion selectivity, kinetic properties, inhibitor sensitivity, and physiological role. In our work, a phylogenetic analysis of a number of Na^+/H^+ antiporters was generated by CLUSTAL W and MEGA 4 software program (Online Resource 2 a). The phylogenetic analysis showed that *VuNHX1* formed a clade with class-I type intracellular Na^+/H^+ (IC-NHX) exchangers, more closely related to legume NHX homologs, and distinct from class-II type IC-NHX exchangers as well as from the cluster of plasma membrane Na^+/H^+ transporters (PM-NHE) (Online Resource 2 a). These results suggested that the product of *VuNHX1* localized on the tonoplast.

A hydropathy plot generated by TMPred software indicated that VuNHX1 with highly hydrophobic N-terminal end consisting of 11 putative hydrophobic regions (Online Resource 2 b), which was different from 9 transmembrane domains in AtNHX1 and OsNHX1 (Fukuda et al. 1999; Yamaguchi et al. 2003), and a longer hydrophilic C-terminal end (Online Resource 2 b). The amiloride binding motif, 84-LFFIYLLPPI-93 in *VuNHX1* is highly conserved among eukaryotic Na^+/H^+ exchangers (Online Resource 1). As shown in Online Resource 1, VuNHX1 was highly conserved in transmembrane domains (TM 1, 3, 5, 7, 8, 10) with other reported legume NHXs. However, comparatively low conservation was observed in case of transmembrane domains (TM 2, 4, 6, 9, 11) and the hydrophilic C-Terminal end. Secondary structure prediction by SOPMA indicated occurrence of alpha helix (49.45 %), extended strand (14.21 %), beta turn (3.69 %), and random coil (32.66 %) as represented in Online Resource 3. The putative post-translational modification sites predicted by ScanProsite software are as follows: 2 *N*-glycosylation sites, 14 phosphorylation sites, 9 *N*-myristoylation sites, and 1 Leucine Zipper site (Online Resource 4).

Functional characterization of *VuNHX1* using yeast mutants

In order to assess whether *VuNHX1* had a role in Na^+/H^+ exchange, we used a yeast mutant AXT3 lacking the potential plasma membrane antiporters, ENA 1–4 and NHA1 required for Na^+ efflux, as well as the vacuolar antiporter *NHX1* for compartmentation of toxic Na^+ in vacuole. Therefore, the lack of these transporters in AXT3 cells renders them sensitive to Na^+ due to the absence of effective protective machinery. Yeast cells transformed with the empty vector pYES2.0 and the construct pYES2.0-*VuNHX1* were compared for their ability to grow under salt

stress, LiCl and KCl. As shown in Fig. 1 a, wild type and mutant yeast cells transformed with either construct displayed normal growth on APGal liquid media, under normal physiological conditions. However, a statistically significant difference ($P \leq 0.05$) in growth pattern was observed in W303-1B wild-type and AXTYES2.0 cells under stress conditions. Under salt stress at 50 mM NaCl, no significant difference in growth was observed between AXTYES2.0 and AXTVuNHX1 cells. However, with increase in NaCl concentration (75 and 100 mM NaCl) a marked difference was observed in their growth. The AXTVuNHX1 cells showed 1.85 and 2.87 times better survival efficiency as compared to AXTYES2.0 cells, under 75 and 100 mM NaCl stress respectively (Fig. 1a). Similarly, pronounced difference under increase in external $[K^+]$ between AXTYES2.0 and AXTVuNHX1 cells was observed at 1 M KCl unlike, 0.5 and 0.75 mM KCl stress. AXTYES2.0 cells were able to survive at a similar rate as that of AXTVuNHX1 cells under 15 and 20 mM LiCl stress (Fig. 1a). But, significant growth difference was observed at 25 mM LiCl suggesting the efficient survival of AXTVuNHX1 than AXTYES2.0 cells owing to the cation/proton antiporter activity of *VuNHX1* (Fig. 1a). The yeast complementation assay was performed on solid AP-Gal minimal medium and similar results were obtained as observed in liquid media supplemented with different concentrations of Na^+, K^+ and Li^+. The survival efficiency of AXT3 strains expressing *VuNHX1* was greater than AXT3 cells transformed with empty pYES2.0 vector (Fig. 1b). Wild-type strain W303-1B was taken as control in each case. Heterologous expression of *VuNHX1* under GAL1-inducible promoter restored salt tolerance in AXTVuNHX1 cells at 50 μg/l hygromycin-B (Fig. 1b). Hygromycin-B, a cationic protein synthesis inhibitor is known to confer hypersensitivity in *Δnhx1 and Δnhx1 Δnha1* mutants, owing to its increased uptake by cells under changes in plasma membrane electrical potential but, no effect on wild-type and Δnha1 mutants with intact *ScNHX1* (Ali et al. 2004; Brett et al. 2005b). Therefore, the growth sensitivity of AXTVuNHX1 cells under a higher concentration of hygromycin-B was least affected indicating the functional complementation of *ScNHX1* by heterologous expression of *VuNHX1* as compared to AXTYES2.0 cells lacking effective salt tolerance machinery.

Na^+ and K^+ distribution in yeast mutants

The total intracellular ion distribution in the yeast cells (W303-1B, AXTTES2.0, AXTVuNHX1) was determined. Under normal physiological and salt stress condition, no significant difference was observed in total $[Na^+]$ in yeast cells, moreover, AXTYES2.0 cells showed relatively higher value than W303-1B and AXTVuNHX1 cells.

