Functional validation of a novel isoform of Na^+/H^+ antiporter from *Pennisetum glaucum* for enhancing salinity tolerance in rice

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Salt stress is an environmental factor that severely impairs plant growth and productivity. We have cloned a novel isoform of a vacuolar Na^+/H^+ antiporter from *Pennisetum glaucum (PgNHX1)* that contains 5 transmembrane domains in contrast to *AtNHX1* and *OsNHX1* which have 9 transmembrane domains. Recently we have shown that *PgNHX1* could confer high level of salinity tolerance when overexpressed in *Brassica juncea.* Here, we report the functional validation of this antiporter in crop plant rice. Overexpression of *PgNHX1* conferred high level of salinity tolerance in rice. Transgenic rice plants overexpressing *PgNHX1* developed more extensive root system and completed their life cycle by setting flowers and seeds in the presence of 150 mM NaCl. Our data demonstrate the potential of *PgNHX1* for imparting enhanced salt tolerance capabilities to salt-sensitive crop plants for growing in high saline areas.

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1. Introduction

Among the various stresses that affect the crop productivity, salinity is a major threat to agriculture. A recent estimate from FAO suggested that around 6% of the world's total land area and 20% of irrigated land is affected by high salinity (FAO 2005). Thus, there is an urgent need for raising crops capable of growing in saline environments to enable agriculture in marginal lands. Studies have established that a high salt concentration in the vicinity of a plant manifests itself by disrupting the ability of the roots for efficient water uptake, thereby leading to perturbation of crucial metabolic reactions inside the cell (Hasegawa *et al* 2000). In contrast with the salt tolerant halophytes, glycophytes display a growth penalty even when exposed to 20–50 mM NaCl stress (Greenway and Munns 1980). Since most of the agriculturally important plants are glycophytes, soil salinity is a significant factor restricting plant growth and depressing yield potential in large areas of the world (Boyer 1982; Zhu 2001).

Mechanisms that confer salt tolerance vary with the plant species, however the basic strategy works towards the maintenance of Na^+ homeostasis in the cytosol (Blumwald 2000). Active exclusion of Na^+ mediated by the plasma membrane localized Na^+/H^+ antiporter *AtSOS1* (Shi *et al* 2000, 2003), higher K^+/Na^+ selectivity as reported for the halophyte *Thellungiella* (Volkov *et al* 2004), the action of alternate transporters such as high affinity K^+ transporters, HKTs (Ren *et al* 2005) or the sequestration of excess sodium into the vacuoles via vacuolar Na^+/H^+ antiporter (NHX), are some of the strategies employed to maintain lower sodium concentrations in the cytosol/achieve ionic balance in the cytosol.

In addition to some of the still unassigned roles (Ohnishi *et al* 2005; Apse *et al* 2003; Yamaguchi *et al* 2001), vacuolar Na^+/H^+ antiporter helps in the regulation of cell volume and maintenance of physiologically optimum cytosolic pH (Dibrov and Fligel 1998). In saline environments, an active vacuolar antiporter utilizes the proton motive force generated by vacuolar ATPases and pyrophosphatases (Pipases) to sequester excess Na^+ into the vacuole, thereby reducing the toxic effects of Na^+ inside the cytosol and utilizing these ions for maintenance of turgor in the vacuole

