Cloning and Functional Characterization of a Vacuolar Na\textsuperscript{+}/H\textsuperscript{+} Antiporter Gene from Mungbean (VrNHX1) and Its Ectopic Expression Enhanced Salt Tolerance in Arabidopsis thaliana

Sagarika Mishra\textsuperscript{1}, Hemasundar Alavilli\textsuperscript{2}, Byeong-ha Lee\textsuperscript{2}, Sanjib Kumar Panda\textsuperscript{3,4}, Lingaraj Sahoo\textsuperscript{1*}

\textsuperscript{1} Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, \textsuperscript{2} Department of Life Science, Sogang University, Mapo-gu, Seoul, Korea, \textsuperscript{3} Department of Life Sciences and Bioinformatics, Assam University, Silchar, India, \textsuperscript{4} Department of Biochemistry & Molecular Biology, Noble Research Centre, Oklahoma State University, Stillwater, OK, United States of America

Abstract

Plant vacuolar NHX exchangers play a significant role in adaption to salt stress by compartmentalizing excess cytosolic Na\textsuperscript{+} into vacuoles and maintaining cellular homeostasis and ionic equilibrium. We cloned an orthologue of the vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiporter gene, VrNHX1 from mungbean (Vigna radiata), an important Asian grain legume. The VrNHX1 (Genbank Accession number JN656211.1) contains 2095 nucleotides with an open reading frame of 1629 nucleotides encoding a predicted protein of 542 amino acids with a deduced molecular mass of 59.6 kDa. The consensus amidolide binding motif (\textsuperscript{84}LFFYYLLPP\textsuperscript{93}) was observed in the third putative transmembrane domain of VrNHX1. Bioinformatic and phylogenetic analysis clearly suggested that VrNHX1 had high similarity to those of orthologs belonging to Class-I clade of plant NHX exchangers in leguminous crops. VrNHX1 could be strongly induced by salt stress in mungbean as the expression in roots significantly increased in presence of 200 mM NaCl with concomitant accumulation of total [Na\textsuperscript{+}]. Induction of VrNHX1 was also observed under cold and dehydration stress, indicating a possible cross talk between various abiotic stresses. Heterologous expression in salt sensitive yeast mutant AXT3 complemented for the loss of yeast vacuolar NHX1 under NaCl, KCl and LiCl stress indicating that VrNHX1 was the orthologue of ScNHX1. Further, AXT3 cells expressing VrNHX1 survived under low pH environment and displayed vacuolar alkalinization analyzed using pH sensitive fluorescent dye BCECF-AM. The constitutive and stress inducible expression of VrNHX1 resulted in enhanced salt tolerance in transgenic Arabidopsis thaliana lines. Our work suggested that VrNHX1 was a salt tolerance determinant in mungbean.

Introduction

Soil salinity poses increasing threat to plant growth and agricultural productivity worldwide [1]. More than 20\% of the cultivated area and nearly half of the world’s irrigated lands are adversely affected by salinity [2]. Enhanced crop production on saline soils, Na\textsuperscript{+} is the predominant toxic ion. Excess accumulation of Na\textsuperscript{+} in cytosol is detrimental to many metabolic and physiological processes, vital for plant growth and productivity, as it causes ion imbalance, hyper osmotic stress, and oxidative damage to plants [4]. To cope with salinity stress, plants have evolved sophisticated mechanisms, including restricted uptake/exclusion of Na\textsuperscript{+} from cell, and compartmentalization of Na\textsuperscript{+} into vacuoles. Na\textsuperscript{+} efflux is catalyzed by a plasma membrane Na\textsuperscript{+}/H\textsuperscript{+} antiporter (NHX) encoded by SOST [5,6] while, a vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiporter catalyzes the sequestration of Na\textsuperscript{+} into vacuoles. Compartmentalization of Na\textsuperscript{+} into vacuole not only provides an efficient mechanism to avert deleterious effects of Na\textsuperscript{+} in cytoplasm, but also allows plant to use Na\textsuperscript{+} as an osmoticum, for maintaining an osmotic potential for driving water into cell [4,7]. Vacuolar compartmentalization of Na\textsuperscript{+} is a critical process in salt adaptation, which is conserved in both halophytes and dicotyledonous and monocotyledonous angiosperms. The expression of most NHXs was induced by NaCl treatment [9]. Overexpression of vacuolar NHX genes suppressed the salt sensitive phenotype of a yeast mutant defective for endosomal and vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiporters and conferred salt tolerance in...
transgenic plants [10,11]. Several reports on improvement of salt tolerance through overexpression of vacuolar NHXs in agriculturally important but glycophytic crops implicate a pivotal function of the NHXs in intracellular compartmentalization of Na\textsuperscript{+} and salt tolerance [3,12]. In legumes, NHX1 has been reported in *Glycine max* [13], *Medicago sativa* [14], *Trifolium repens* [15], *Lotus tenus* [16], *Caragana korshenskii* [17] and recently by our lab, in *Vigna unguiculata* (GenBank. Acc. No. JN641304.2). However, no salt-tolerant genes including NHX yet reported from mungbean.

Mungbean (*Vigna radiata* L. Wilezek) is an important grain legume widely cultivated in south, east and south-east Asian countries for its protein rich grains. Salinity is recognized as major constraint in the production of mungbean [18,19]. Mungbean is moderately drought tolerant [20] and therefore, this distinctive trait is of great benefit to countries for its protein rich grains. Salinity is recognized as major constraint in the production of mungbean [18,19]. Mungbean is moderately drought tolerant [20] and therefore, this distinctive trait is of great benefit to countries for its protein rich grains.

**Materials and Methods**

**Plant Material and Stress Treatment**

Mungbean (*Vigna radiata* L. Wilezek cv. K-851) seeds were surface sterilized with 0.2% mercuric chloride and rinsed three times with distilled water. The seeds were germinated in dark chamber for 2 days, transferred to Hoagland’s nutrient medium, grown hydroponically in a controlled growth chamber at 25°C, 80% relative humidity with a 16 hr/8 hr photoperiod and photosynthetic flux intensity of 300 μmol m\textsuperscript{2} s\textsuperscript{-1} for 14 days. For salt stress treatment, these two weeks old mungbean seedlings grown under hydroponic conditions were transferred to 200 mM NaCl solution for 12 hrs and roots were harvested, frozen immediately, and stored at −80°C until further use.

