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Research article

# Expression of wild rice *Porteresia coarctata PcNHX1* antiporter gene (*PcNHX1*) in tobacco controlled by *PcNHX1* promoter (*PcNHX1p*) confers $Na^+$-specific hypocotyl elongation and stem-specific $Na^+$ accumulation in transgenic tobacco

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ABSTRACT

Soil salinization is a major abiotic stress condition that affects about half of global agricultural lands. Salinity leads to osmotic shock, ionic imbalance and/or toxicity and build-up of reactive oxygen species. $Na^+/H^+$ antiporters (*NHXs*) are integral membrane transporters that catalyze the electro-neutral exchange of $K^+/Na^+$ for $H^+$ and are implicated in cell expansion, development, pH/ion homeostasis and salt tolerance. *Porteresia coarctata* is a salt secreting halophytic wild rice that thrives in the coastal-riverine interface. *P. coarctata NHX1* (*PcNHXI*) expression is induced by salinity in *P. coarctata* roots and shows high sequence identity to *Oryza sativa NHX1*. PcNHX1 confers hygromycin and $Li^+$ sensitivity and $Na^+$ tolerance transport in a yeast strain lacking sodium transport systems. Additionally, transgenic *PcNHX1* expressing tobacco seedlings (*PcNHX1* promoter) show significant growth advantage under increasing concentrations of NaCl and MS salts. Etiolated *PcNHX1* seedlings also exhibit significantly elongated hypocotyl lengths in 100 mM NaCl. *PcNHX1* expression in transgenic tobacco roots increases under salinity, similar to expression in *P. coarctata* roots. Under incremental salinity, transgenic lines show reduction in leaf $Na^+$, stem specific accumulation of $Na^+$ and $K^+$ (unaltered $Na^+/K^+$ ratios). *PcNHX1* transgenic plants also show enhanced chlorophyll content and reduced malondialdehyde (MDA) production in leaves under salinity. The above data suggests that *PcNHX1* overexpression (controlled by *PcNHX1p*) enhances stem specific accumulation of $Na^+$, thereby protecting leaf tissues from salt induced injury.

## 1. Introduction

Soil salinity is one of the primary causes of crop loss worldwide. Every year, about 1.5 million hectares of agriculture lands are affected by high salinity and rendered unsuitable for crop production (Munns, 2005; Carillo et al., 2011). Plants have evolved numerous biochemical and physiological mechanisms to overcome the limitations imposed by salinity stress (Zhu, 2003). $K^+$ is a major macronutrient required for plant growth (Barragán et al., 2012). Under salinity stress, soil $Na^+$ competes $K^+$ for uptake in roots by transporter systems, since both hydrated ions have almost the same ionic radius (Munns and Tester, 2008; Sairam and Tyagi, 2004). One strategy involves sequestration of $Na^+$ in the vacuoles, limiting exposure of cytoplasmic components to toxic $Na^+$ concentrations (Tester and Davenport, 2003). Plant vacuolar $Na^+/H^+$ antiporters (NHX) are ubiquitous membrane proteins that were initially thought to play a primary role in sequestering $Na^+$ in the vacuole, exchanging $Na^+$ for $H^+$ across vacuolar membranes (Blumwald et al., 2000; Jiang et al., 2010; Bassil et al., 2012a,b). *Arabidopsis AtNHX1*, the first tonoplast $Na^+/H^+$ exchanger identified in plants, mediates $Na^+/H^+$ exchange activity in plant vacuoles. Overexpression of *AtNHX*confers salt tolerance in *Arabidopsis* plants and salt tolerance correlates with increased vacuolar $Na^+/H^+$ exchange activity and vacuolar sodium accumulation (Apse et al., 1999). Improved salt tolerance of a variety of plant species expressing vacuolar NHX-like proteins from various sources has been reported (reviewed in Blumwald et al., 2000; Apse and Blumwald, 2007; Pardo et al., 2006). Higher $Na^+$ contents in tissues of transgenic *Arabidopsis* and tomato overexpressing *AtNHX1* have been reported (Apse et al., 1999; Zhang et al., 2008). Subsequent work has shown *AtNHX1* mediates both $Na^+/H^+$ and $K^+/H^+$ exchange in tonoplast vesicles from transgenic tomato plants, in

*Abbreviations:* NHX, $Na^+/H^+$ antiporter; GUS, β-Glucuronidase; ORF, Open reading frame; AP, Arginine phosphate; DW, Dry weight

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artificial proteoliposomes containing AtNHX1 protein and in vacuoles of a yeast mutant strain lacking the endogenous $Na^+/H^+$ and $K^+/H^+$ antiport activities at the tonoplast (Jiang et al., 2010).

Increasing data however suggests that plant NHX antiporters play crucial roles in $K^+$ homoeostasis. A T-DNA knockout of *AtNHX1* shows impaired $Na^+/H^+$ and $K^+/H^+$ exchange in leaf vacuoles, altered leaf development and up-regulated transcripts for high affinity $K^+$ uptake transporters (Apse et al., 2003; Sottosanto et al., 2004). *Arabidopsis nhx1/nhx2* knockout mutants exhibit reduced growth, cell size, leaf turgor, acidified vacuoles, reduced $K^+/H^+$ antiporter activity in leaf vacuolar vesicles, reduced root and leaf vacuolar $K^+$ content, delayed stomatal closure, defective male reproductive organs (including non-dehiscent anthers) that is partly rescued by addition of external $Na^+$ (Bassil et al., 2011a; Barragán et al., 2012).

Halophytic plants serve as excellent model systems for understanding basic mechanisms of salinity tolerance (Flowers and Colmer, 2008). *Porteresia coarctata* (= *Oryza coarctata*) Roxb. Tateoka is a halophytic distant wild rice relative. It occurs as a mangrove associate along the coastal belts of India and Bangladesh (Jagtap et al., 2006). It is a highly tolerant to salinity and can survive in salinity as high as 20-40 dS/m (Bal and Dutt, 1986). *P. coarctata* shows better root and shoot growth, increased leaf biomass, higher relative water content under increasing salt concentrations compared to cultivated rice varieties IR64 and Pokkali (Bal and Dutt, 1986; Sengupta and Majumder, 2009). *P. coarctata* secretes salt through microhairs on its adaxial leaf surface, maintaining a low leaf $Na^+/K^+$ ratio even under high salinity, with $Na^+$ and $Cl^-$ accumulating in the vacuoles of the microhairs under salinity (Flowers et al., 1990). We have previously reported the isolation of a diurnally regulated vacuolar antiporter from *P. coarctata* (*PcNHX1*; Kizhakkedath et al., 2015). In the present study, the function of PcNHX1 protein was examined in a yeast mutant deficient in sodium transport. The ability of *PcNHX1*(under the transcriptional control of *PcNHX1p*) to impart salinity tolerance *in planta* was also examined in transgenic tobacco at both seedling and vegetative stages.

## 2. Materials and methods

### 2.1. Plant materials

*P. coarctata* plants used in this study were collected from Karaikal, Tamil Nadu, India. *P. coarctata* growth conditions are according to Kizhakkedath et al. (2015). For salinity stress, two month old acclimatized *P. coarctata* tillers (0.5X MS; liquid) were transferred to 0.5X MS with 150 mM NaCl and root samples were collected at 12 hourly intervals [(0; no salinity), 12 h, 24 h, 36 h, 48 h, 96 h/48 h salt withdrawal] and used for total RNA isolation.