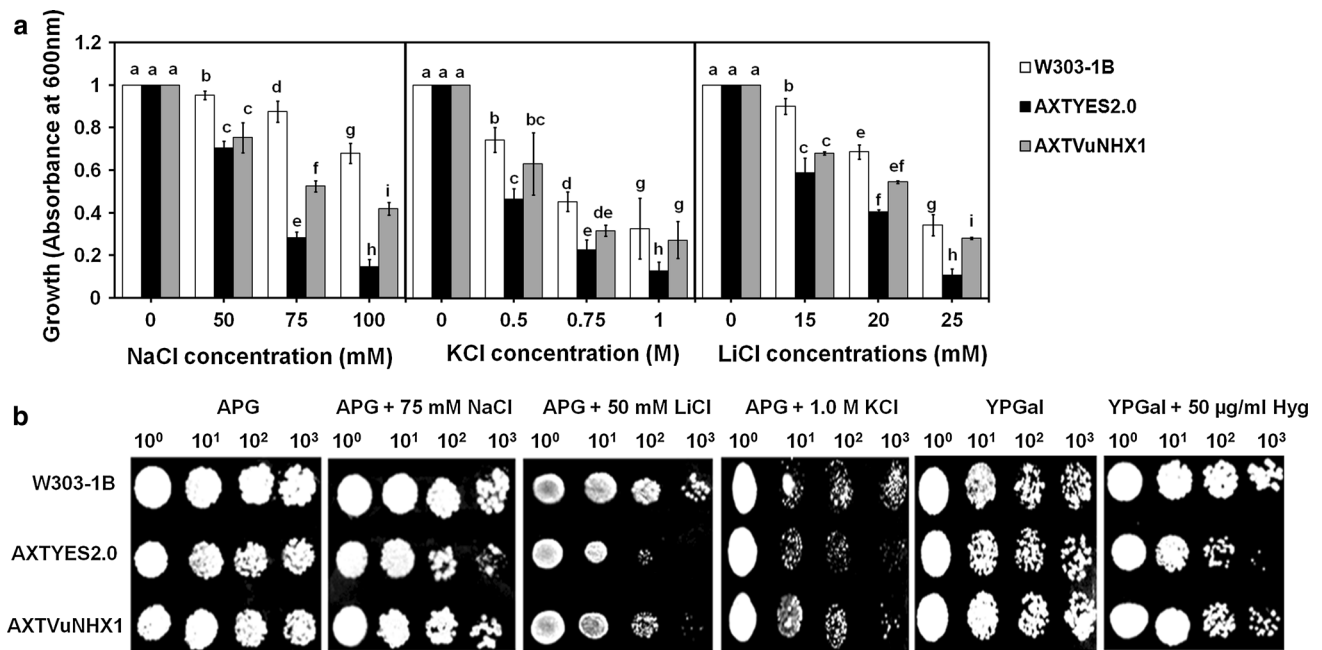Fig. 1 **a** Heterologous expression of *VuNHX1* in yeast mutant. Wild type (W303-1B) and Δ *ena1-4* Δ*nha1* Δ*nhx1* mutant (AXT3) strains used for the complementation assay were transformed with pYES2.0 vector only and pYES*VuNHX1* recombinant vector and labeled as AXTYES2.0 and AXTVuNHX1, respectively. Cation sensitivity assay of transformed yeast strains under various concentrations of NaCl (0, 50, 75, 100 mM), KCl (0, 0.5, 0.75, 1.0 M), and LiCl (0, 15, 20, 25 mM). Saturated seed cultures for each strain was diluted to an OD$_{600}$ of 0.006 and inoculated to liquid APGal medium (pH 5.5) supplemented with or without above mentioned concentrations of NaCl, KCl, and LiCl. Growth was observed at 30 °C after 3 days and absorbance recorded at 600 nm. Data are means of 3 independent events (n = 3) and SE are plotted in the *graph*. Statistically significant values at *P* ≤ 0.05 are indicated as *different letters* using Bonferroni analysis. **b** 10^1-, 10^2-, and 10^3-fold serial dilutions of saturated seed cultures of each strain were spotted onto APGal media (pH 5.5) supplemented with or without 75 mM NaCl, 50 mM LiCl, 1.0 M KCl and YPGal media (pH 5.5) supplemented with or without 50 µg/ml Hyg. The plates were incubated at 30 °C for 3 days

However, statistically significant difference ($P \leq 0.05$) was observed in total [K$^+$] in yeast cells. The W303-1B and AXTVuNHX1 exhibited 2.8 and 1.9 times higher accumulation of total K$^+$, respectively than AXTYES2.0 cells (Fig. 2). This could be due to the activity of antiporters other than Na$^+$/K$^+$/H$^+$ Nhx1 in vacuole involved in K$^+$ transport. Further, ion analysis studied in vacuolar and cytoplasmic region for understanding of distribution of ions in yeast strains revealed no significant difference in vacuolar Na$^+$ accumulation under normal physiological condition (Fig. 2). However, K$^+$ accumulation was found significantly higher in W303-1B (2.7 times) and AXTYESVuNHX1 (2.1 times) as compared to AXTYES2.0 (Fig. 2). Under salt stress, AXTYES2.0 cells exhibited 2.1 and 1.9 times lower vacuolar Na$^+$ than wild-type and Δ*ena* Δ*nha1* Δ*nhx1* mutant expressing *VuNHX1* (Fig. 2). This could be attributed to the fact that AXTYYES2.0 cells lacked vacuolar antiporter *ScNHX1* involved in sequestration of cations. Further, under salt stress the vacuolar K$^+$ accumulation was 2.4 and 1.94 times higher in W303-1B and AXTVuNHX1 as compared to AXTYES2.0 cells. The comparatively higher K$^+$ in AXTVuNHX1 than

AXTYES2.0 cells indicated the possible role of *VuNHX1* in compartmentalizing K$^+$ along with Na$^+$ in vacuole. Higher [Na$^+$] was observed in the cytoplasmic region of AXTYES2.0 cells as compared to W303-1B and AX-TVuNHX1 cells owing to lack of plasma membrane and vacuolar antiporters involved in exclusion and compartmentalization of excess toxic ions.