**Molecular cloning of VrNHX1 cDNA by RACE approach**

Total RNA was isolated from salt-treated roots of mungbean using AMBION RNAqueous Kit (Ambion, Carlsbad, CA, USA). One microgram of RNA was used for cDNA synthesis using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was amplified with a pair of degenerate primers (Deg FP: 5'-TAT(A/T)ATATT-CATGTC/GCCA(A/G)GT(A/G)-3' and Deg RP: 5'-GCAATT(G/A)/GCCA(A/G)/GTAATG(A/T)GA-CAT(A/G)/CAG -3') designed from the conserved region of transmembrane domains of plant NHX antiporters submitted in NCBI database. The PCR condition was: 94°C for 3 min; 94°C for 30 sec, 52°C for 30 sec, 72°C for 30 sec with 30 cycles, and a final extension at 72°C for 10 min. Based on the resulting partial fragment, gene specific primers were designed for amplification of 5'- and 3'-untranslated regions of *VrNHX1*.

The 5' RACE was performed using the 5' RACE System for Rapid Amplification of cDNA Ends Kit, Version 2.0 (Invitrogen, Carlsbad, CA, USA). Briefly, five micrograms of RNA was used for first strand cDNA synthesis using a gene specific primer (GSP1: 5'-GTGCTTTTTTCACCTGAAACCCAGG -3') and Superscript II reverse transcriptase (Invitrogen). cDNA was purified using SNAP column to remove unincorporated dNTPs and primer, that might interfere in the homopolymeric tailing of cDNA. Terminal access enzyme was used to add dCTPs to 3' end of cDNA. The d-tailed cDNA was amplified using abridged anchor primer (AAP: 5'-GGCCACGGGTGACGACTGATCAGTAC -3') and gene specific primer (GSP2: 5'-ACCTGAAAACCCAGCATTTACAT-3'). The PCR condition was: 94°C for 3 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec with 30 cycles, and a final extension at 72°C for 10 min. Further, nested PCR was performed using abridged universal anchor primer (AUAP: 5'-GGCCACGGGTGACGACTGATCAGTAC -3') and nested gene specific primer (GSP3: 5'-GGTTATATGAA-GAAAAGATCTTCTC -3') using the first PCR product as template. The PCR condition was: 94°C for 3 min; 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min and 30 sec with 30 cycles, and a final extension of 72°C for 10 min. The PCR product was diluted 10 times (1:10) and used as template for nested 3' RACE-PCR. The nested 3' RACE-PCR was carried out using gene specific primer (GSP4: 5'-AGTGGCATCCTCCTACTGATTCTTTTTTG -3') and abridged universal anchor primer (AUAP: 5'-GGCCACGGGTGACGACTGATCAGTAC -3').

The PCR condition was: 94°C for 3 min; 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and 30 sec with 30 cycles, and a final extension of 72°C for 10 min. The above PCR products were cloned to TA cloning vector pTZR/T (Thermo Fisher Scientific, Waltham, MA, USA) sequenced and contiguous sequences aligned to obtain full length of *VrNHX1* cDNA.

**Bioinformatic analysis of VrNHX1**

Multiple sequence alignment and phylogenetic analysis were performed using Clustal W [21]. A phylogenetic tree was constructed using neighbor joining method and reliability of the tree was analyzed with bootstrap analysis with 500 replicates using MEGA4 (Molecular Evolutionary Genetics Analysis): Tree Explorer software [22]. Hydrophobicity plot and transmembrane domain prediction was performed using TMPred software [23]. Post-translational modification of *VrNHX1* was predicted by searching for conserved motifs of N- and O-glycosylation and N-myristoylation sites using ScanProsite [24].

**Southern hybridization for VrNHX1 copy number in mungbean genome**

Twenty μg of genomic DNA was used for gene copy analysis of *VrNHX1* and digested with restriction endonucleases EcoRI and HindIII. Digested genomic DNA was electrophoretically fractionated on 0.8% agarose gel and blotted onto Zeta-Probe membrane (Bio-Rad, Hercules, CA, USA). The blot was hybridized with DIG-labeled 1.6 kb PCR product, corresponding to the coding region of *VrNHX1*. Southern hybridization was carried out using 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 hrs. Washing and detection was performed according to instructions of the DIG Labeling and Detection system (Roche Diagnostics, Mannheim, Germany).

Heterologous expression of VrNHX1 in yeast mutant

Functional complementation assay was performed in yeast strains, W303-1B (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura3-1) and AXT3 (A enol- 4: HIS3 Anuha1:: LEU2 Anuha1::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, 2% Glucose) and APGal synthetic minimal media (10 mM arginine, 8 mM phosphoric acid, 2 mM MgSO4, 1 mM KCl, 0.2 mM CaCl2, 2% Galactose, trace vitamins, and minerals; pH 4.0) supplemented with appropriate amino acids as indicated.

The CDS of VrNHX1 was cloned into yeast expression vector pYES2.0 (Invitrogen, Carlsbad, CA, USA) with restriction sites of KpnI and BamHI. The yeast strains were transformed with pYES2.0 empty vector (labeled as AXTYES2.0 strain) or pYES2.0 (Invitrogen, Carlsbad, CA, USA) with restriction sites of KpnI and BamHI and APGal synthetic recombinant plasmid (labeled as AXTVrNHX1 strain) by Lithium acetate method [23] and selected on SC ura- medium.

For growth assay, precultured cells were grown till OD600 of 1.0, diluted to an OD600 of 0.006, and inoculated to liquid APGal ura- synthetic minimal media supplemented with different concentrations of NaCl, KCl, and LiCl and grown at 30°C for 48 hrs. For complementation assays, liquid cultures (OD600 0.8) of each strain were serially diluted to 10, 100 and 1000 fold and spotted on APGal solid media supplemented with or without 50, 100 and 150 mM NaCl, 0.5 M KCl, 25 mM LiCl and YPGal media supplemented with 50 μg/ml hygromycin. Plates were maintained at 30°C. Growth was monitored after 3 days.