### 2.2. qRT-PCR analysis of PcNHX1 expression in P. coarctata

Total RNA was isolated from *P. coarctata* roots using TRI reagent (Sigma-Aldrich). cDNA was synthesized from 1 µg of Total (M-MLV Reverse Transcriptase; Invitrogen). Diluted cDNA (1:10; 2 µl) was used as template for PCR amplification. qRT-PCR analysis was carried out using a pair of PcNHX1 RT Fwd2/PcNHX1 RT Rev2 and Actin 1 Fwd/Actin1 Rev primers (200 nm each). β-Actin was used as a house-keeping gene. PCR cycling conditions: 95 °C (10′), 40 cycles [denaturation: 95 °C (15s), annealing and extension at 60 °C (1′)] in a 96-well optical reaction plate (Applied Biosystems, USA). Each qRT-PCR reaction was performed in triplicates, in order to evaluate data reproducibility for two biological replicates. *PcNHX1* expression in root was analyzed using StepOne™ software by comparative Ct ($2^{-\Delta\Delta Ct}$) quantitation method, with values representing 'n'-fold difference relative to house-keeping control.

### 2.3. Functional characterization of PcNHX1 using salt sensitive yeast (Saccharomyces cerevisiae) mutant strains

The *Saccharomyces cerevisiae* triple mutant AB11c (*Mat a; ade2-1; leu2-3; his3-11,15; trp1Δ2, ura3-1, ena1-4:HIS3, nhx1:TRP1, nha1:LEU2*) and wild type strain W303 (*Mat a; ura3-52; trp1Δ2; leu2-3,112; his3-11; ade2-1*; Marešová and Sychrová, 2005) were used in this study. The *PcNHX1* ORF was cloned in *HindIII/BamH1* sites of p*YES2* vector. *OsNHX1* ORFs (*OsiNHX1*: isolated from *O. sativa indica* var IR64; *OsiNHX1* isolated *from O. sativa japonica* leaf tissues by RT-PCR) were amplified from first strand cDNA using *Kpn* I OsNHX1 Fwd/*BamH*I OsNHX1 Rev primers. Fragments were cloned in pTZ57 R/T (Fermentas) and sequenced. *PcNHX1, OsjNHX1* and *OsiNHX1* ORFs were digested with restriction enzymes mentioned above and cloned into the yeast expression vector p*YES2* (Invitrogen). Plasmids were transformed into yeast strain according to the manufacturer's instructions. p*YES2* empty vector was transformed into mutant (AB11c) as well as wild type (W303) strains (negative control). Transformed yeast cultures were grown either in YNB medium (30 °C) or arginine phosphate (AP) medium (pH 6.5; 2% glucose) to an $Abs_{600}$ of 0.8 (48 h). Cells were harvested in cold (centrifugation; 3000 × *g*; 5′), re-suspended in sterile water (1 ml). Ten-fold serial dilutions were prepared for each strain and 5 µl of each dilution were spotted onto YNB containing hygromycin B (0, 75 or 100 µg/ml) or AP medium (pH 4.0; 2% galactose) containing NaCl (0, 25 or 50 mM) or LiCl (0, 0.5 or 1 mM) and grown at 30 °C for 2 days. For cation tolerance assays in liquid medium, starter cultures of transformed strains were grown inoculated in AP medium as mentioned above. The $Abs_{600}$ of starter cultures was measured and diluted appropriately with AP medium [(pH 4.0; 2% galactose) with NaCl (0, 25 or 50 mM) or LiCl (0, 0.1 or 0.2 mM)], such that the initial $Abs_{600}$ of the cultures was 0.09. The strains were grown at 30 °C for 36 h, after which final $Abs_{600}$ was recorded.

### 2.4. Generation of pCAMBIA 1301: PcNHX1p:PcNHX1 transgenic Nicotiana tabacum (tobacco) lines

*PcNHX1* cDNA was cloned directionally in the *BamH1* site of the binary vector p*CAMBIA 1301* under the transcriptional control of the *PcNHX1* promoter (*XbaI/HindIII* sites of MCS' pCAMBIA*1301:PcNHX1p:PcNHX1*; Supplementary Fig. S1A) and transformed into tobacco *via Agrobacterium* mediated transformation. Five independent *PcNHX1* transgenic lines ($T_0$) were selected on hygromycin (50 mg/L) and T-DNA integration was confirmed by PCR (*PcNHX1*promoter Fwd4/*PcNHX1* Exon Rev 4). PCR and GUS positive $T_0$ plants were grown to maturity (green-house) and selfed to obtain $T_1$ seeds (Supplementary Figs. S1B and C). Segregation analysis using hygromycin as selection marker, suggested that for *PcNHX1* lines L2, L3, L7 and L8, values were in the range of 2.7–3.3:1 (close to expected 3:1 Mendelian ratio; Supplementary Table 1), suggestive of single copy insertion of the T-DNA. All four lines were selfed to obtain $T_2$ seeds.

### 2.5. PcNHX1 tobacco transgenic seedling performance under increasing salinity and MS medium strength

Surface sterilised non-transgenic control and *PcNHX1* transgenic ($T_2$) seeds were plated on agar containing medium with (i) 0, 100 or 150 mM NaCl or (ii) MS medium of increasing strength (0.5X, 1X, 1.5X or 2X), were grown at25 ± 1 °C (8 h dark/16 h light) for 25 and 10 days respectively. Seedling fresh weight, root length, and first leaf pair span were recorded.

### 2.6. Etiolation response of PcNHX1 tobacco transgenic seedlings under increasing salinity

The protocol of Bassil et al. (2011b) was modified to measure the etiolation response of *PcNHX1* transgenic seedlings under salinity.

Surface sterilized seeds were plated on modified Spalding medium (with 1 mM KCl) containing increasing NaCl (0, 50,75 or 100 mM) and kept in dark. Seedling hypocotyl length was measured on the 11th day.

### 2.7. Effect of salinity on PcNHX1 tobacco transgenic lines at vegetative stage

Eight week old non-transgenic and *PcNHX1* transgenic plants were transferred to green house and acclimatized for two weeks. Incremental NaCl stress was given to ten week-old control and transgenic *PcNHX1* plants (50, 75, 100 and 125 mM every second day; Supplementary Fig. S2). On 11th day, leaf (top, second and third leaf), root and stem samples were collected for $Na^+$, $K^+$ estimation and qRT-PCR analysis. A separate set of plants were subjected to salinity stress and leaf tissues (top, second and third leaf) were used for chlorophyll and malondialdehyde (MDA) estimation.

#### 2.7.1. qRT-PCR analysis of PcNHX1 expression in tobacco transgenic lines

Total RNA isolation and cDNA synthesis methods are detailed above. Primer pairs PcNHX RT Fwd2/PcNHX RT Rev2, NtNHX2 Fwd1/NtNHX2 Rev1 (endogenous gene) and NtUbi Fwd/Ubi Rev primer (housekeeping gene) were used to amplify fragments of sizes 134 bp (*PcNHX1*), 153 bp (*NtNHX2*) and 125 bp (*NtUbi*; housekeeping gene; internal control) respectively (Table 1). *PcNHX1* expression in transgenic tobacco leaves and root was analyzed using StepOne™ software by comparative Ct ($2^{-\Delta\Delta Ct}$) quantitation method, with values representing 'n'-fold difference relative to housekeeping control.

#### 2.7.2. $Na^+/K^+$ measurement in PcNHX1 transgenic tobacco lines

NaCl treated (treated) and control (untreated) transgenic *PcNHX1* leaf, root and stem tissues were dried in a hot air oven (60°; 2 days). Dry weight was recorded. 50 mg of dried powder was acid digested (nitric acid; 4 ml) on a hotplate at 120 °C (10′). Samples were cooled to room temperature and filtered through Whatman paper (Grade 1). Sample volume was made up to 10 ml using HPLC grade water and diluted appropriately. $Na^+$, $K^+$ content was estimated by atomic absorption spectroscopy (Perkin Elmer, AAnalyst 200 atomic absorption spectrometer).