Copy number analysis of *VuNHX1*

The copy number of *NHX* gene in the cowpea genome was determined by Southern blot analysis. The cowpea genomic DNA was digested separately with three restriction enzymes, EcoRI, HindIII and PstI and then blotted onto membrane and hybridized to a probe specific to 3′-region of *VuNHX1* CDS that lacked all three restriction sites. The results indicated presence of a single copy of *NHX1* in cowpea genome as revealed from a single hybridization signal in HindIII digested genomic DNA (Fig. 3). The occurrence of two and three signals in case of *Eco*RI and *Pst*I digested genomic DNA respectively could be due to presence of multiple sites of *Eco*RI and *Pst*I in *VuNHX1*.
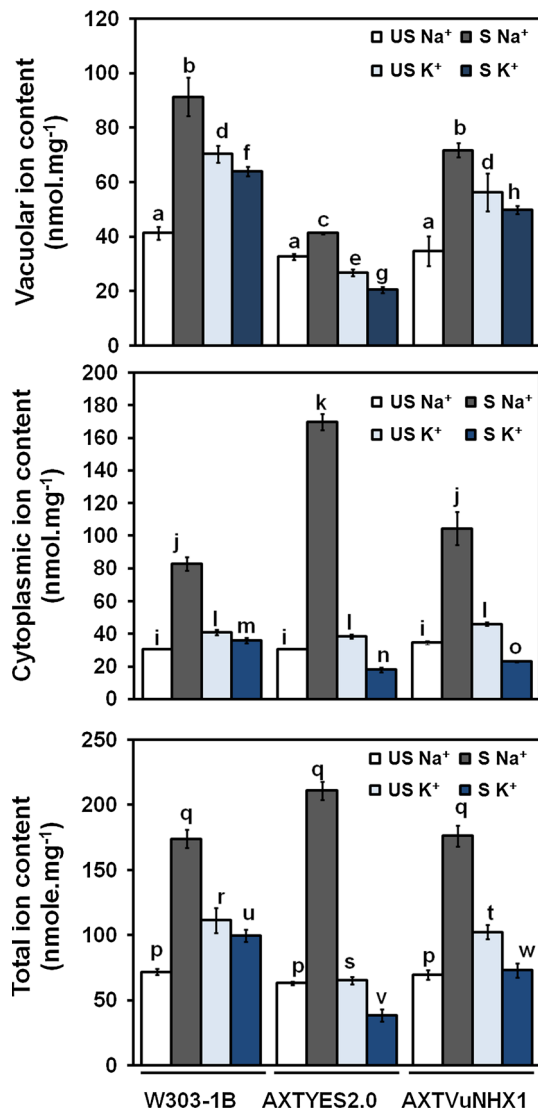
Fig. 2 Total intracellular ion estimation in yeast strains. Wild type (W303-1B) and Δ ena1-4 Δnha1 Δnhx1 mutant (AXT3) strains used for the complementation assay were transformed with pYES2.0 vector only and pYES*VuNHX1* recombinant vector and labeled as AXTYES2.0 and AXTVuNHX1, respectively. Yeast cells were grown in APG medium (pH 4.0) with 1 mM KCl supplemented in presence (stressed, *S*) or absence of 70 mM NaCl (unstressed, US) and harvested at a cell density of 0.3. Total intracellular, vacuolar and cytoplasmic Na$^+$ and K$^+$ content was determined as described in the materials and methods section. Data are means of 3 independent events (n = 3) and SE are plotted in the *graph*. Statistically significant values at $P \leq 0.05$ are indicated as *different letters* using Bonferroni analysis

Fig. 3 Genomic DNA Southern Blot analysis of *VuNHX1*. Cowpea genomic DNA (20 µg) was digested with *Eco*RI, *Hin*dIII and *Pst*I and hybridized with DIG-labeled probe corresponding to the CDS of *VuNHX1* (0.9 kb)

control and treated seedlings, and used for semi-quantitative RT-PCR assays. The results showed varied response of *VuNHX1* under salt, cold and dehydration stress at whole plant level. Under salt stress, a gradual increase in the transcript level was observed after 6 h, in leaves and roots of treated cowpea seedlings (Fig. 4a). The expression of *VuNHX1* in cowpea seedlings subjected to salt stress was significantly upregulated (Fig. 4b) indicating the association of *NHX1* with salt stress response. The expression of *VuNHX1* was also found upregulated under dehydration stress with strong induction at 6 and 12 h of osmotic stress (Fig. 4b), and the results were in agreement with the fact that high saline condition known to mimic osmotic stress in plants. Interestingly, a 3.75 fold increase in *VuNHX1* expression was observed under cold stress at 6 h and the expression decreased by 2.2 fold with further increase in cold stress period relative to the seedlings under normal condition (Fig. 4b).The increased expression level under cold stress could be accounted for the cross-talk between NHX1 and stress components involved in low temperature stress.

Expression analysis of *VuNHX1* under abiotic stress

To understand the potential role of *VuNHX1* in abiotic stress, the expression of this gene was assessed under different stress conditions. Cowpea seedlings were subjected to salt, dehydration or cold treatment for various time periods (0, 6, 12, and 24 h), total RNA was extracted from
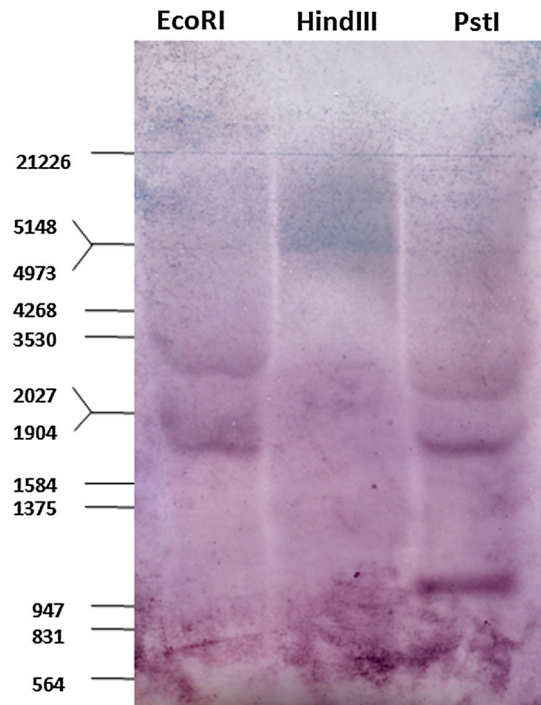
Ion accumulation in salt stressed cowpea seedlings