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

Intracellular ion was extracted from yeast strains grown in liquid APGal media, pH 4.0 supplemented without or with 75 mM NaCl [26]. Briefly, cells were harvested at an OD600 of 0.3-0.4, centrifuged at 3000 g/3 min, washed twice in ice-cold 10 mM MgCl2, 10 mM CaCl2 and 1 mM HEPES buffer and resuspended in the same buffer. The relationship between cell density and NaCl concentrations of NaCl, KCl, and LiCl and grown at 30°C 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 in flame photometer (Systronics, MP, India).

Vacuolar pH estimation and fluorescence imaging

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Yeast cells were grown in APGal medium (pH 5.0) to an OD600: 0.25-0.5, pelleted, and washed with deionized distilled water. Further, the yeast cells were incubated with 50 μM 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) (Molecular Probes, Eugene, Oregon) for 30 min, centrifuged, washed thrice and resuspended in APGal medium (pH 5.0) and immediately used for fluorescence measurement. Single emission fluorescence measurement at 490 nm excitation wavelength and absorbance at 600 nm were measured using LS 53 Fluorescence Spectrophotometer (Perkin Elmer, Waltham, MA, USA). The calibration curve for fluorescence intensities at different pH was obtained for each strain [27]. Briefly, the yeast strains (W303-1B, AXTYES2.0, AXTVrNHX1) were incubated in experimental medium containing 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 M ammonium acetate, 10 mM NaN3, 10 mM KCl, 2- deoxy glucose, 50 μM carbonyl cyanide m-chlorophenylhydrazone, titrated to five different pH values within the range of 4.0 to 8.0. Background subtracted I490 values were normalized to cell density for each strain, labeled as NI490 and plotted against pH values. For vacuolar pH estimation, experimental NI490 values corresponding to each strain was analyzed with the calibration curve for each strain.

For vacuolar pH imaging the yeast cells were grown, pelleted to be suspended in the same medium with 50 μM BCECF-AM pH specific dye as above. For fluorescence imaging, 100 μl of BCECF-loaded yeast suspension was plated onto glass cover slips precoated with concavalin-A (Sigma-Aldrich, St. Louis, MO, USA) and placed on glass slides. Fluorescence images were captured in Nikon eclipse Ti-U Fluorescence microscope (Nikon, Chiyoda, Tokyo, Japan).

Expression analysis of VrNHX1 using semi-quantitative RT-PCR

Expression analysis under salt stress: Two different stages of growth in mungbean seedlings i.e. early and mid stage, were considered for expression analysis under salt stress (200 mM NaCl). Mungbean seedlings were germinated, grown in Hoagland’s nutrient medium for five and ten days, in case of early and mid stage respectively, and transferred to 200 mM NaCl solution for salt stress assay. Leaves and roots of salt treated early and mid stage mungbean respectively, were harvested at time intervals 0, 6, 12, 18, 24, and 48 hrs. Similarly, expression pattern for VrNHX1 in response to different forms of abiotic stress such as salt (200 mM NaCl), dehydration (200 mM mannitol) and cold stress (4°C) was also studied at different time intervals 0, 6, 12, and 24 hrs) for mid-stage (10 days old) mungbean seedlings. Total RNA was extracted using RNAeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands) and reverse transcribed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Semi-quantitative RT-PCR was performed using gene specific primers (RF: 5’-GTATTTCCACTGGCGTATGTTATTTTG-3’ and RR: 5’-GCACTAATTACACAGCACCCTCTCGG-3’). The PCR condition was: 94°C for 3 min; 94°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec for 28 cycles, and a final extension at 72°C for 10 min. Housekeeping VrTabulins-β primers (FN: 5’-GGTTCGACTGATCCTCGATGTATTTTG-3’ and RR: 5’-CCACGCTAATTACGCTGGCA-TACTCTG-3’) were used as an internal control. The PCR condition was: 94°C for 3 min; 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec for 28 cycles, and a final extension at 72°C for 10 min. Semi-quantitative RT-PCR was repeated three times. The PCR products were analyzed in 2% agarose gel stained with 10 mg/ml ethidium bromide.
Measurement of total ion content in salt stressed mungbean seedlings

Leaves and roots of untreated and salt-treated early and mid stage mungbean seedlings were harvested at different time intervals (0, 6, 12, 18, 24, 48 and 72 hrs). The samples were dried, digested with concentrated HNO₃ at 90°C for 1 hr and centrifuged at 12,000 rpm for 10 min [28]. The suspension was diluted with sterile milliQ water and analyzed for Na⁺ and K⁺ content in flame photometer.

Binary vector preparation and plant transformation

The 1.6 kb CDS of VrNHX1 was cloned into standard plant binary vector pCAMBIA2301 (11.6 kb) flanked by cauliflower mosaic virus CaMV 35S promoter and terminator at PsI restriction site. The resulting recombinant binary vector was labeled as pCAMBIA2301-35S::VrNHX1 (13.9 kb). Further, a 0.98 kb promoter fragment of AtRD29A (DQ071887.1) was amplified from A. thaliana genomic DNA and cloned into EcoRI digested recombinant binary vector pCAMBIA2301-35S::VrNHX1 (13.9 kb) by replacing the 0.4 kb 35S promoter fragment from 35SP::VrNHX1::35S Ter cassette. The resulting binary vector was named pCAMBIA2301-35S::VrNHX1 (14.4 kb).