#### 2.7.3. Chlorophyll and malondialdehyde estimation in PcNHX1 transgenic tobacco lines

For chlorophyll estimation, 100 mg of leaf tissue was homogenized in ice cold 80% acetone. Sample absorbance was measured at 663 and 645 nm. Total chlorophyll as well as Chlorophyll *a* and *b* contents were estimated according to Arnon (1949). Malondialdehyde (MDA) content was estimated according to Heath and Packer (1968). Briefly, 0.5 g of fresh leaf was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 1000 rpm (5′). To 1 ml of supernatant, 4 ml of a TCA: Thiobarbituric acid mix [20%: 0.5%; (w/v) TB] was added and incubated at 95 °C for 30′, chilled on ice, and centrifuged at 4000 × *g* (10′). Absorbance measurements at 532 and 600 nm were used to estimate MDA equivalents.

**Table 1**
Primers used in this study.

| Primer Name | Sequence 5′ - 3′ |
|---|---|
| *PcNHX1* promoter Fwd 4 | TGTAAACACATGGAGTTGTCTCC |
| PcNHX1 Exon Rev 4 | CCTGGTTGAGGACCTGCAATGT |
| PcNHX1 RT Fwd 2 | GAGAGGAGCTGTGTCGATTGC |
| PcNHX1 RT Rev 2 | TCATCCCAAACACCATAGTGCT |
| PcActin Fwd | GAAAGGAAGTACAGTGTCTGGATTG |
| PcActin Rev | AAGCATTTCCTGTGCACAATGGAT |
| NtNHX2 Fwd | AGATTACCTTGCAATTGGAGC |
| NtNHX2 Rev 1 | TCTGGACAGCATTGAACAGAA |
| NtUbi Fwd | TCCAGGACAAGGAGGGTATC |
| NtUbi Rev | CATCAACAACAGGCAACCTAG |
| HindIII ORF Fwd | CCC**AAGCTT**ATGGGGCTGGAGGTGGCGGC |
| BamH1 ORF Rev | GCG**GGATCC**TCATTGTCCTTCATGGACGCTC |
| Kpn1 OsNHX1 Fwd | GG**GGTACC**ATGGGGATGGAGGTGGCGGC |
| BamH1 OsNHX1 Rev | GCG**GGATCC**TCATCTTCCTCCATGGCTC |

Restriction sites are bold underlined.

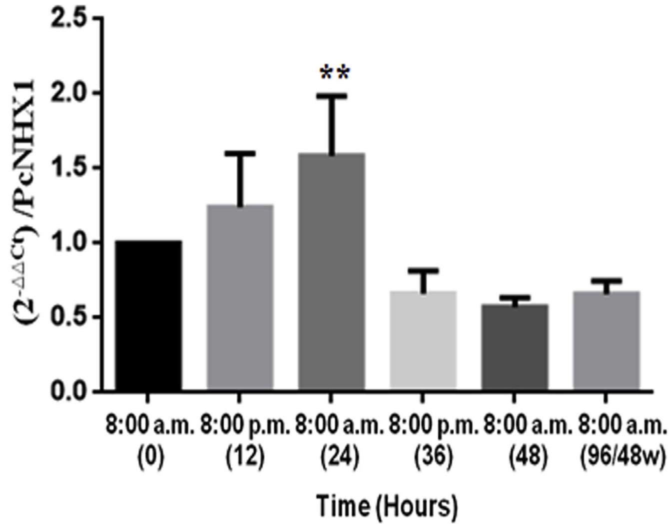


**Fig. 1. *PcNHX1* expression in *P. coarctata* roots under salinity.** qRT-PCR analysis of *PcNHX1* expression in 150 mM NaCl treated *P. coarctata* root tissues (samples collected at 12 hourly intervals [(0; no salinity), 12 h, 24 h, 36 h, 48 h, 96 h/48 h salt withdrawal] and used for total RNA isolation. $2^{-\Delta\Delta Ct}$ values shown are the mean of qRT-PCR estimations (n = 6) with internal replicates (three each) from two independent biological sample sets. Error bars indicate standard deviation (s.d.) in *PcNHX1* expression. Significance was calculated using One way ANOVA (Tukey test):**P < 0.01.

### 2.8. Statistical analysis

The data has been subjected to analysis of variance (ANOVA) using GraphPad (v. 6.1). Mean comparisons were made using Tukey's HSD multiple comparison of mean at $P < 0.05$ or Dunnett's (two way ANOVA) analysis.

## 3. Results

### 3.1. PcNHX1 expression analysis in P. coarctata root tissues

In *P. coarctata* roots, *PcNHX1* expression increased gradually up to 24 h (1.5 fold) and subsequently reduced to half of the initial expression at 36 h-48 h of salinity treatment and also upon salt withdrawal (Fig. 1).

### 3.2. Assessment of PcNHX1 function in Saccharomyces cerevisiae mutant strain AB11c lacking sodium-extruding transport systems

OsNHX1 and PcNHX1 show 96% identity at the amino acid level (Kizhakkedath et al., 2015; Supplementary Fig. S3). Hence, the ability of PcHNX1 *vis-à-vis* OsNHX1 to confer hygromycin and cation tolerance in a sodium transport defective strain (AB11c; Marešová and Sychrová, 2005) was examined. *PcNHX1, OsjNHX1* and *OsiNHX1* exacerbated the hygromycin sensitive phenotype of AB11c cells in a concentration dependent manner (Fig. 2A). Further, *PcNHX1* exacerbated the hygromycin sensitive phenotype more than *OsjNHX1* and *OsiNHX1*. In contrast, *pYES-PcNHX1* transformed AB11c cells showed better growth in the presence of increasing NaCl (25 and 50 mM) concentrations (acidic pH) compared to the same mutant strain grown in medium lacking NaCl in both plates and liquid cultures (Fig. 2B and D). However, there appeared to be no significant difference between the ability of *PcNHX1*-,