The Na$^+$ and K$^+$ content in leaves and roots of untreated and salt-treated cowpea seedlings were measured after
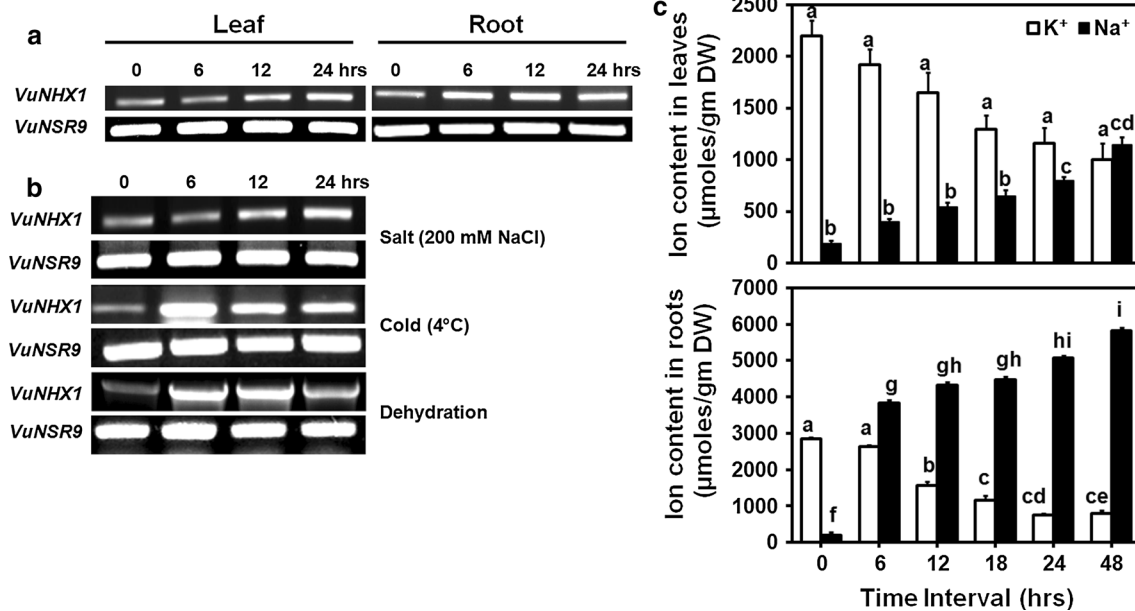
Fig. 4 **a** Semi-quantitative RT-PCR for studying expression patterns of *VuNHX1* under salt stress (200 mM NaCl). Total RNA was isolated from leaves and roots of 10 days old cowpea seedling under 200 mM NaCl treatment at time intervals of 0, 6, 12, and 24 h. **b** Semi-quantitative RT-PCR for studying expression patterns of *VuNHX1* under different abiotic stress conditions such as salt, cold and drought stress. Total RNA was isolated from 10 days old cowpea seedling under 200 mM NaCl, Cold (4 °C), and dehydration treatment at time intervals of 0, 6, 12, and 24 h. PCR fragments of 260 and 292 bp size corresponding to *VuNHX1* and *NSR9* gene were fractionated electrophoretically on 2 % agarose gel stained with 10 mg/ml ethidium bromide. **c** Total intracellular ion measurement in leaves and roots of mid stage cowpea seedlings. Na^+ and K^+ content in leaves and roots of unstressed and salt stressed cowpea seedlings harvested at time intervals of 0, 6, 12, 24, and 48 h was measured using flame photometer. Values indicate mean $\pm$ SE (n = 3). Statistically significant values at $P \leq 0.05$ are indicated as *different letters* using Bonferroni analysis

different time intervals. As shown in Fig. 4c, under normal physiological condition, the Na^+ content was almost similar in both leaves as well as roots of cowpea seedlings. Under salt stress, the Na^+ accumulation was significantly increased in roots than leaves and the increase was concomitant with increase in exposure time (Fig. 4c). Significant difference ($P \leq 0.05$) in Na^+ content was observed after 6 h of salt stress in roots and 18 h of stress in leaves relative to unstressed condition (Fig. 4c). The Na^+ accumulation was increased by 9.3-fold in roots and 6.1-fold in leaves, after 48 h of salt treatment (Fig. 4c). However, no significant difference was observed in $[K^+]$ in leaves under salt stress, unlike in roots after 6 h of salt stress. As compared to control condition, 2.9- and 33.7-fold higher Na^+/K^+ values were observed in case of leaves and roots, respectively, at 48 h of salt stress. The restriction of movement of Na^+ to leaves could possibly the reason for enhanced Na^+ accumulation at 48 h of salt stress in cowpea seedlings, and a higher K^+/Na^+ ratio of 2.1 in leaves as compared to 0.12 in roots. Maintenance of higher K^+ in leaves is essential for cellular and ionic homeostasis to attain unhindered growth under hypersalinity and hyperosmotic condition.

Growth of transgenic *Arabidopsis* overexpressing *VuNHX1* under salt stress

To validate the function of *VuNHX1* in model plant, *Arabidopsis* during salt stress, independent T_4 homozygous *Arabidopsis* lines expressing *VuNHX1* either through constitutive CaMV35S promoter (Fig. 5a) or stress-responsive AtRD29A promoter (Fig. 5b), were subjected to salt stress. The difference in their growth and survival was monitored. The effect of salt stress on germination was studied with *Arabidopsis* lines (WT; 35S::*VuNHX1*, line #4 and RD29A::*VuNHX1*, line #13) after one week exposure to 150 mM NaCl. Wild-type and transgenic lines exhibited no apparent difference in growth under normal physiological condition (Fig. 5c). However, under salt stress, the transgenic lines (#4 and # 13) exhibited significantly better growth and survival as compared to WT (Fig. 5c).