The recombinant plant binary vectors, pCAMBIA2301-35S::VrNHX1 (13.9 kb) and pCAMBIA2301-35S::VrNHX1 (14.4 kb) were transferred into A. tumefaciens GV3101 strain via electroporation at 1250 V with capacitance of 25 mF and resistance of 400 ohm. The constructs were used for transformation of Arabidopsis thaliana (ecotype Columbia) via floral dipping method [29]. The T1 transgenic lines were screened on 1/2 MS medium (Duchefa, Haarlem, Netherlands) supplemented with 50 mg/l kanamycin (Duchefa, Haarlem, Netherlands). The transgenic selections were continued until T3 generation to obtain homozygote transgenic lines with a single T-DNA locus (35S::VrNHX1 or RD29A::VrNHX1).

RNA extraction and Real Time PCR of transgenic Arabidopsis lines

Total RNA was extracted from wild-type (WT) and T3 independent 35S::VrNHX1 and RD29A::VrNHX1 transgenic lines using RNeasy Plant Mini Kit (Qiagen), quantified in Nanovue Plus Spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and cDNA was prepared using Revert Aid First Strand cDNA Synthesis Kit. The gene specific forward primer (VrRTF: 5'-TGATTCAATCCATCGACCAA-3') and 35S poly-A reverse primer (TerparR: 5'-GCGAAACCC-TATAAGAACCTAATTC-3') were used for amplification of a 0.283 kb fragment of VrNHX1::35S poly-A in transgenic A. thaliana plants. Housekeeping (UBQ1FP: 5'- AGAGCTGTGCAACTGGAGAAGA-3' and UBQ1RP: 5'-ACAA-GAAAACAAAACCTATCCAAGG) primers were used to amplify a 150 bp fragment of AtUbiquitin to be used as an internal control. Real time PCR was performed using USB VeriQuest SYBR Green qPCR Master Mix (2X) (Affymetrix, Santa Clara, CA, USA) and primers at a final concentration of 200 nM in 7500 Real-Time PCR System (Applied Biosystem, Foster City, California, USA) following the manufacturer’s protocol. 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 level of transgene VrNHX1 in wild-type (WT) and transgenic 35S::VrNHX1 and RD29A::VrNHX1 Arabidopsis lines was estimated by normalizing VrNHX1 expression values with respect to housekeeping AtUBQ1 expression values in each case.

Salt tolerance assays of transgenic Arabidopsis lines

Wild-type (WT) and T3 transgenic 35S::VrNHX1 and RD29A::VrNHX1 Arabidopsis seeds were germinated on ½ MS medium [30] in growth chamber maintained at 22°C and 60% relative humidity with a 16 hr/8 hr photoperiod under controlled conditions. Studying germination efficiency under salt stress: The WT and T3 transgenic 35S::VrNHX1 and RD29A::VrNHX1 lines were germinated on ½ MS medium supplemented with or without 150 mM NaCl and kept at 4°C for 3 days, prior to, transfer to growth chamber. The germination efficiency was studied after 10 days of salt stress.

Measurement of growth parameters under salt stress: The 4 days old germinated seedlings were transferred to ½ MS liquid medium supplemented with or without 200 mM NaCl for 5 days. For measurement of chlorophyll content, shoot samples were homogenized in 93% 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 [31]. 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 (trichloroacetic acid). The homogenate was boiled for 30 min at 95°C and centrifuged at 10,000 g for 5 min. Absorbance values were recorded at 532 nm and values corresponding to non-specific absorption at 600 nm were subtracted [32]. 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 filtered through Whatman filter paper (No. 2) and 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 [33]. Mean data was collected from three replicates (n = 3) for wild-type (WT) and T3 kanamycin selected transgenic Arabidopsis lines.

Measurement of Na⁺ and K⁺ in transgenic Arabidopsis lines

The germinated seedlings were initially grown in ½ MS medium (0.5% agar) for 5 days and then subsequently transferred to soilrite and grown for 2 weeks. The WT and T3 transgenic lines were subjected to salt stress for a period of 2 weeks by watering them with ½ MS nutrient liquid media supplemented with 250 mM NaCl. The whole plant was harvested for Na⁺ and K⁺ estimation using method described elsewhere [30]. Mean data was collected from three replicates (n = 3) for wild-type (WT) and T3 kanamycin selected transgenic Arabidopsis lines.

Statistical analysis

Statistical comparison between the variances was determined by ANOVA (Analysis of variance) and significant differences between

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mean values were determined by Bonferroni analysis. Statistically significant mean values were denoted as "*" (P≤0.05).

Results

Isolation and in-silico analysis of VrNHX1

A VrNHX1 cDNA of 2095 nucleotides in length (Genbank Accession number JN656211.1), with an open reading frame of 1,629 bp was obtained by RACE-PCR approach. It encodes a polypeptide of 542 amino acid residues with an estimated molecular mass 59.60 kDa and isoelectric point 6.76, predicted using ExPaSy bioinformatic tools for protein structure analysis (http://www.expasy.org/tools/). Multiple sequence alignment of deduced amino acid sequences of VrNHX1 revealed that it has 97.42% sequence identity with Vigna unguiculata, 92.25% with Glycine max, 88.48% with Caragana korshinskii, 87.27% with Lotus tenuis, 87.25% with Trifolium repens, 87.06% with Medicago sativa, and 86.72% with Cicer aritinum (Fig. 1 and S1). Phylogenetic relationship analysis performed using MEGA4 software indicated that VrNHX1 clustered into Class-I type IC-NHX legume NHX homologs, more closely to VuNHX1 and GmNHX1 (Fig 1). The hydropathy plot of VrNHX1 protein predicted by TMpred software indicated highly hydrophobic N-terminal end with 11 putative transmembrane domains and a longer hydrophilic C-terminal end inside the vacuolar lumen (Fig. S2). The amiloride binding motif 84-LFFIYLLPPI-93, a classic inhibitor of Na+/H+ antiporters [34] and also highly conserved among eukaryotic Na+/H+ exchangers, was detected in TM3 region (Fig. S1). The prediction of putative post-translational modification sites by ScanProsite software indicated presence of two potential N-glycosylation (ASN_glycosylation) sites, fifteen phosphorylation sites for protein kinase CK2 and protein kinase C, ten N-myristoylation sites, and one Leucine Zipper site (Table S1).