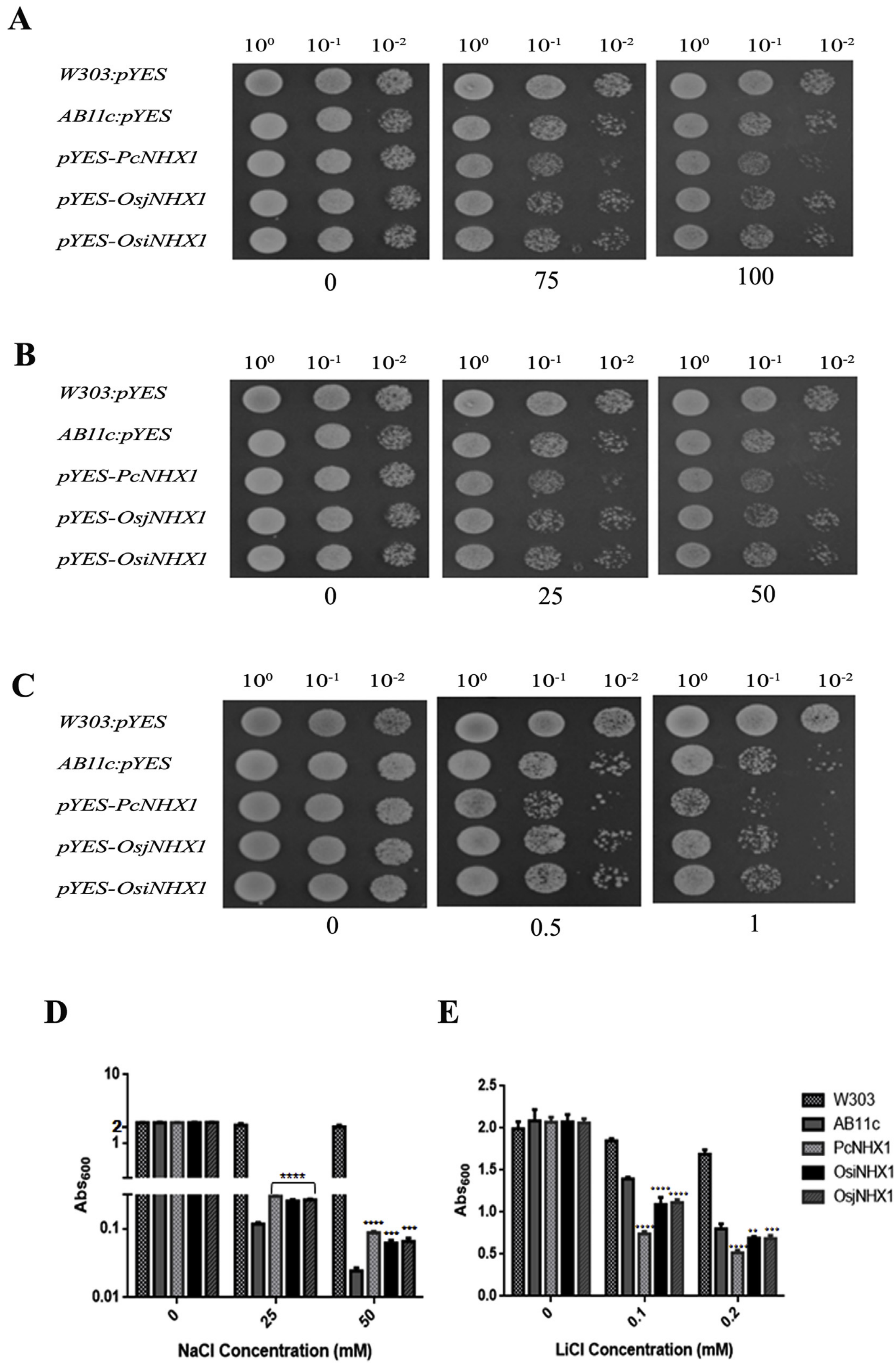


**Fig. 2. Growth of sodium transport-deficient yeast AB11c cells (transformed with *PcNHX1*, *OsiNHX1* or *OsjNHX1*) in the presence of Hygromycin B, NaCl, LiCl containing medium.** *PcNHX1, OsjNHX1* and *OsiNHX1* (cloned in *pYES2* vector) were transformed into mutant strain AB11c. Serially diluted cultures (indicated) of AB11c/W303 transformed with *pYES2, pYES-PcNHX1, pYES-OsiNHX1* and *pYES-OsjNHX1* plated on **(A)** Hygromycin B: 0, 75 or 100 µg/ml (YNB medium), **(B)** NaCl: 0, 25 or 50 mM (AP medium) and **(C)** LiCl: 0, 0.5 or 1 mM (AP medium). AB11c/W303 transformed with *pYES2, pYES-PcNHX1, pYES-OsiNHX1* and *pYES-OsjNHX1*were grown in liquid medium (AP) with increasing **(D)** NaCl concentrations (0, 25 or 50 mM) or **(E)** LiCl concentrations (0, 0.1 or 0.2 mM). The data shown is the mean of three biological replicates n = 3, each with three internal replicates. Significance was calculated using Two way ANOVA (Dunnett's test): **P < 0.01, ***P < 0.001, ****P < 0.0001.

*OsjNHX1*- and *OsiNHX1*-transformed strains to confer NaCl tolerance. Further, all three transformed strains did not confer NaCl tolerance comparable to wild type cells, suggesting the complementation effect is only partial. In LiCl containing medium (0.5 or 1 mM for plate; 0.1 or 0.2 mM for liquid cultures), at acidic pH, the growth of *pYES-PcNHX1* transformed AB11c cells significantly was reduced compared to AB11c cells transformed with *pYES2*, *pYES-OsiNHX1* or *pYES-OsjNHX1* (Fig. 2C and E).

### 3.3. Salinity tolerance of PcNHX1 transgenic tobacco lines at the seedling stage

Seeds of control (C) and *PcNHX1* transgenic lines L2, L3, L7, L8 were germinated on MS (IX) medium with 2% sucrose containing various concentrations of NaCl (0, 100 or 150 mM). In medium lacking NaCl, non-transgenic control and *PcNHX1* transgenic plants showed similar germination efficiencies (radicle emergence). Germination was delayed in both non-transgenic control and *PcNHX1* transgenic lines with increasing concentrations of NaCl. In 100 and 150 mM NaCl, radicle emergence was seen to occur two days earlier in *PcNHX1* transgenic lines (100 mM: fifth day; 150 mM: eighth day) than in non-transgenic control plants (100 mM: seventh day; 150 mM: tenth day). On day 25, fresh weight, root length and first pair leaf span were recorded for both non-transgenic control as well as *PcNHX1* transgenic seedlings. In 150 mM NaCl containing medium, control and *PcNHX1* transgenic leaves showed curling. Fresh weight of *PcNHX1* transgenic seedlings was significantly increased at 150 mM NaCl compared to non-transgenic control (Fig. 3A). *PcNHX1* transgenic lines also showed significantly increased root length at 100–150 mM NaCl (Fig. 3B). First leaf pair span was also significantly enhanced at 100–150 mM NaCl for *PcNHX1* transgenic lines (Fig. 3C). In contrast, fresh weight and root lengths of non-transgenic control vis-à-vis transgenic lines decreased steadily with increasing salinity.

### 3.4. Transgenic tobacco seedling performance under increasing MS strength

In 0.5X MS, radicle emergence was observed on the second day for both control and *PcNHX1* transgenic lines. On the tenth day, a fully expanded first leaf pair and a well established primary root with prominent root hairs were visible (Supplementary Fig. S4). In 1X, 1.5X and 2X MS medium, radicle emergence occurred earlier in *PcNHX1* transgenic lines (1X, 1.5X: third day; 2X: fifth day) while in non-transgenic control plants radicle emergence was delayed by a day (1X, 1.5X: fourth day; 2X: sixth day). With increasing MS strength, leaf growth and root length were reduced. Root hairs were significantly reduced in both non-transgenic control as well as *PcNHX1* transgenic seedlings (1X, 1.5X MS). In 1.5X and 2X MS, leaf succulence was pronounced and a distinct curving of the root to avoid medium penetration was seen in both non-transgenic control as well as *PcNHX1* transgenic seedlings. On the tenth day, transgenic *PcNHX1* lines showed significantly better growth (fresh weight, root length, first leaf pair span length) at all MS concentrations examined relative to control seedlings (Fig. 3D–F).