The physiological growth parameters (root length and lateral root development) were monitored in WT and independent T_4 homozygous transgenic *Arabidopsis* lines expressing *VuNHX1* constitutively (Line #4, 35S::*VuNHX1*) and in inducible manner (Line #13, RD29A::*VuNHX1*) that were subjected to 150 or 200 mM NaCl. The
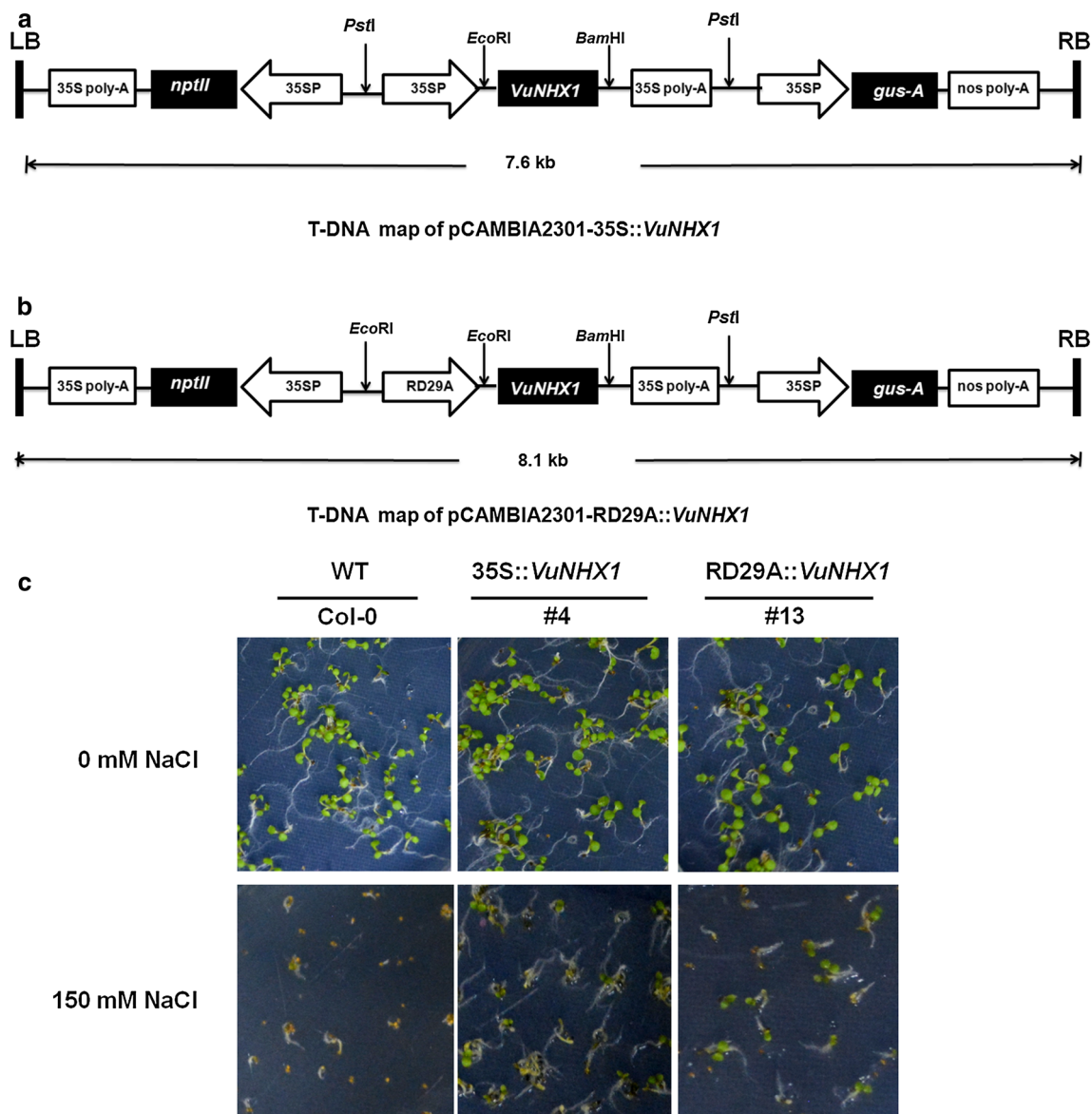
Fig. 5 T-DNA region (7.6 kb) of **a** pCAMBIA2301-35S::*VuNHX1* and **b** pCAMBIA2301-RD29A::*VuNHX1* (14.4 kb). Restriction enzyme PstI and EcoRI used for cloning 35SP::*VuNHX1*::35STer cassette (2.3 kb) and RD29A::*VuNHX1*::35STer cassette (2.8 kb) into plant binary vector pCAMBIA 2301 is also highlighted. *LB* left border, *RB* right border; 35SPromoter, cauliflower mosaic virus 35S promoter; RD29A promoter, stress indicible AtRD29A promoter; CaMV 35S poly-A, cauliflower mosaic virus 35S terminator; nos poly-A, nopaline transferase terminator; *nptII*, neomycin phosphotransferase; *intron-gus-A*, intron interrupted β-glucuronidase; *Vu-NHX1*, *Vigna unguiculata* NHX1. **c** Study on effect of salt stress (150 mM NaCl) on germination efficiency of wild-type WT (col-0) and transgenic *Arabidopsis* 35S:*VuNHX1* (#4) and RD29A::*VuNHX1* (#13) lines

difference in root length was measured after 7 days of salt stress. Under control unstressed condition, no significant difference was observed between WT and transgenic *Arabidopsis* lines (Fig. 6a). However, a significant difference ($P \leq 0.05$) in root length was observed in transgenic *Arabidopsis* lines under 150 and 200 mM NaCl stress as compared to WT plants. Under salt stress, root growth inhibition was significantly lower in transgenic *Arabidopsis* lines (35S::*VuNHX1* and RD29A::*VuNHX1*) than WT (Fig. 6a, b). Lateral root development was affected by

salinity stress. Under 200 mM salt stress, significantly ($P \leq 0.05$) higher lateral root number was observed in transgenic lines (#4 and #13) as compared to WT (Fig. 6b).