The Southern hybridization analysis revealed presence of single copy of VrNHX1 in mungbean genome (Fig. 2). Two hybridization signals, one each for HindIII and EcoRI digested mungbean genome were detected, possibly due to the occurrence of a single HindIII site in VrNHX1 (1.6 kb). Occurrence of a single EcoRI site in genome fragment of VrNHX1 was accounted for getting two signals as probe lacked any EcoRI site.

Functional characterization of VrNHX1 using salt sensitive yeast mutant

Previous work showed that heterologous expression of Na+/H+ antiporter genes in yeast mutant AXT3 could partly suppress its hypersensitivity to hygromycin and restore salt tolerance. The similar method was exploited to initially characterize the function of VrNHX1. The AXTVrNHX1 cells displayed enhanced Na+, K+ and Li+ tolerance with statistically significant improvement in their survival at NaCl (75 and 100 mM) (Fig. 3 A) and 0.5 M KCl (Fig. 3 B), in contrast to AXTYES2.0 cells. Expression of VrNHX1 in AXT3 cells under GAL1-inducible promoter restored salt tolerance upto 100 times dilution in 75 and 100 mM NaCl (Fig. 4 A), and better survival at 1000 times dilution range in 75 and 100 mM NaCl (Fig. 4 B). VrNHX1 has been suggested to ameliorate sensitivity of yeast cells by sequestering hygromycin-B, a cationic aminoglycoside antibiotic in vacuole. Therefore, yeast mutant lacking NHX1 is more susceptible to hygromycin treatment [27]. VrNHX1 expression showed suppression of hygromycin (50 μg/ml) sensitivity in AXTVrNHX1 cells (Fig. 4 C).
cells exhibited higher K\(^+\) values as compared to AXTYES2.0, indicating the improved ability of AXTVrNHX1 cells in maintaining a higher intracellular K\(^+\)/Na\(^+\) ratio for ionic homeostasis. The total ion content in yeast cells was in accordance with distribution of Na\(^+\) and K\(^+\) in cytoplasm and vacuole.

**Vacular pH estimation and imaging**

2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM), a widely used cell-permeant and pH-sensitive fluorescent indicator was used to measure the change in vacuolar pH of yeast mutant expressing VrNHX1 grown under low pH environment. The study on the effect of low pH on growth efficiency of yeast cells showed that growth of AXTYES2.0 cells was highly affected with a 70.66% reduction in growth as compared to W303-1B. Moreover, AXTVrNHX1 mutant showed improved growth under acidic condition (Fig. S3). Vacuolar pH was estimated following calibration curve plotted for each strain (Fig. S4). An acidic vacuolar pH of 5.4 was observed for AXTYES2.0 cells whereas, a pH value 5.9 and 6.2 was recorded for AXTVrNHX1 and W303-1B cells, respectively in response to low pH stress condition (Fig. 6 A). Similarly, fluorescence images provided acidic vacuolar pH values for AXTYES2.0 cells and expression of VrNHX1 alkalinized the vacuolar compartment (Fig. 6 B).

**Expression pattern of VrNHX1 under abiotic stress by Semi-quantitative RT-PCR**

The expression of VrNHX1 was studied by semi-quantitative RT-PCR, in roots and leaves of mungbean seedlings at early (five days old) and mid (ten days old) growth stages exposed to salt stress (200 mM NaCl) for different time interval (0, 6, 12, 18, 24 and 48 hrs). The results indicated that transcript levels of VrNHX1 were induced by NaCl in both roots and shoots of early and mid stage mungbean seedlings, indicating the potent role of VrNHX1 in salt tolerance mechanisms in mungbean. In case of early seedling stage, higher expression level of VrNHX1 was observed in leaves at 12, 24, and 48 hrs and in roots after 6 hrs (Fig. 7 A). The differential expression of VrNHX1 in roots and leaves was also observed in mid stage seedlings, with a significant accumulation observed at 48 hrs in leaves whereas, some basal level of VrNHX1 transcript was observed in roots under normal condition which further increased steadily with salt stress treatment period (Fig. 7 A).

To determine whether the expression of VrNHX1 was also induced by dehydration (200 mM Mannitol) and cold (4\(^\circ\)C), mid-stage (10 days old) seedlings were given the respective stress treatments for different time intervals (0, 6, 12, and 24 hrs). The VrNHX1 expression varied with salt, cold and drought stress. The accumulation of VrNHX1 transcript under salt, cold and dehydration stress reached its peak at 24 hours (Fig. 7 B). The results indicated that osmotic and low temperature stress is involved in the up-regulation of VrNHX1 in addition to an ion-specific signaling component in mungbean. The VrNHX1 expression analysis revealed involvement of cross talk between salinity, low temperature and osmotic stress in mungbean.

**Figure 2. Copy number analysis of VrNHX1 in mungbean genome.** Mungbean genomic DNA (20 \(\mu\)g) was digested with EcoRI and HindIII, and hybridized with DIG-labeled probe corresponding to the CDS of VrNHX1. Hybridization signals are indicated as arrows. doi:10.1371/journal.pone.0106678.g002

**Figure 3. Cation sensitivity assay of transformed yeast strains (W303-1B, AXTYES2.0, AXTVrNHX1) under various concentrations of NaCl, KCl, and LiCl.** 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 various concentrations of (A) NaCl (0, 50, 75, 100 mM), (B) KCl (0, 0.5, 0.75, 1.0 M), and (C) LiCl (0, 15, 20, 25 mM). Growth was observed at 30\(^\circ\)C after 3 days and absorbance recorded at 600 nm. Data are means of 3 independent events (n = 3) and standard errors are plotted in the graph. Statistically significant values at P\(\leq\)0.05 are indicated as **"***, using Bonferroni analysis. doi:10.1371/journal.pone.0106678.g003
Figure 4. Heterologous expression of VrNHX1 in yeast mutant. Wild type (W303-1B) strain was used as a control, Δ ena1-Δ nhx1 mutant (AXT3) strain was transformed with null pYES2.0 (labeled as AXTYES2.0 strain) and pYES/VrNHX1 recombinant vector (labeled as AXTVrNHX1) were used for complementation assay. 10-fold serial dilutions of saturated seed cultures of each strain were spotted onto APGal media (pH-5.5) supplemented with or without (A) 50, 75 and 100 mM NaCl, (B) 25 mM LiCl, and (B) 0.5 M KCl. (C) Hygromycin sensitivity assay was performed by spotting 10-fold serial dilutions of saturated seed cultures of each strain onto YPGal media (pH-5.5) supplemented with or without 50 μg/ml Hyg. The plates were incubated at 30°C for 3 days.