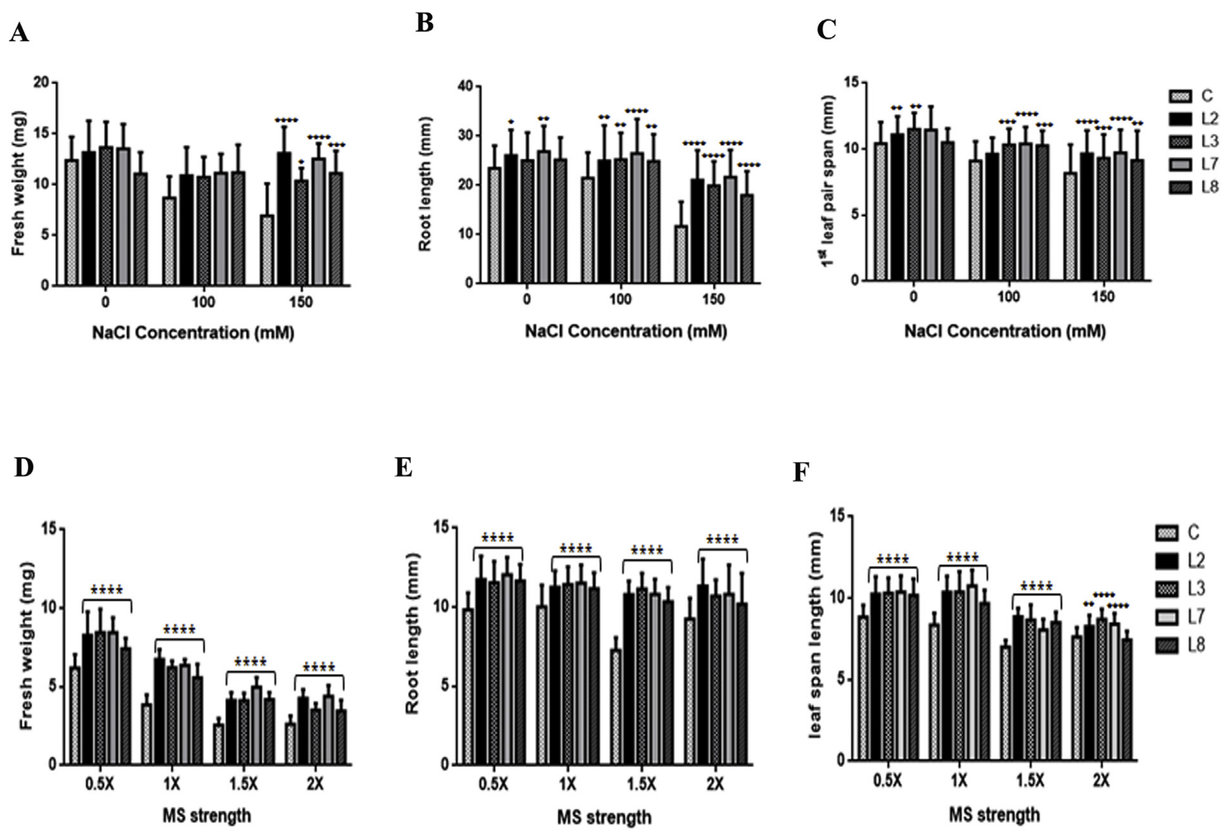


**Fig. 3. Effect of salt stress in *PcNHX1* overexpressing tobacco lines in the seedling stage.** Measurement of **(A)** fresh weight, **(B)** root length and **(C)** first leaf pair span under NaCl in non-transgenic control and $T_2$ *PcNHX1* transgenic seeds of lines L2, L3, L7 and L8 plated on MS medium with increasing NaCl concentrations (0, 100 or 150 mM). Measurements were taken on the 25th day. Bars indicate mean of three biological replicates ± SE (n = 54/treatment/line). Measurement of **(D)** fresh weight **(E)** root length and **(F)** first leaf pair span under increasing MS Strength (0.5X, 1X, 1.5X or 2X) in non-transgenic control and $T_2$ *PcNHX1* transgenic seeds of lines L2, L3, L7 and L8. Measurements were taken on the tenth day. Bars indicate mean of three biological replicates ± SE (n = 27/treatment/line). Significance was calculated using Two way ANOVA (Dunnett's test): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

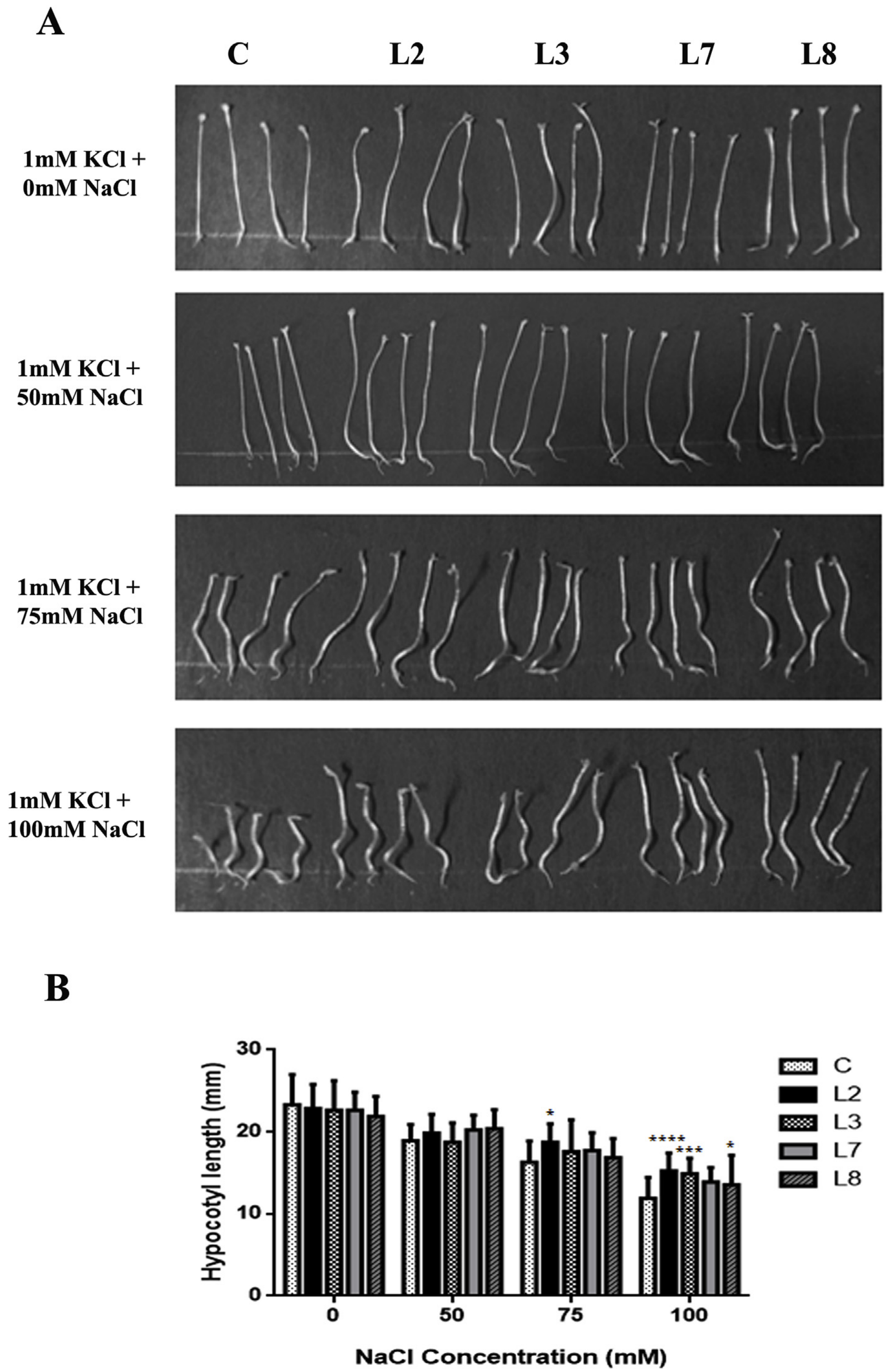


**Fig. 4. Etiolation response of *PcNHX1* transgenic lines under increasing NaCl.** Seeds of non-transgenic control and $T_2$ *PcNHX1* transgenic seeds of lines L2, L3, L7 and L8 were plated on Spalding medium (1 mM KCl) with **(A)** 0, 50, 75 or 100 mM NaCl and germinated under dark for eleven days. **(B)** Hypocotyl lengths were measured on the eleventh day. Bars indicate mean of 3 replicates ± SE (n = 21/treatment/line). Significance was calculated using Two way ANOVA (Dunnett's test): *P < 0.05, ***P < 0.001, ****P < 0.0001.

### 3.5. Etiolation response (PcNHX1 transgenic tobacco) under increasing salinity

With increasing salinity and limiting $K^+$, hypocotyl lengths of both non-transgenic control as well as *PcNHX1* transgenic seedlings were found to decrease. However, etiolated hypocotyls of *PcNHX1* transgenic seedlings showed more growth with increasing salinity compared to non-transgenic control and this difference was significant at 100 mM NaCl. At 75 or 100 mM, etiolated hypocotyls of both non-transgenic control and *PcNHX1* seedlings showed wavy growth (Fig. 4).