The effect of salt stress on mature *Arabidopsis* plants was studied by subjecting the mature WT and transgenic lines (Line #4, 35S::*VuNHX1* and Line #13, RD29A::*Vu-NHX1*) to salt stress (200 mM NaCl) and growth and physiological parameters were recorded. Under salt stress, plant growth was found inhibited in WT with symptoms of salinity induced leaf senescence whereas, transgenic plants
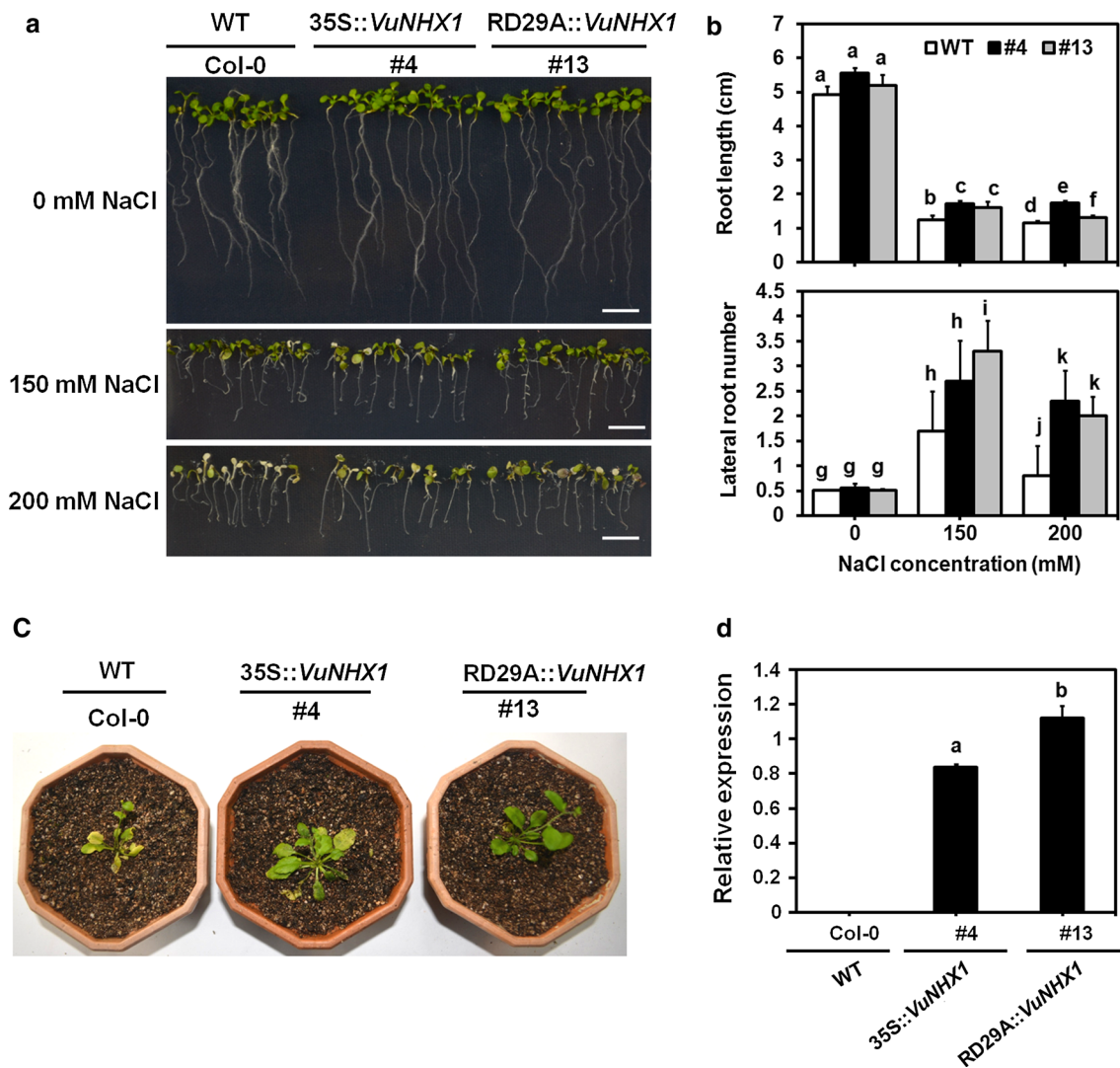
Fig. 6 a Study of root growth inhibition in wild type (WT, Col-0) and transgenic *Arabidopsis* lines (*Line #4*, 35S::*VuNHX1* and *Line #13*, RD29A::*VuNHX1*) upon salt stress. Excessive NaCl-induced root growth inhibition in Col-0 wild-type (WT) plants was observed as compared to transgenic lines. **b** Root length (cm) and lateral root number was measured after exposure of WT and transgenic lines to 150 and 200 mM NaCl stress for 1 week. Values indicate mean ± SE (n = 10). Statistically significant values at $P \leq 0.05$ are indicated as *different letters* using Bonferroni analysis. **c** Salt tolerance assay in wild type (WT, Col-0) and transgenic *Arabidopsis* plants expressing *VuNHX1* constitutively (*Line #4*, 35S::*VuNHX1*) and inducibly (*Line #13*, RD29A::*VuNHX1*) subjected to 200 mM NaCl treatment for 2 weeks. **d** Relative transgene expression level of *VuNHX1* in transgenic *Arabidopsis* lines. No transgene expression was observed in WT. A 0.28 kb fragment of *VuNHX1*::35SployA and 0.150 kb fragment of *AtUBQ1* was amplified in case of transgenic *Arabidopsis* lines

(35S::*VuNHX1* and RD29A::*VuNHX1*) exhibited better survival and comparatively less salinity induced leaf senescence (Fig. 6c). Further, real-time PCR analysis indicated a 1.34-fold higher expression of *VuNHX1* in line #13 (RD29A::*VuNHX1*) as compared to line #4 (35S::*Vu-NHX1*) (Fig. 6 d) indicating the stress inducible expression of *VuNHX1* provided enhanced salt tolerance in comparison to constitutive expression.

The leaves from WT and transgenic lines (#4 and #13) were analyzed for Na^+ and K^+ accumulation, chlorophyll, malondialdehyde (MDA) and proline content, after subjecting the plants to salt stress for 2 weeks. Under normal physiological condition, no difference in Na^+ content was observed between WT and transgenic lines however, transgenic lines #4 and #13 accumulated 1.06 and 1.34 times higher Na^+ than WT, respectively after salt stress. Transgenic line #4 exhibited 1.93 times and line #13 1.66 times higher accumulation of K^+ than WT. Consequently, transgenic lines #4 and #13 maintained a 1.8 and 1.2 fold higher K^+/Na^+ ratio, respectively than WT (Fig. 7). The maintenance of K^+ homeostasis under salinity is essential for regulation of plant growth and survival.