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Figure 5. Total intracellular ion estimation in yeast strains W303-1B, AXTYES2.0 and AXTVrNHX1. Yeast cells were grown in APG medium (pH 4.0) with 1 mM KCl supplemented in presence (stressed) or absence of 75 mM NaCl (unstressed) 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 standard errors are plotted in the graph. Statistically significant values at P≤0.05 are indicated as ‘*’, using Bonferroni analysis.

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Na⁺ and K⁺ measurement in salt stressed mungbean seedlings

The measurement of Na⁺ and K⁺ content in leaves and roots of untreated and salt-treated mungbean seedlings at different time intervals (0, 6, 12 18, 24, 48 and 72 hrs) showed that under salt stress, Na⁺ accumulation increased in leaves/roots by 1.28/2.1, 1.1/2.3, 2.1/4.36, 4.8/4.3, 4.1/4.54 times whereas, K⁺ accumulation decreased by 3.4/4.5, 1.6/1.78, 1.59/2.43, 2.2/3 and 2.1/3.5 times as compared to control condition at 6, 12, 18, 24 and 48 hrs, respectively in early stage mungbean seedlings (Fig. 8 A). Similarly, in mid stage seedlings, Na⁺ accumulation in leaves/roots

Figure 6. Measurement of vacuolar pH in yeast strains. (A) Vacuolar pH was measured for BCECF-AM loaded yeast strains W303-1B, AXTYES2.0 and AXTvNHX1 as described in materials and methods following the calibration curve (Figure S4). Mean and SEs are plotted for three independent events (n = 3) in each case. Statistically significant values at P≤0.05 are indicated as *′′, using Bonferroni analysis. (B) Accumulation of pH-sensitive fluorescent BCECF dye in yeast vacuoles was measured. The yeast strains were grown in APGal media (pH 5.0), resuspended in minimal medium with BCECF-AM dye for 30 min at 30°C. Yeast cells were visualized by Nikon eclipse Ti-U Fluorescence microscope (Nikon) at excitation wavelength of 440 nm. Bar scale: 50 μm. doi:10.1371/journal.pone.0106678.g006

Figure 7. Expression analysis of VrNHX1 in early and mid stage mungbean seedlings under various abiotic stresses. (A) Semi-quantitative RT-PCR for studying expression patterns of VrNHX1 under salt stress was performed. Total RNA was isolated from leaves and roots of early (5 days) and mid stage mungbean seedlings (10 days) under 200 mM NaCl treatment at time intervals of 0, 6, 12, 18, 24, and 48 hrs. (B) Semi-quantitative RT-PCR for studying expression patterns of VrNHX1 under different abiotic stress conditions such as salt, cold and dehydration stress was studied. Total RNA was isolated from mid stage mungbean seedlings under (A) 200 mM NaCl, (B) Cold (4°C), and (C) 200 mM Mannitol treatment at time intervals of 0, 6, 12, and 24 hrs. PCR fragments of 566 bp and 422 bp size corresponding to VrNHX1 and VrTubß were fractionated electrophoretically on 2% agarose gel stained with 10 mg/ml ethidium bromide. doi:10.1371/journal.pone.0106678.g007
also increased by 1.1/1.4, 1.4/2.4, 4/3.3, 4.5/3.5, 9.8/4.2 and 7.1/4.7 times whereas, K⁺ accumulation decreased by 1.05/1.1, 1.03/1.66, 1.1/3.57, 1.34/3.2, 1.36/4.07, 1.77/4.03 times as compared to control condition at 6, 12, 18, 24, 48 and 72 hrs, respectively (Fig. 8 B). The overall higher accumulation of Na⁺ (μmoles/g DW) in roots as opposed to leaves indicated the restriction of movement of toxic Na⁺ to the aerial part of the plant as a plausible mechanism to confer salinity tolerance in mungbean.

**Ectopic expression of VrNHX1 resulted in enhanced salt tolerance in transgenic Arabidopsis**

In order to characterize VrNHX1 functionally in planta, T₃ homozygous Arabidopsis lines expressing VrNHX1 under the control of constitutive CaMV35S promoter or a stress-responsive RD29A promoter were generated using the binary constructs pCAMBIA2301-35S::VrNHX1 (Fig. S5 A) and pCAMBIA2301-RD29A::VrNHX1 (Fig. S5 B), respectively, to study their performance under salt stress. The germination efficiency was studied in transgenic lines 1 (35S::VrNHX1) and 4 (RD29A::VrNHX1) after exposure to 150 mM NaCl stress for 10 days. Under normal condition, no difference was observed in WT and transgenic lines (Fig. 9 A). However, the transgenic lines exhibited better survival and germination efficiency than WT under salt stress (Fig. 9 A). Further, inhibition of root growth in WT and transgenic lines under salt stress (150 mM NaCl) was studied (Fig. 9 B). Transgenic lines 1 and 4 exhibited 2.65 and 3 times higher root length respectively, than WT (Fig. 9 C). The effect on physiological parameters was monitored in 10 days old wild-type (WT) and independent transgenic Arabidopsis lines expressing VrNHX1 constitutively (Lines 1–3, 35S::VrNHX1) and inducibly (Lines 4–6, RD29A::VrNHX1) under 200 mM NaCl stress for 5 days, by analyzing the total chlorophyll, malondialdehyde (MDA) for lipid peroxidation and proline content. Under normal physiological condition, no qualitative and statistical difference was observed between wild-type and transgenic Arabidopsis lines (Fig. 10). However, under salt stress (200 mM NaCl), WT showed leaf senescence while transgenic Arabidopsis lines (Lines 1–3, 35S::VrNHX1 and Lines 4–6, RD29A::VrNHX1) showed better growth and survival (Fig. 10 A). The transgenic lines showed higher chlorophyll (18–20 mg/ml) and proline (4.3–6 μmoles/g FW) content than WT (Fig. 10 B). The 35S::VrNHX1 lines showed 1.35 times higher proline than RD29A::VrNHX1 lines. A lower lipid peroxidation was detected in transgenic lines as WT showed 1.33 times higher malondialdehyde (MDA) content (Fig. 10 B).