### 3.6. qRT-PCR analysis of PcNHX1 and NtNHX2 (endogenous transporter gene) in transgenic lines under salinity

qRT-PCR analysis showed that *PcNHX1* expression is significantly increased in NaCl treated leaf tissues of transgenic lines L3 and L8 compared to untreated transgenic lines. In contrast in line L7, *PcNHX1* expression was lower in NaCl treated sample compared to untreated

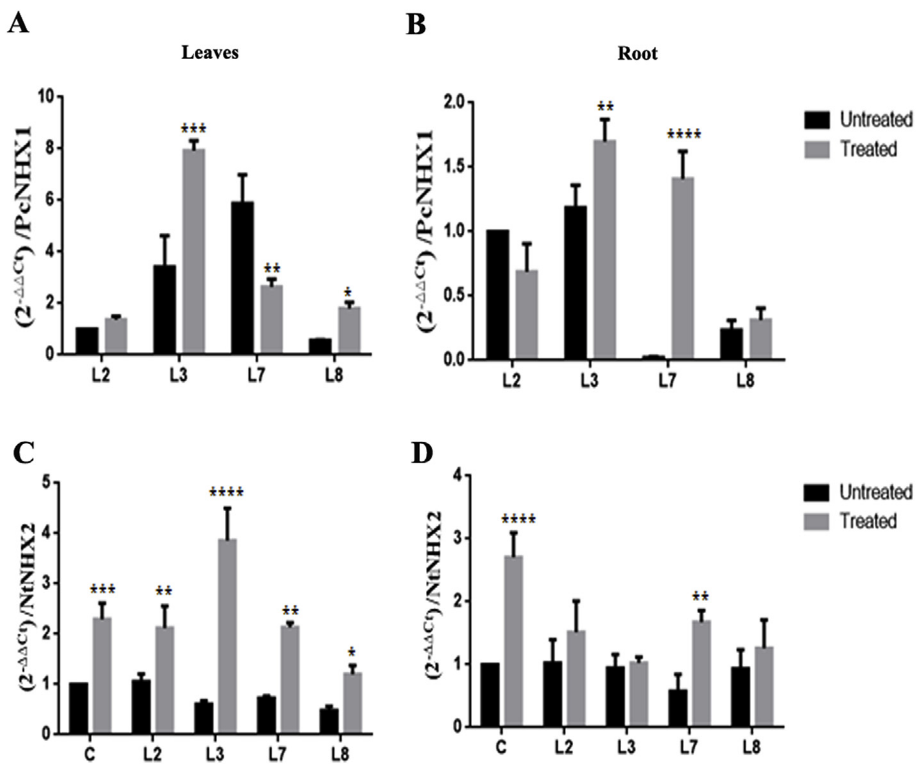


**Fig. 5. qRT-PCR analysis of (A) *PcNHX1* (B) *NtNHX2* in leaves and roots of *PcNHX1* transgenic lines.** Expression analysis of incrementally NaCl treated non-transgenic control and *PcNHX1* transgenic lines. Data shown for one biological set. Bars indicate mean of three internal replicates ± SE (n = 3). Significance was calculated using one way ANOVA (Dunnett's test): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Untreated: unstressed non-transgenic control and *PcNHX1* lines; Treated: Salinity treated non-transgenic control and *PcNHX1* lines.

plants (Fig. 5A). In roots, *PcNHX1* expression was increased in NaCl treated transgenic lines L3, L7 and L8 compared to untreated transgenic lines. In contrast, *PcNHX1* expression was downregulated in transgenic line L2 under incremental NaCl treatment compared to untreated L2 plants (Fig. 5B).

Expression of an endogenous endomembrane vacuolar antiporter (*NtNHX2)* was also assessed in *PcNHX1* transgenic lines under salinity. In leaf tissues of control (non-transgenic plants) as well as transgenic *PcNHX1* lines (L2, L3, L7, L8), *NtNHX2* expression was significantly increased under salinity. However, only in line L3, *NtNHX2* expression was highly up-regulated (Fig. 5C). In roots, however *NtNHX2* expression is upregulated in non-transgenic plants under salinity; however in salinity treated *PcNHX1* transgenic lines *NtNHX2* expression is reduced compared to salinity treated non-transgenic plants (Fig. 5D).

### 3.7. Estimation of tissue $Na^+$, $K^+$ content of PcNHX1 transgenic lines under salinity

In leaves and roots, $Na^+$ content was more in treated plants compared to untreated plants (Fig. 6A and C). In contrast, in stem tissues, $Na^+$ content was lowered in treated plants compared to untreated plants (Fig. 6E). In leaf tissues, $Na^+$ content in treated transgenic line L8 was reduced and significantly in L2 and L7 (reduced by half compared to treated non-transgenic control). Roots of salinity treated transgenic *PcNHX1* lines did not show any significant difference in $Na^+$ content compared to salinity treated non-transgenic control plants. Stem tissues of salinity treated transgenic plants on the other hand, show significant elevation of $Na^+$ content in lines L7 and L8 (marginal in line L2). $K^+$ content of leaf, root and stem tissues in both non-transgenic control as well as *PcNHX1* transgenic lines was reduced by salinity treatment (Fig. 6B, D and F). In leaf tissues of transgenic *PcNHX1* lines, $K^+$ content was reduced by approximately 20%. Line L3 showed significant elevation root $K^+$ content under salinity while stem tissues of transgenic lines L3, L7 and L8 showed significant increase in $K^+$ content under salinity. $Na^+/K^+$ ratios were estimated for all tissues under salinity (Supplementary Fig. S6). In both leaf and root tissues, $Na^+/K^+$ ratios increased under salinity. However, the data suggests there was no significant difference between salinity treated non-transgenic and transgenic leaf tissues (except lines L3 and L8) and a marginal decrease in root $Na^+/K^+$ ratios in transgenic lines. Unlike root and leaf tissues, $Na^+/K^+$ ratios in stem tissues of transgenic *PcNHX1* lines are unaltered by salinity.

### 3.8. Salinity effects on chlorophyll content and lipid peroxidation in PcNHX1 transgenic lines

Chlorophyll content of control non-transgenic plants and *PcNHX1* plants was almost equal under untreated conditions Leaves of all *PcNHX1* transgenic lines showed increased chlorophyll retention (significant for lines L2, L7 and L8) under salinity stress vis-à-vis the salinity treated non-transgenic control (Fig. 7A). MDA content of control non-transgenic plants and *PcNHX1* plants was similar under untreated conditions. Under salinity treatment, there was a significant decrease in leaf MDA content of transgenic lines (lines L2, L7 and L8) compared to non-transgenic control.

## 4. Discussion

Recent studies have indicated that NHX proteins mediate primarily $K^+/H^+$ antiport (to a lesser extent $Na^+/H^+$ exchange) across the membrane by utilizing the proton gradient as a driving force, with vacuolar $K^+$ sequestration providing the turgor force for cell expansion and plant growth (Bassil et al., 2012b). Isolation and characterization of *PcNHX1* has been reported previously (Kizhakkedath et al., 2015). The PcNHX1 amino acid sequence shows 96% identity with OsNHX1 (Fukuda et al., 2004). A comparative assessment of PcNHX1 function *vis-à-vis* OsNHX1 was carried out in the sodium transporter-deficient yeast strain, AB11c. The lack of all three transporters in AB11c leads to a mild depolarization of cell plasma membranes under basal conditions (Kinclova-Zimmermannova et al., 2005). AB11c cells also show sensitivity to hygromycin and this has been attributed to a defective sequestration of toxic cations in intracellular compartments (Kinclova-Zimmermannova et al., 2005). Overexpression of*PcNHX1*, *OsjNHX1* or *OsiNHX1* appears to exacerbate the hygromycin sensitive phenotype of