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for cell expansion and growth (Barkla and Blumwald 1991). It has been reported that endogenous levels of Na^+/H^+ antiporter remain low in salt sensitive plants such as rice even when subjected to salt stress (Fukuda *et al* 1998). Heterologous overexpression of NHX protein from different plant species confers tolerance to varying limits suggesting that one of the reasons for salt sensitivity in plants maybe the lack of sufficient *NHX* gene product(s) that may differ in regulation and activity. Orthologs of Na^+/H^+ antiporter genes have been isolated from both glycophytes and halophytes (Hasegawa *et al* 2000; Munns 2005). Overexpression of *Arabidopsis thaliana AtNHX1* conferred enhanced salt tolerance in *Arabidopsis* (Apse *et al* 1999), and several other plant species such as tomato (Zhang and Blumwald 2001), *Brassica napus* (Zhang *et al* 2001), *Triticum aestivum* (Xue *et al* 2004) and maize (Xiao-Yan *et al* 2004). Overexpression of *Oryza sativa OsNHX1* in rice plants (Fukuda *et al* 1999, 2004) and transfer of *Gossypium hirsutum GhNHX1*, in tobacco (Wu *et al* 2004) has been shown to confer salt tolerance. Overexpression of *Hordeum brevisubulalum*, *HbNHX1* in tobacco rendered transgenic plants tolerant to both salt and drought stress (Lu *et al* 2005). Genes encoding for Na^+/H^+ antiporter have also been isolated from halophytes such as *Mesembryanthemum crystallinum* (Chauhan *et al* 2000), *Atriplex gmelini* (Hamada *et al* 2001), *Sueda salsa* (Ma *et al* 2004) and *Beta vulgaris* (Xia *et al* 2002). Introduction of *Atriplex gmelini AgNHX1*, conferred only limited salinity tolerance to salt sensitive rice plants (Ohta *et al* 2002). Together these studies have demonstrated the potential for use of specific vacuolar antiporters as a candidate gene in imparting salt tolerance capabilities.

Pennisetum glaucum is a glycophyte with a natural ability to withstand relatively higher levels of drought, salinity and heat stress. We isolated vacuolar Na^+/H^+ antiporter gene (*PgNHX1*) from *P. glaucum* (GenBank accession No. DQ071264) and have recently shown its importance in enhancing salinity tolerance in *Brassica* (Rajagopal *et al* 2007). Here we report the functional validation of *PgNHX1* in rice. Overexpression of PgNHX1 resulted in extensive root growth and improved the ability of transgenic rice plants to withstand salt without adversely affecting plant growth and development. Also, transgenic rice seeds were able to germinate and grow in the presence of 100 mM NaCl raising the possibility of using the saline hit soils for growing rice.

2. Materials and methods

2.1 *Cloning of PgNHX1 in plant transformation vector*

The full length cDNA clone encoding the *PgNHX1* ORF (Accession No. DQ071264) was PCR amplified using forward (5'gCC ggA TCC AAT ggC TGT gTT CAg CAg gAC AT 3' with *Kpn*I site) and reverse primer (5' AgT CgC ggC CgC TCA CCA AAA ACA TgT CTT CAT 3' with *Spe*I site). The 1413 bp amplified product was cloned as the *Kpn*I and *Spe*I fragment in a pCAMBIA1300 based plant transformation vector under the control of ABA responsive promoter flanked by the MAR sequence (Matrix Attachment Region) on either end to get pCAM-ABA-NHX.

2.2 *Generation of transgenic rice plants*

For rice transformation, the recombinant plasmid, pCAM-ABA-NHX, was transferred into *Agrobacterium tumefaciens* (LBA4404) by the liquid nitrogen freeze-thaw method as described in Singla-Pareek *et al* (2003). Rice calli *(Oryza sativa* cv PB1) were transformed with *PgNHX1* gene via *Agrobacterium* mediated transformation and the transformed calli were selected on hygromycin (50 mg/l) following the procedure described previously (Garg *et al* 2002).

2.3 *PCR, Southern and Northern blot analysis*

Putative transformed plants were screened by PCR analysis using rice genomic DNA from untransformed and various transgenic lines as template and *PgNHX1* forward and reverse primers. For Southern blot hybridization, 10 μg of genomic DNA from PCR positive rice lines was digested with *Kpn*I and *Spe*I enzymes (cloning site for *PgNHX1* gene in pCAM-ABA-NHX vector), blotted onto the nylon membrane and probed using DIG labeled *PgNHX1* gene according to the standard protocol (Roche Diagnostics Inc). To check the expression levels of *PgNHX1* transcript, total RNA was extracted from shoot tissues of transgenic and wild-type (non-transgenic) rice seedlings subjected to 100 μM ABA for 4 h (to induce the ABA inducible promoter driven expression of *PgNHX1*) following the standard protocol (Chomczynski *et al* 1997). Northern blot was prepared using 30 μg total RNA and was probed with DIG labeled *PgNHX1* cDNA.