Effect of salt stress was studied in mature WT and transgenic lines (Line 1, 35S::VrNHX1 and Line 4, RD29A::VrNHX1). The transgenic lines displayed better survival efficiency while WT exhibited leaf senescence and growth inhibition upon salt stress (200 mM NaCl) (Fig. 11 A). Transgenic Arabidopsis 35S::VrNHX1 plants displayed constitutively high expression of VrNHX1 under both control (unstressed) and salt stress conditions, whereas RD29A::VrNHX1 lines showed high induction of VrNHX1 only after stress treatment with basal expression levels under normal conditions (Fig. 11 B). The total Na⁺ and K⁺ accumulated in transgenic lines was higher than WT. Further, transgenic 35S::VrNHX1 and RD29A::VrNHX1 lines exhibited 1.3 and 1.14 times higher Na⁺/K⁺ ratio, respectively, as compared to WT (Fig. 11 C, D).

**Discussion**

This is the first report on isolation and functional characterization of a vacuolar Na⁺/H⁺ antiporter (VrNHX1) from mungbean. Phylogenetic analysis and evolutionary relationship revealed that...
**Figure 9. Effect of salt stress on germination efficiency and root growth of transgenic Arabidopsis lines.** (A) The wildtype (WT, col-0) and transgenic (line 1, 35S::VrNHX1 and line 4, RD29A::VrNHX1) seedlings were observed for germination score after 10 days exposure to salt stress (150 mM NaCl). (B) Root growth inhibition in wild type (WT, Col-0) and transgenic Arabidopsis (Line 1, 35S::VrNHX1 and Line 4, RD29A::VrNHX1) plants upon salt stress (150 mM NaCl) was studied. The 4 days old germinated seedlings were transferred to 150 mM NaCl stress for a period of 7 days and (C) root length measured was plotted in graph. Values indicate means ± SE (n = 10). Statistically significant values at P≤0.05 are indicated as "*", using Bonferroni analysis.

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**VrNHX1** shared highest homology with reported legume Na⁺/H⁺ antiporters belonging to the Class-I type NHX exchanger group. The potential structural and functional similarity between yeast and plant endosomal Na⁺/H⁺ exchanger, serves as a valuable tool for validation of novel plant Na⁺/H⁺ exchangers for their role in salt tolerance [35,36]. Restored growth of AXTVrNHX1 cells in presence of high concentrations of Na⁺, K⁺, and Li⁺ and suppression of hygromycin sensitivity indicated the functional complementation of ScNHX1 by heterologous expression of VrNHX1. The Na⁺ distribution pattern in vacuolar and cytoplasmic fractions of AXTVrNHX1 cells as compared to AXTYES2.0 cells, indicated the potent role of VrNHX1 as a vacuolar Na⁺/H⁺ antiporter limited to vacuolar sequestration of alkali cations for establishing ion homeostasis. Similar findings were reported in functional complementation of OsNHX1 in AXT3 mutant [37]. Moreover, VrNHX1 expression in AXTVrNHX1 showed enhanced K⁺ distribution within vacuolar fractions which was in accordance with the results obtained in heterologous expression of AtNHX1 [10] and TNHX1 [38] in AXT3 mutant. It was also observed that cytoplasmic K⁺ fractions were lower in AXTYES2.0 cells as compared with AXTVrNHX1 cells and W303-1B wild type cells. Alkalization of endolytic compartments has been reported to be mediated by ScNHX1 which serves as a leak pathway for H⁺, thus, regulating the pH level for efficient survival against external acid stress [27,39]. In our studies, we observed that growth sensitivity of AXTVrNHX1 cells was lower than AXTYES2.0 cells under external acidic pH environment. Vacular acidification was reduced in AXTVrNHX1 cells under low pH indicating the role of VrNHX1 in extrusion of excess H⁺ by its ion specificity.

Differential regulation of Na⁺ uptake, extrusion, compartmentalization, radial transport to stele, loading and unloading into xylem is responsible for the varied response of plants against salinity stress. Under salt stress, VrNHX1 expression was induced in both leaves and roots of mungbean seedlings with concomitant higher expression in roots than leaves in both early and mid stage seedlings. This result was in accordance with previous reports on expression of ZmNHX1, AeNHX1, AlNHX1, and ThNHX1 [40–43] and contrary to reports of expression OsNHX1, AgNHX1, SsNHX1, PeNHX1, MsNHX1, TrNHX1, ZjNHX1, ZxNHX, and DmNHX1 [17,44–51] which had higher expression in leaves/shoots. The expression pattern of VrNHX1 under various abiotic stress conditions in mungbean revealed gradual increase in expression under salt stress (200 mM NaCl) after 24 hrs, cold stress (4°C) at 12 hrs and dehydration stress (200 mM mannitol) after 24 hrs. The result was contrary to the previous reports on expression pattern of PeNHX1 and ThNHX1 [42,48] under cold stress that showed decrease in the transcript accumulation. No change in expression pattern of AtNHX1 under cold stress has been reported [52]. Up-regulation of VrNHX1 under cold stress can be attributed to the other unknown functional mechanisms that still remain to be deciphered. However, involvement of NHX1 in conferring freezing tolerance has been reported in transgenic A. thaliana overexpressing ScNHX1, although the exact mechanism has not been explained [53]. Water deficit and altered water potential along with ionic imbalance are known to be primary effects of salt stress [4,8]. We found under dehydration stress the expression pattern of VrNHX1 in mungbean seedlings similar to previous reports on expression of GmNHX1, ThNHX1 and EgNHX1.
which displayed up-regulation under dehydration stress [13,42,54]. However, contrasting results have been reported for expression of PeNHX1 and AtNHX1 [48,52]. Physiological response under salt stress, indicated higher Na⁺ accumulation in roots than shoots in early and mid stage mungbean seedlings, in contrast to the reports in T. repens,
Z. japonica, H. caspica, Z. xanthoxylum, D. morifolium [17,49–51,55] that showed preferential accumulation of Na\(^+\) in leaves/shoots. This indicated that higher K\(^+\)/Na\(^+\) ratio is maintained in leaves owing to sequestration of higher Na\(^+\) in root vacuoles thus, restricting their movement to the aerial part of plant. Combined together, increased \(VrNHX1\) transcript level coupled with higher sequestration of Na\(^+\) in roots can be attributed as the tolerance mechanism of mungbean under salt stress.