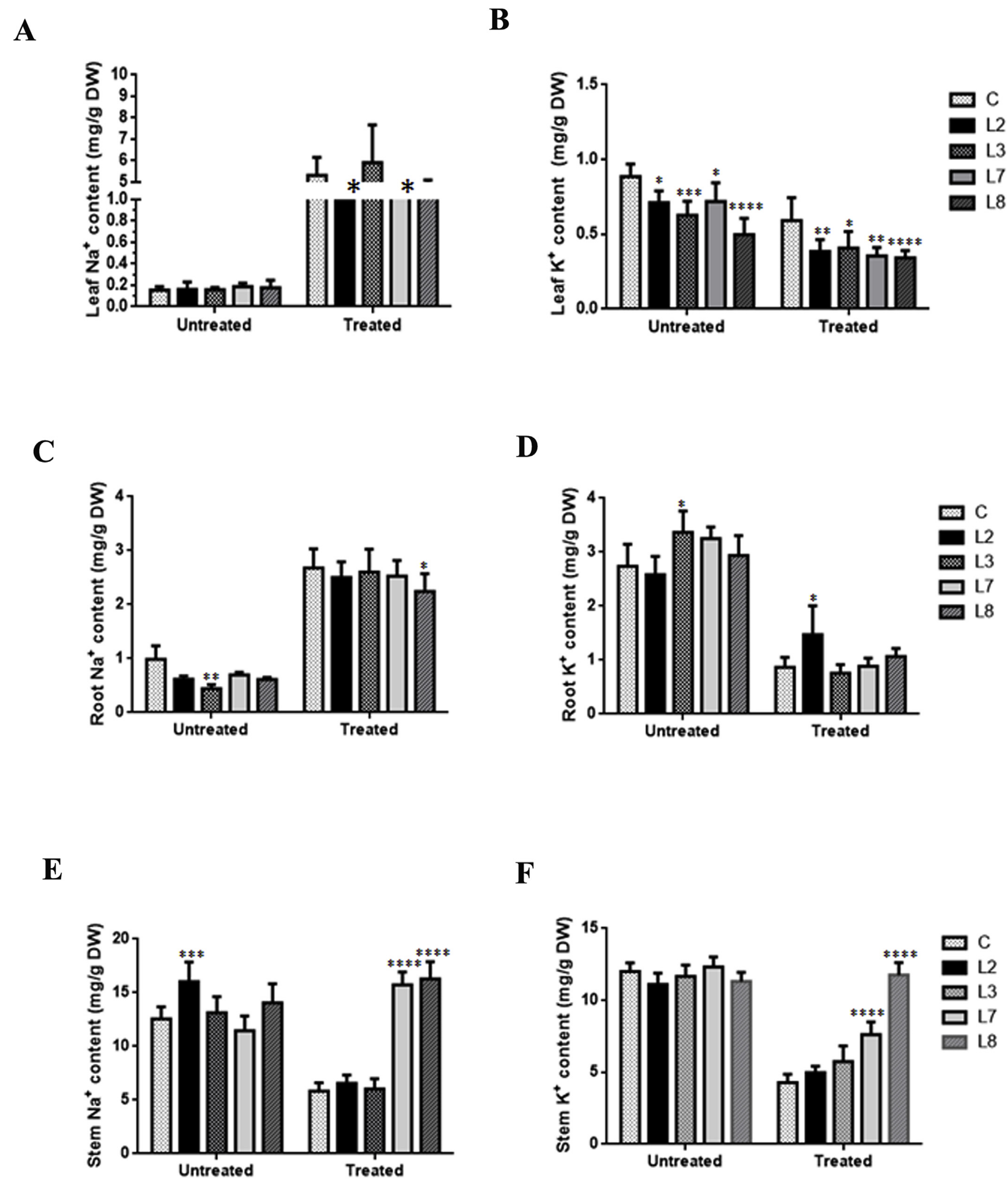


**Fig. 6. $Na^+/K^+$ measurements in *PcNHX1* transgenic lines under salinity.** $Na^+/K^+$ content in **(A)** Leaf **(B)** root and **(C)** stem estimated by atomic absorption spectrometry. Bars indicate mean ± SE (n = 5/treatment/line). Significance was calculated using (Dunnett's test): *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Untreated: unstressed non-transgenic control and *PcNHX1* lines; Treated: Salinity treated non-transgenic control and *PcNHX1* lines.

AB11c cells, possibly increasing toxic cation accumulation in cells. Overexpression of OsjNHX1 in yeast R100 (Δnhx1) cells confers hygromycin tolerance (Fukuda et al., 2011), similar to *PcNHX1* overexpression in YDR456w (Δnhx1:kanMX; EUROSCARF; data not shown). Overexpression of *PcNHX1, OsjNHX1* or *OsiNHX1* cells partially restores growth under increasing NaCl concentrations at pH 4.0.

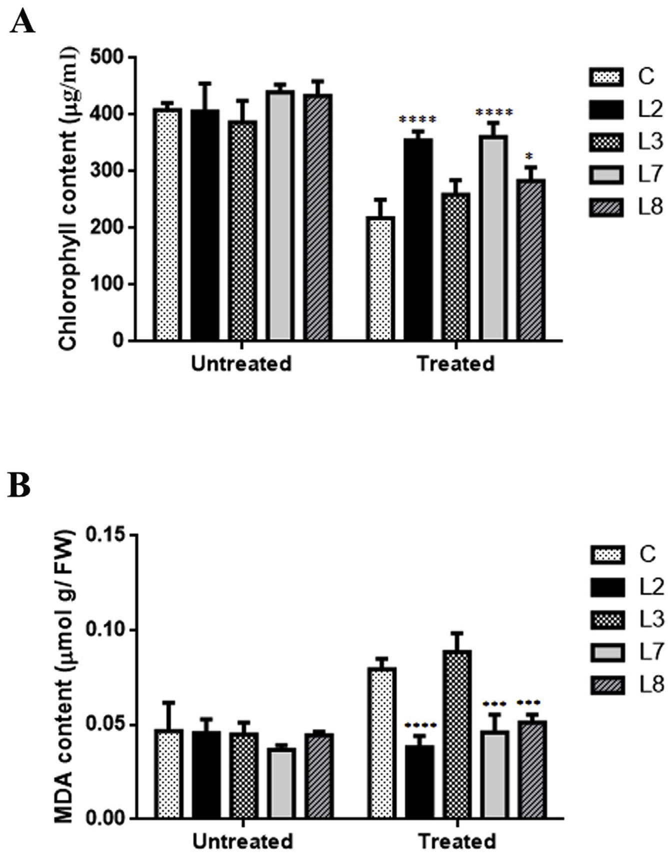


**Fig. 7. Estimation of (A) chlorophyll content and (B) malondialdehyde production [MDA] in top, second and third leaf of non-transgenic control and *PcNHX1* transgenic lines L2, L3, L7 and L8.** Stress treatments for non-transgenic control and *PcNHX1* transgenic lines indicated in Fig. S2. Bars indicate mean ± SE (n = 3/treatment/line). Significance was calculated using (Dunnett's test): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Untreated: unstressed non-transgenic control and PcNHX1 lines; Treated: Salinity treated non-transgenic control and *PcNHX1* lines.

