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 (PcNHX1) 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 (NHXs) 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 Nax⁺/H⁺ exchanger identified in plants, mediates Na⁺/H⁺ exchange activity in plant vacuoles. Over-expression of AtNHX1 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
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+ homeostasis. A T-DNA knockout of AnNHX1 shows impaired Na+/H+ and K+/H+ exchange in leaf vacuoles, altered 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-desicant anthers) that is partly rescued by addition of external Na+ (Bassist 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 P. coarctata (under the transcriptional control of P. coarctata) 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 acclimated 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 (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 Fwd/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−ΔΔCt) quantitation method, with values representing ‘n’-fold difference relative to house-keeping control.

2.3. Functional characterisation 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, nhx1Δ; Trp1, nhl1Δ; 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 pYES2 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 1 OsNHX1 Fwd/BamH1 OsNHX1 Rev primers. Fragments were cloned in pT7Z5 R/T (Fermentas) and sequenced. PcNHX1, OsiNHX1 and OsiNHX1 ORFs were digested with restriction enzymes mentioned above and cloned into the yeast expression vector pYES2 (Invitrogen). Plasmids were transformed into yeast strain according to the manufacturer’s instructions. pYES2 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 Abs600 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 Abs600 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, 0.5 or 1 mM)), such that the initial Abs600 of the cultures was 0.09. The strains were grown at 30 °C for 36 h, after which final Abs600 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 pCAMBIA 1301 under the transcriptional control of the PcNHX1 promoter (XbaI/HindIII sites of MCS of pCAMBIA1301:PCNHX1p:PCNHX1; Supplementary Fig. S1A) and transformed into tobacco via Agrobacterium mediated transformation. Five independent PcNHX1 transgenic lines (T0) 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 T0 plants were grown to maturity (green-house) and selfed to obtain T1 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 T2 seeds.

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

Surface sterilised non-transgenic control and PcNHX1 transgenic (T2) 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 at 25 ± 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 greenhouse 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 were 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 (2ΔΔCt) 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, AAAnalyst 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 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, AAAnalyst 200 atomic absorption spectrometer).

Table 1

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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−ΔΔCt 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 PcNHX1 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 OsjNHX1 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 OsjNHX1. 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, OsiNHX1 and OsjNHX1 (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 T2 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 T2 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.
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 control samples.

Fig. 4. Etiolation response of PcNHX1 transgenic lines under increasing NaCl. Seeds of non-transgenic control and T2 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 < 0.01, ***P < 0.001, ****P < 0.0001.
3.7. Estimation of tissue Na⁺, K⁺ content of PcNHX1 transgenic lines compared to salinity treated non-transgenic plants (Fig. 5D).

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 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, OsNHX1 or OsNHX1 appears to exacerbate the hygromycin sensitive phenotype of
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. 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.
membrane (TM) domain of Ser410 (PcNHX1) the presence of two consecutive amino acids, in PcNHX1, OsjNHX1 or OsiNHX1 and may contribute to breaking a hydrophobic residue in PeNHX3 is replaced by a polar residue in Li+ transport. Alignment of the PeNHX3 sequence with PcNHX1, et al. (2014) have implicated the eleventh TM domain of PeNHX3 in phenotype significance. Untreated: unstressed non-transgenic control and Treated: Saliency treated non-transgenic control and PcNHX1 lines; Treated: Sodium 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 harbours silencer/enhancer elements (van Leuween et al., 2001; Kohli et al., 2006). Salinity also upregulates expression in 
PcNHX1p 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 (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 (Basil et al., 2011b; Barragan 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+ (Basil et al., 2012b). Further, nhx1 nhx2 mutants show increased shoot growth in minimal K+ medium containing sub-toxic Na+ ion content (Barragan et al., 2012). Vacular 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 over-expressing 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 halo-phytes, Na+ serves as an energetically cheap osmotomic 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 Pcnxh1 plants, PcNHX1 expression is also induced by salinity treatment in roots (The variation in expression in Pcnxh1 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 internal 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, Olias 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 dampened 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 PcNHX1:pGUS 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 SiSOSI directly stems specific Na⁺ accumulation, preventing Na⁺ from reaching leaf tissues (Olías et al., 2009). Increased transport of Na⁺ to the leaves.

References

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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


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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.

References