Ectopic expression of \(VrNHX1\) conferred salt tolerance in transgenic \(Arabidopsis\) lines. Both, 35S::\(VrNHX1\) and RD29A::\(VrNHX1\) homozygous T\(_2\) lines displayed better growth response in comparison to WT. Salt stress affects the photosynthetic system components including chlorophyll contents [56]. The reduction in chlorophyll content was less in transgenic lines (35S::\(VrNHX1\) and RD29A::\(VrNHX1\)) as compared to WT. Lipid peroxidation is mediated by increase in accumulation of reactive oxygen species (ROS) under salinity stress [57]. Therefore, the extent of lipid peroxidation was measured using malonaldehyde (MDA), a by-product of lipid peroxidation. Transgenic lines showed lower extent of MDA generation as compared to WT indicating protection against membrane damage process. Metabolic response against salt stress, generally includes generation of proline, an osmoprotectant and compatible osmolyte, as a protective measure in plants [4]. Transgenic lines expressed higher proline content in response to salt stress. Proline is also known as a potent ROS scavenger [58] which might also be correlated with the lower levels of generation of ROS, thus rendering reduced lipid peroxidation in transgenic plants as compared to WT. Similar result was also reported for proline content in transgenic \(Arabidopsis\) lines overexpressing \(DmNHX1\) [51]. The regulation of K\(^+\)/Na\(^+\) ratio to maintain K\(^+\) homeostasis for proper cellular and enzymatic functioning is an essential mechanism against salinity stress in plants [59]. Our results demonstrated that the transgenic lines (35S::\(VrNHX1\) and RD29A::\(VrNHX1\)) maintained a higher K\(^+\)/Na\(^+\) ratio than WT plants under salt stress indicating effective tolerance in transgenic lines under salt stress. The phenotypical, physiological and expressional analysis using quantitative real-time PCR concluded that the transgenic RD29A::\(VrNHX1\) line displayed comparable higher survival and growth than 35S::\(VrNHX1\) lines under salt stress and can be further exploited in crop plants.

The expression of \(VrNHX1\) under constitutive and inducible promoter enhanced salt tolerance in transgenic \(Arabidopsis\) lines. \(AtNHX1\) is one of the most effective genes in improving plant salt tolerance, however, it played a dominant role mainly in leaf. Our result suggested that \(VrNHX1\) might play an important role in the root resistance to Na\(^+\) toxicity. Therefore, we could assume that overexpression of \(VrNHX1\) in crop plants might generate enhanced salt tolerance.
Supporting Information

Figure S1 Multiple sequence alignment was performed for amino acid sequences of plant NHX proteins using CLUSTAL W. The GenBank Accession numbers for NHX proteins are: VrNHX1 (AE050758.1), Vigna unguiculata; GmNHX1 (AAY43006.1), Glycine max; CkNHX1 (ABG89337.1), Caragana korshinskii; MsNHX1 (AA804487.1), Medicago sativa; CaNHX1 (ADL28385.1), Cicer arietinum; MsNHX1; LtNHX1 (ACE73822.1), Lotus tenuis. “**” indicates identical amino acid (AA) residues. “.” indicates conservative AA substitutions and “.” represents semi-conservative AA substitutions in the sequence alignment. The transmembrane region of VrNHX1 as indicated by TM 1–11.

Figure S2 Prediction of transmembrane helices of VrNHX1 (AE050758.1). The hydropathy plot was generated using TMPred online software. The positive values indicate putative transmembrane domains as indicated as TM 1–11.

Figure S3 Growth measurement of yeast strains under low pH. Yeast strains were grown in synthetic medium APGal (pH 4.0) and absorbance was measured at 600 nm. The data shown above are normalized to growth under normal condition (APGal, pH 7.0). W303-1B: Wild type strain, AXTYES2.0: AXT3 mutant harboring null pYES2.0 plasmid, AXTVrNHX1:- AXT3 mutant harboring null pYES2.0 plasmid, AXTVrNHX1:- AXT3 mutant harboring null pYES2.0 plasmid. AXTVrNHX1 recombinant plasmid. Data represent an average of three independent experiments ±SD. (TIF)

Figure S4 Calibration curve for pH sensitive BCECF fluorescent dye was plotted using standards ranging from pH 4.0–8.0. Yeast strains (W303-1B, AXTYES2.0, AXTVrNHX1) grown in APGal medium (pH 4.0) were loaded with BCECF dye as described in materials and methods, fluorescence intensity was measured at 440 and 490 nm, background values (measured with only cell extract and only BCECF dye) were subtracted and the ratio was plotted for each pH value. The data from the three yeast stains were pooled and mean ratio values were plotted with a fitted non-linear graph.

(TIF)

Table S1 The putative post-translational modification sites predicted by ScanProsite software for VrNHX1.

(DOCX)

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Author Contributions

Conceived and designed the experiments: LS BhL SKP. Performed the experiments: SM HA. Analyzed the data: LS BhL SM. Contributed reagents/materials/analysis tools: LS SM HA BhL. Wrote the paper: SM LS. Supervised the study: LS BhL. Provided critical revision of the manuscript for important intellectual content: BhL.

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