All cells grew equally in medium lacking NaCl, also at pH 4.0, suggesting the effect is not related to the acidic pH of the medium. No differential growth phenotype of AB11c cells overexpressing *PcNHX1*, *OsjNHX1* or *OsiNHX1* was observed in medium containing $K^+$ relative to mutant AB11c cells. Cation transporter function has been linked to the presence of two consecutive amino acids, 'ND', in the fifth transmembrane (TM) domain of *Populus euphratica* NHX3 (PeNHX3; Wang et al., 2014), also conserved in PcNHX1, OsjNHX1 or OsiNHX1 (Supplementary Fig. S3). Overexpression of *PcNHX1*, *OsjNHX1* or *OsiNHX1* (AP medium; pH 4.0) exacerbates the $Li^+$ sensitive phenotype of AB11c cells. Further, *PcNHX1* appears to enhance the $Li^+$ sensitive phenotype significantly more than either *OsjNHX1* or *OsiNHX1*. Wang et al. (2014) have implicated the eleventh TM domain of PeNHX3 in $Li^+$ transport. Alignment of the PeNHX3 sequence with PcNHX1, OsjNHX1 or OsiNHX1 sequences suggests that the eleventh TM domain sequence is largely conserved (Supplementary Fig. S3). PcNHX1, OsjNHX1 or OsiNHX1show three amino acid changes from the PeNHX3 sequence in TM11 domain: $Ile_{400\ (PcNHX1)}\rightarrow Met_{398(PeNHX3)}$; $Ser_{410\ (PcNHX1)}\rightarrow Ala_{408\ (PeNHX3)}$; $Gln_{414\ (PcNHX1)}\rightarrow Asn_{412\ (PeNHX3)}$. Of these, the $Ser_{410\ (PcNHX1)}\rightarrow Ala_{408\ (PeNHX3)}$ may have more significance, as 'Ala', a hydrophobic residue in PeNHX3 is replaced by a polar residue in PcNHX1, OsjNHX1 or OsiNHX1 and may contribute to breaking a transmembrane helical domain.

The *PcNHX1* gene was overexpressed in transgenic tobacco under the transcriptional control of the previously characterized *PcNHX1* promoter (Kizhakkedath et al., 2015). *PcNHX1* tobacco seedlings (*PcNHX1* promoter) show significant growth advantage under increasing concentrations of NaCl and MS salts (increased ionic strength). *PcNHX1* expression in transgenic seedlings was confirmed by RT-PCR (Supplementary Fig. S5). This growth advantage was clearly visible in the absence of stress application (faster germination, increased fresh weight, first leaf pair span). Upon application of NaCl stress, seedlings showed increased fresh weight, root length and first leaf pair span. Increased hypocotyl elongation in *PcNHX1* transgenic seedlings under salinity limiting $K^+$ conditions was also $Na^+$-dependent. Replacing $Na^+$ with $K^+$ in the medium did not confer the hypocotyl elongation phenotype in etiolated *PcNHX1* seedlings, suggesting the effect was $Na^+$-specific (data not shown). *AtNHX1* knockout mutants show delayed seedling establishment and impaired leaf development in the presence of NaCl (Apse et al., 2003). More recently, *AtNHX1* and *AtNHX2* have been established as vacuolar $K^+/H^+$ exchangers, sequestering $K^+$ in the vacuoles to drive turgor-related growth processes (Bassil et al., 2011b; Barragán et al., 2012). In *Arabidopsis nhx1 nhx2* mutants, inclusion of NaCl in the growth medium restores flowering and seed setting partially. Hypocotyls of *nhx1 nhx2* seedlings grown in 30 mM $Na^+$ elongate more than *nhx1 nhx2* grown in control media with limiting $K^+$ (Bassil et al., 2012b). Further, *nhx1 nhx2* mutants show increased shoot growth in minimal $K^+$ medium containing sub-toxic $Na^+$ ion content (Barragán et al., 2012). Vacuolar localization of *PcNHX1* has been shown previously (Kizhakkedath et al., 2015). The above data suggests that $Na^+$ ions can partially substitute for the lack of $K^+$ accumulation in contributing to vacuolar cell expansion related processes (increased leaf span and hypocotyl length) in *PcNHX1* overexpressing lines. Maintaining high $K^+$ in the cytoplasm is essential for a number of enzymatic activities (Zeng et al., 2015). Under salinity stress, $Na^+$ competes with $K^+$ to enter plant cells, mainly through $K^+$ transport pathways at the plasma membrane (Zeng et al., 2015). In halophytes, $Na^+$ serves as an energetically cheap osmoticum that helps balance the plant's osmotic potential against the hypertonic soil solution, thereby alleviating the water deficit that is imposed due to a saline environment (Shabala, 2013).*P. coarctata* grows in soils constantly exposed to fluctuating salinity (coastal-riverine interface; Sengupta and Majumder, 2010). *P. coarctata* leaves secrete excess NaCl through specialized microhairs found on the adaxial leaf surface (Flowers et al., 1990). $Na^+$-specific activity in yeast cells expressing *PcNHX1* and $Na^+$-dependent growth observed in *PcNHX1* transgenic seedlings suggests that it functions in vacuolar sequestration of $Na^+$ in *P. coarctata*.

Incremental salinity treatment of *PcNHX1* transgenic lines resulted in lowered leaf $Na^+$ content, unaltered root $Na^+$ and increased stem $Na^+$ and $K^+$ content (and unaltered $Na^+/K^+$ ratios). *PcNHX1p*-directed GUS activity is detectable in all plant parts and is highest in stem and roots (inducible by salinity in both tissues) followed by leaf tissues (Kizhakkedath et al., 2015). In root tissues, salinity-induced *PcNHX1p*-directed GUS expression is detected in the cortex, vasculature and root tips. In transgenic *PcNHX1* plants, *PcNHX1* expression is also induced by salinity treatment in roots (The variation in expression in *PcNHX1* transgenic lines L2 (root) and L7 (leaf) vis-à-vis other lines under salinity may be due to (i) differential post transcriptional regulation of *PcNHX1* mRNA in the lines. (ii) promoter methylation (ii) differential influences of flanking plant DNA sequences (position effect) superimposed on basic promoter activity (especially if the flanking DNA sequence harbours silencer/enhancer elements (van Leuween et al., 2001; Kohli et al., 2006). Salinity also upregulates *PcNHX1* expression in *P. coarctata*. The data presented above suggests that root-specific expression of *PcNHX1* during salinity plays a role in initial storage following uptake. However, since root $Na^+$ is unaltered in salinity treated *PcNHX1* lines, it is possibly transferred in the transpiration stream to the stem by other $Na^+$ transporting systems (eg. SOS1, HKT1,4; Olías et al., 2009; Suzuki et al., 2016) where it is stored. Increased stem specific $Na^+$ content in *PcNHX1* lines under salinity is consistent with elevated *PcNHX1p*- directed GUS activity in stems of tobacco plants under salinity (Kizhakkedath et al., 2015). In *PcNHX1p:GUS* promoter fusion lines, *PcNHX1* expression is unaltered by salinity and shows a diurnal rhythm that is damped by NaCl treatment in *P. coarctata* leaves (Kizhakkedath et al., 2015). Lowered $Na^+$ content in leaves of *PcNHX1* lines correlates with reduced GUS activity in leaves of PcNHX1p:GUS transgenic lines under salinity (relative to stem and root). Stem specific $Na^+$ storage in *PcNHX1* lines contributes to reduced $Na^+$ content in actively photosynthesizing tissues (leaves). In tomato, the plasma membrane antiporter *SiSOS1* directs stem specific $Na^+$ accumulation, preventing $Na^+$ from reaching leaf tissues (Olías et al., 2009). Increased stem specific *PcNHX1* expression also correlates with increased $Na^+$-specific hypocotyl elongation in etiolated *PcNHX1* seedlings. Thus, differential tissue-specific expression of *PcNHX1* directed by the *PcNHX1* promoter (both derived from halophytic *P. coarctata*) under salinity controls $Na^+$ partitioning in plant organs and reduces leaf $Na^+$ accumulation in a glycophytic species like tobacco (Supplementary Fig. S7).

## 5. Conclusion

*PcNHX1* is a $Na^+$-specific $Na^+/H^+$ antiporter from *P. coarctata*. Under salinity, increased stem specific expression of *PcNHX1* in *P. coarctata* contributes to accumulation of $Na^+$ in the stem and reduced transport of $Na^+$ to the leaves.

## Contributions PPB

V.J.: Yeast complementation studies, qRT-PCR analysis, cloning and sequencing of PCR products.

V.J./K.K./S.P.: Stress experiments and biochemical analysis.

G.V.: supervised experimental design.

V.J./K.K./S./G.V./A.P. wrote the manuscript together.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.014.

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