2.4 *Leaf senescence assay for salinity stress tolerance*

Healthy and fully expanded younger most leaves (of similar age) from WT and T1 generation transgenic plants (90 d old) were briefly washed in deionized water, and 0.5 cm long segments were finely cut from the upper half of the leaf and floated in a 5 ml solution of NaCl (150, 300 and 600 mM, 96 h) or sterile distilled water (which served as experimental control) for the leaf senescence assay (Fan *et al* 1997). The effect of salt stress treatment on leaf segments were assessed by observing phenotypic changes and quantified by estimating their chlorophyll content (Arnon 1949). The experiment was repeated thrice with three different transgenic lines.

2.5 *Salinity stress tolerance of transgenic rice plants*

T1 generation seeds from three transgenic lines were germinated on Murashige and Skoog (MS) medium (Sigma) supplemented with 50 mg/l hygromycin. Half of the surviving seedlings (10 d old) were transferred into hydroponic system containing 150 mM NaCl and their root and shoot growth was monitored for next 15 d. The other half of the surviving seedlings were transferred to pots containing vermiculite and 20 d old seedlings were transferred to soil pots and grown further in a greenhouse (10 h light/14 h dark photoperiod, 26°C ± 2°C and 60–70% relative humidity). After one week, 50% of the transgenic plants were irrigated with 150 mM NaCl (conductivity of 14–15 dS/m) till the completion of their life cycle. The remaining 50% seedlings continued to grow in water and served as experimental controls. The relative growth of the seedlings in the presence of continuous salt stress *vis a vis* control conditions was monitored.

2.6 *Seed germination assay*

Seeds from non transgenic control plants or transgenic lines from T2 generation were germinated in 100 mM NaCl solution and 50 μg/ml hygromycin B. Root and shoot lengths of individual plants were measured 10 days post germination. Data represent mean + SE of three independent experiments.

3. Results and discussion

3.1 *Production of transgenic rice by overexpression of PgNHX1*

The *PgNHX1* was cloned in pCAMBIA1300 based binary vector as the *Kpn*I and *Spe*I fragment under the control of ABA inducible promoter to give pCAM-ABA-NHX recombinant vector (figure 1a). Four-weeks-old scutellum-derived callus of indica rice PB1 was cocultivated with *Agrobacterium* LBA4404 carrying the *PgNHX1* recombinant plasmid pCAM-ABA-NHX. A total of 12 independent transgenic lines were obtained which were transferred to pots and raised to maturity to obtain T1 seeds (figure 1b). Morphologically, no noticeable difference was observed in the transgenic plants vs non-transgenic plants. PCR analysis confirmed the presence of stable transgene in all 12 independent lines when total genomic DNA from various independent transformed lines was used as the template and end sequences of *PgNHX1* were used as the primers in the PCR analysis (data not shown). Genomic Southern blot analysis using DIG labeled *PgNHX1* probe also indicated the transgenic nature of these lines showing the presence of *PgNHX1* of expected size i.e. 1.4 kb (figure 1c). However, no cross hybridizing band was seen in the wild-type plants. Northern blot analysis of representative transgenic and wild-type plants subjected to 100 μM ABA showed presence of *PgNHX1* transcript in abundance, while no *PgNHX1*