The salt stress apparently penalized the photosynthesis machinery in WT as evident from 1.9 fold lower
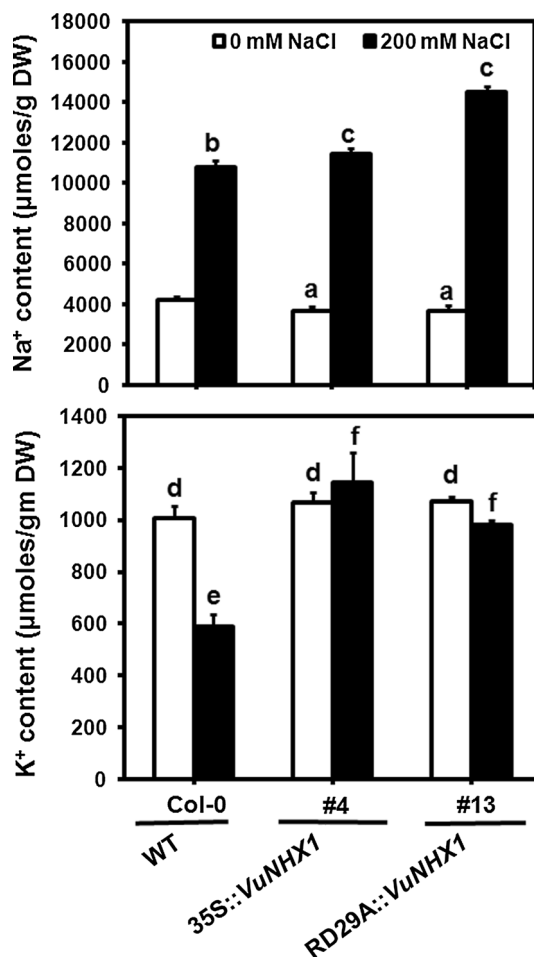
Fig. 7 Na^+ and K^+ content (µmoles/g DW) was estimated in leaves of salt stressed (200 mM NaCl) and unstressed (0 mM NaCl) WT and transgenic lines (*Line #4*, 35S::*VuNHX1* and *Line #13*, RD29A::*Vu-NHX1*), as described in "Materials and methods" section. *Values* indicate mean ± SE (n = 3). Statistically significant values at $P \le 0.05$ are indicated as *different letters* using Bonferroni analysis

chlorophyll content in WT as compared to transgenics (Fig. 8). The MDA content was found 1.2 fold lower in transgenics as compared to WT under salt stress (Fig. 8) indicating lower lipid peroxidation in *Arabidopsis* over-expressing *VuNHX1*. A 1.53 fold higher proline content in transgenic line #4 than WT revealed better osmoprotection under salt stress in transgenic *Arabidopsis* overexpressing *VuNHX1*. In general, the transgenic line (#4) expressing *VuNHX1* under constitutive promoter exhibited higher proline accumulation than transgenic line (#13) that expressed *VuNHX1* under stress inducible promoter (Fig. 8).

Discussion

In this report, a vacuolar Na^+/H^+ antiporter gene, *VuNHX1* was isolated from cowpea, a dry land grain legume with

wide adaptability to varied climatic conditions. Cowpea is known as a drought hardy crop and has been reported to have moderate tolerance to salinity (Murillo-Amador et al. 2006; Duzdemir et al. 2009). Sequence analysis revealed that it encoded an AtNHX1-like antiporter protein, and consequently named as *VuNHX1*. The *VuNHX1* is the first orthologue of *AtNHX1* isolated from cowpea. Phylogenetic analysis of predicted VuNHX1 protein revealed it as Na^+/H^+ antiporter located in the vacuole and clearly distinct from those located at the endosomal or plasma membrane. The results indicated that *VuNHX1* was evolutionary closer and shared a high homology with legume *NHX1*. The topological analysis showed *VuNHX1* comprised of 11 transmembrane domains, in addition to the putative amiloride binding motif at TM3, common to vacuolar Na^+/H^+ antiporters. These results suggest that VuNHX1 corresponds to a Na^+/H^+ antiporter localized in the vacuole. The protein homology and topology suggested that Vu-NHX1 might have similar function and regulation role in salt tolerance as that of dicot glycophyte plants. Southern analysis of cowpea genomic DNA revealed presence of a single copy of *VuNHX1*, unlike the earlier reports of occurrence of multiple copies NHX1 in other plants (Wang et al. 2003; Qingxia et al. 2009).