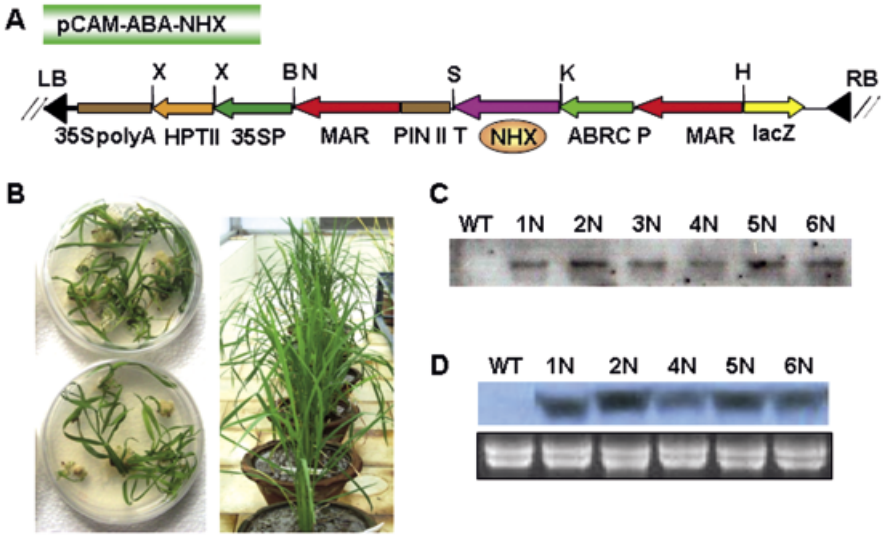

Figure 1. Agrobacterium mediated transformation of rice (PB1) with *PgNHX1* cloned into plant transformation vector. (**A**) *pCAM-ABA-NHX* plasmid (having *NHX* at *Spe*I and *Kpn*I site) used for *Agrobacterium* transformation. (**B**) Steps involved in transgenic rice regeneration, from left to right are: regenerating calli on selection media plate, putative transgenics transferred to soil pots for better and fast growth. (**C**) Testing of six *PgNHX1* independent transgenic lines for the presence of *PgNHX1* transgene by Southern blot hybridization. (**D**) Analysis of *PgNHX1* transcript accumulation in wild-type (WT) and transgenic lines (1N to 6N) by Northern blot hybridization after exposing the seedlings to 100 μM ABA.

transcript was detected in the WT plants indicating that the transgene is being expressed at a higher rate under stress conditions in the transgenic plants (figure 1d).

3.2 *PgNHX1 transgenic rice plants tolerate high levels of salinity*

The experiments described in the following text were carried out on three transgenic lines (1N, 2N and 5N) and we observed almost similar results for all. Leaf disc senescence assay of wild-type (untransformed) versus transgenic T2 generation plants was performed as a bioassay for estimation of salt tolerance potential (Singla-Pareek *et al* 2003). Leaf segments (0.5 cm) from untransformed and *PgNHX1* transgenics were floated on saline solutions of different concentrations for 96 h to investigate the effect of overexpression of *PgNHX1* in ameliorating the toxic effect of NaCl. In these tests, generally higher concentration of NaCl is used to see the results in a shorter period of time and these concentrations do not reflect the tolerance limits of plants in soil conditions. The *PgNHX1* overexpressing lines showed a clear advantage in overcoming the deleterious effect brought in by NaCl toxicity in a concentration dependent manner. Results shown in figure 2a indicate that the leaf segments from transgenic lines stayed green *in vitro* even up to 600 mM NaCl. The non-transgenic lines showed extensive bleaching reflecting symptoms of injury due to stress while the transgenic lines did not appear to be affected under similar conditions. Biochemical investigations for the estimation of chlorophyll (which was taken as an index of the damage done to the photosynthesis apparatus under stress) indicated that under high salinity conditions, the transgenic lines could retain as high as 60% of chlorophyll as compared to the non-stress conditions. This is in contrast to only 20% chlorophyll retention by the non-transgenic lines under similar conditions (figure 2b). This documented the usefulness of *PgNHX1* overexpressing lines over non-transgenic lines to survive under toxic NaCl levels.