The role of *VuNHX1* was assessed by complementation of yeast wild type and mutant AXT3 strains. Yeast endosomal Na^+/H^+ exchanger shares functional similarity with plant Na^+/H^+ exchanger, and thereby provide an excellent platform to validate the function of plant Na^+/H^+ exchangers in salt tolerance (Darley et al. 2000; Quintero et al. 2000). Plasma membrane and endosomal Na^+/H^+ antiporters in yeast are involved in ion homeostasis, and cellular pH maintenance. Therefore, $\Delta ena\Delta nha1\Delta nhx1$ mutant shows high sensitivity to increased level of Na^+ and K^+ at low pH condition. Additionally, the $\Delta nhx1$ mutant is sensitive to hygromycin-B, a cationic aminoglycoside antibiotic due to defective sequestration in vacuolar lumen (Brett et al. 2005b). Heterologous expression of plant endosomal Na^+/H^+ antiporters, *AtNHX1* (Hernandez et al. 2009), *MzNHX1* (Qingxia et al. 2009), *MsNHX1* (Bao-Yan et al. 2008), *TSNHX1* (Gouiaa et al. 2012), *AeNHX1* (Qiao et al. 2007), *CmNHX1* (Wang et al. 2011), *ThNHX1* (Wu et al. 2009), *PgNHX1* (Rajagopal et al. 2007) in mutant AXT3 have earlier allowed to complement its salt sensitive phenotype. Expression of *VuNHX1* in $\Delta ena\Delta nha1\Delta nhx1$ mutant enhanced its growth abilities under low osmolarity media and in presence of high concentrations of Na^+, K^+, and Li^+ as compared to AXT3 mutant harboring only pYES2.0. Suppression of hygromycin sensitivity further supported the role of *VuNHX1* in functional complementation of yeast vacuolar NHX exchanger, Sc*NHX1*. The total Na^+ and K^+ content in AXT3 cells transformed with pYES2.0 and pYES*VuNHX1* were in accordance with the
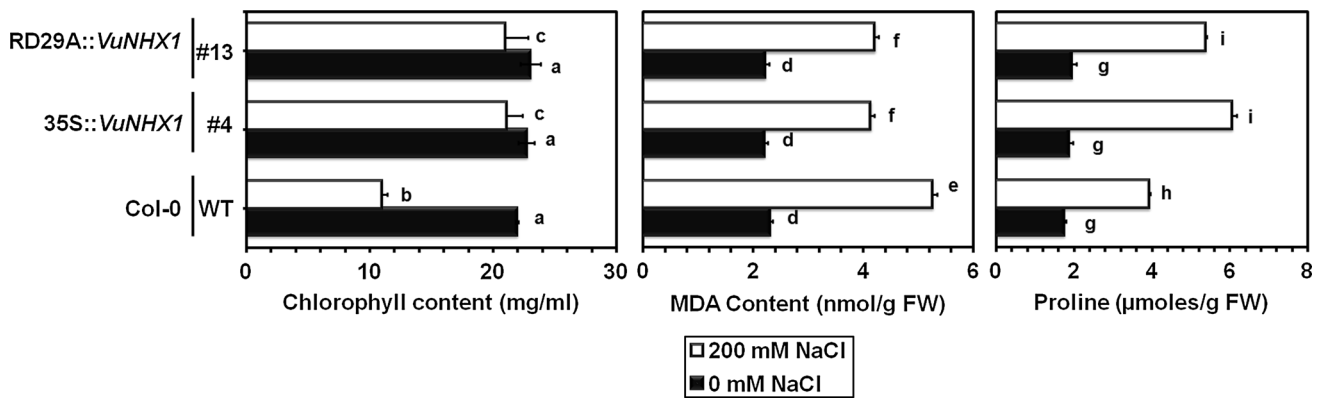
Fig. 8 Physiological analysis of WT (Col-0) and transgenic *Arabidopsis* plants expressing *VuNHX1* constitutively (*Line #4*, 35S::*VuNHX1*) and inducibly (*Line #13*, RD29A::*VuNHX1*) upon salt stress. Changes in chlorophyll, MDA and proline content were estimated and analyzed as explained in "Materials and methods" section. *Values* indicate mean ± SE (n = 3). Statistically significant values at $P \leq 0.05$ are indicated as *different letters* using Bonferroni analysis

function of *VuNHX1*. The results suggested that the role of *VuNHX1* is probably limited to vacuolar sequestration of alkali cations for establishing ion homeostasis. Similar findings were reported for *OsNHX1* expressed in $\Delta ena\Delta nha1\Delta nhx1$ mutant (Kinclova-Zimmermannova et al. 2004). Moreover, *VuNHX1* expression in AXT3 showed enhanced K^+ distribution within both cytoplasmic and vacuolar fractions which was in accordance with the suggested vacuolar cation/proton activity.

In this study, it was observed that *VuNHX1* expression is responsive to salt stress and upregulation of *VuNHX1* was higher in roots than in leaves. Differential distribution of Na^+ in plants is a deciding factor for enhanced salt tolerance (Tester and Davenport 2003). Higher expression in roots was reported for *ZmNHX* (Zorb et al. 2005), *ThNHX1* (Wu et al. 2009) and *AlNHX1* (Zhang et al. 2008) whereas, lower expression reported for *TrNHX1* (Tang et al. 2010), *MsNHX1* (Bao-Yan et al. 2008) and *DmNHX1* (Zhang et al. 2012). In cowpea, in addition to salt stress, dehydration and cold stress also induced the expression of *VuNHX1*. However, down-regulation of *NHX* has been reported under cold stress in *P. glaucum* (Rajagopal et al. 2007) and *T. halophila* (Wu et al. 2009) with no expression in *A. thaliana* (Shi and Zhu 2002). In cowpea, the upregulation of *VuNHX1* in response to dehydration and cold stress is indicative of possible cross-talks between various abiotic stress. Up-regulation of *NHX1* under dehydration stress has been reported for *EgNHX1* (Baltierra et al. 2012), *GmNHX1* (Li et al. 2006), and *OsNHX1* (Fukuda et al. 2004). Cowpea under salt stress accumulated higher Na^+ in roots than shoots and the content increased with increase in stress period. The results were in agreement with the semi-quantitative expression data indicating that higher Na^+ were sequestered in root vacuoles and thereby restricting their movement to the aerial part that maintained a higher K^+/Na^+ ratio. The *VuNHX1* upregulation and higher sequestration of Na^+ in roots was indicative to the salt stress response associated with *NHX1* in cowpea.

Inhibition of seed germination, plant growth and induced senescence are accelerated by salt stress (Lee and Zhu 2010). In our results, transgenic *Arabidopsis* expressing *VuNHX1* through stress inducible promoter (Line #13) or constitutive promoter (Line #4) exhibited comparable degree of salt tolerance. The transgenic lines (#4 and #13) showed significantly higher germination rate, root length and lateral root emergence than WT. The higher Na^+ displayed in leaves of transgenic lines (#4 and 13) was possibly due to the efficient compartmentalization of Na^+ by *VuNHX1*. The transgenic lines showed reduced leaf senescence, chlorophyll and MDA content indicating an active protection against salt and oxidative stress. The higher K^+ accumulation in transgenic plants under salt stress could be due to $Na^+/K^+/H^+$ activity of *VuNHX1*. The maintenance of higher K^+/Na^+ ratio in transgenic plants was essential for ionic homeostasis under salt stress. Our results clearly demonstrated that transgenic *Arabidopsis* plants expressing *VuNHX1* had better survival under salt stress.

In summary, we have isolated a novel vacuolar Na^+/H^+ antiporter gene from cowpea for the first time, which allowed a yeast mutant strain lacking *Scnhx1* to suppress its Na^+ sensitive phenotype. Additionally, we showed that *VuNHX1* expression increased significantly in response to salinity, dehydration as well as cold stress, even though most studies associate its role to salt stress. Moreover, *VuNHX1* stable expression in *A. thaliana* plants subjected to high salt concentration improved their performance in comparison to wild type plants. These results suggested *VuNHX1* overexpression in cowpea through cisgenic and in other legumes by transgenic approach might confer enhanced tolerance to salinity, and possibly to other stress through cross talk triggering.

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