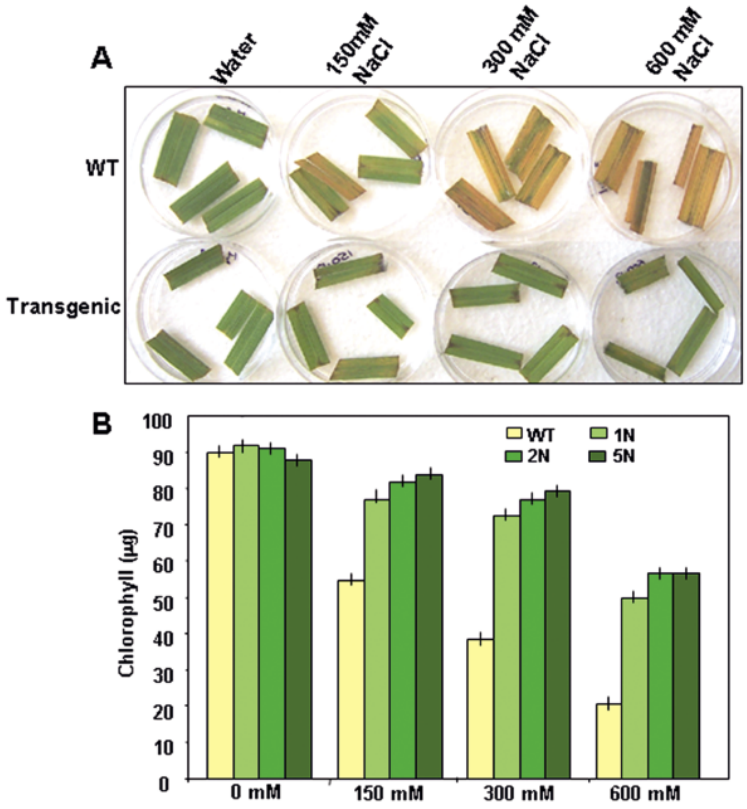

Figure 2. Retardation of salt stress-promoted senescence in detached leaves of transgenic rice plants overexpressing *PgNHX1*, indicating the tolerance at cellular level towards toxic levels of salt. Phenotypic differences (**A**) and chlorophyll content (μg/g of fresh weight) (**B**) from sodium chloride-treated leaf segments of WT and *PgNHX1* transgenic plants (line# 2N shown here) after incubation in 150 , 300 and 600 mM solutions of NaCl for 96 h are shown. Leaf segments floated in water served as the experimental control. The standard deviation in each case is represented by the vertical bar in each graph (n=3). Note the difference in the retention of chlorophyll in WT and *PgNHX1* transgenic rice.

3.3 *Stable integration and functional analysis of PgNHX1 in T1 transgenic rice*

The T0 transgenic plants overexpressing *PgNHX1* completed their life cycle and set normal viable seeds. To check the genetic stability of the introduced transgene, T1 seeds of transgenic plants obtained after selfing the T0 plants were germinated on hygromycin containing MS media. Five d old surviving seedlings were checked for the presence of transgene by PCR using the *PgNHX1* gene specific primers (data not shown) that confirmed the transgenic status of the surviving seedlings. To check for the relative salt tolerance at the early vegetative phase, half of the surviving seedlings (10 d old) were transferred into hydroponic system containing 150 mM NaCl and their shoot and root growth was monitored for next 15 d. It was found that *PgNHX1* overexpressing transgenic rice plants grew further in the presence of 150 mM NaCl while the growth of the WT plants was greatly affected (figure 3a). The transgenic plants developed extensive and larger root system in the presence of 150 mM NaCl as compared to the WT plants (figure 3b). In a recent study, it has been established that overexpression of AVP1 (vacuolar pyrophosphatase from *Arabidopsis*) in *Arabidopsis* results in elongation of roots and it has been documented that extended growth in root is associated with increased perturbations in specific hormonal contents such as auxins (Li *et al* 2005). However, it needs further detailed investigation to search for involvement of PgNHX1 in such vital processes.

For scoring the relative salt tolerance at the late vegetative phase, the 5 d old seedlings were transferred to pots containing vermiculite and after 15 d, seedlings were transferred to soil pots and grown further in a greenhouse (10 h light/14 h dark photoperiod, 26°C ± 2°C and 60-70% relative humidity). After one week, 50% of the transgenic plants were irrigated with 150 mM NaCl (Conductivity of 14–15 dS/m) till the completion of their life cycle. The remaining 50% seedlings continued to grow in water and served as experimental controls. The relative growth of the wild-type and transgenic seedlings in the presence of continuous salt stress was monitored. After 10 d of 150 mM NaCl treatment, the T1 transgenic plants showed significant salt tolerance as they grew normally while the wild-type plants showed typical yellowing of leaves (figure 3c). In fact, the *PgNHX1* overexpressing plants could complete their life cycle in the presence of 150 mM NaCl while the wild-type plants could not do so. A representative picture of one of the transgenic lines is shown in figure 3c for clarity of presentation; results for the other two lines were very similar to the specific line presented here. The wild-type plants

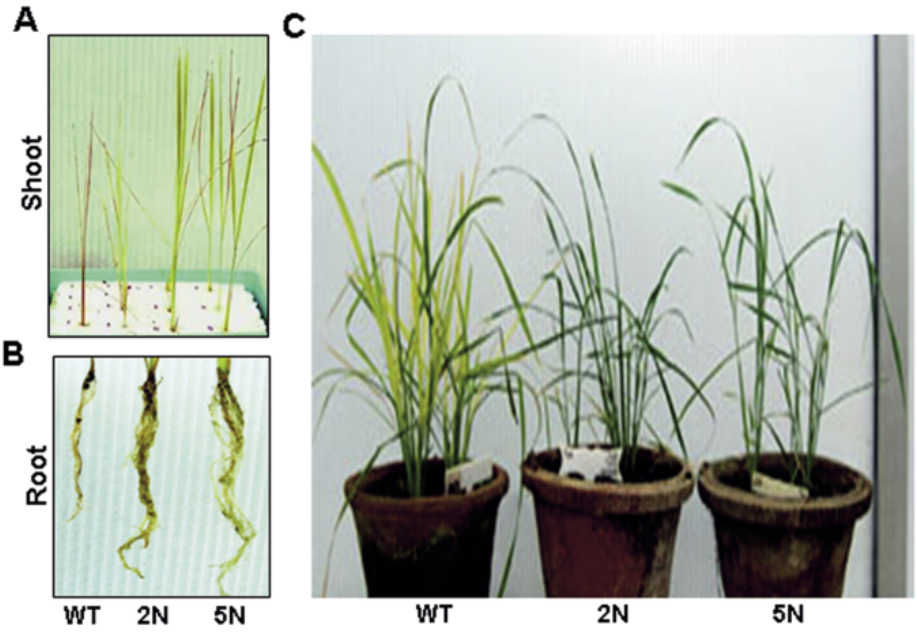

Figure 3. Salt tolerance of T1 generation rice plants overexpressing *PgNHX1* at early and late vegetative phase. (**A**) Shoot and (**B**) root growth in the presence of 150 mM NaCl shown for wild-type and transgenic lines (line # 2N and 5N) at the early vegetative phase. Note the extensive root growth in the transgenic lines. (**C**) Relative salt tolerance of wild type and transgenic plants at the late vegetative phase. After 25 d of growth under normal conditions, the seedlings were irrigated with 150 mM NaCl (conductivity of 14–15 dS/m) till the completion of their life cycle. Note the typical yellowing of leaves in the wild-type plants while those of transgenic plants stayed green.

displayed severe chlorosis probably due to the inability of their cells to compartmentalize excess of sodium into the vacuoles leading to toxicity.

Our results corroborate with earlier finding on salinity tolerance of plants overexpressing *AtNHX1* in various systems such as *Arabidopsis* (Apse *et al* 1999), and *Brassica napus* (Zhang *et al* 2001). Overexpression of *AgNHX1* in salt sensitive rice rendered the plant to tolerate upto 300 mM of NaCl, however, for a period of 3 days only (Ohta *et al* 2002). The transgenic rice plants overexpressing *OsNHX1* were able to survive a stress of 50-100 mM NaCl for 7-weeks (Fukuda *et al* 2004). Transgenic *Brassica juncea* plants overexpressing *PgNHX1* could withstand 300mM salt stress till the seed setting stage and exhibited normal growth phenotype without much loss of seed yield (Rajagopal *et al* 2007). In contrast, the yield of transgenic wheat overexpressing *AtNHX1* was reduced to 50% compared to 65% in the untransformed plants in presence of saline soils with an ECe of 10 dS/m (Xue *et al* 2004). These results indicate that *PgNHX1* is an efficient candidate gene for improving salt tolerance and opens up the possibility to engineer salt sensitive cultivars of other crop plants as well.

3.4 *Seed germination of wild-type and PgNHX1 (T2) transgenic rice in 100 mM NaCl*

Salt stress generally leads to growth reduction by a decrease in the water potential (Munns *et al* 1993). To test the effect of higher concentrations of NaCl on the seed germination of transgenic plants, seeds from transgenic lines (T2 generation) and wild-type plants were germinated on 100 mM NaCl and shoot/ root length was measured following 10 d post germination. The *PgNHX1* transgenic seeds germinated and grew well in the presence of 100 mM NaCl while the percentage of germination was highly reduced for the wild-type seeds under similar conditions (figure 4a). Salt stress severely inhibited growth patterns of wild-type plants (in terms of root and shoot growth under salinity stress) while transgenic plants showed performed relatively much better under similar conditions (figure 4b,c). It has been shown in pea, that continued stress of 50 or 150 mM NaCl for 24 h resulted in the death of root cells due to lack of increase in vacuolar volumes (Mimura *et al* 2003). The overexpression of *PgNHX1* could help in providing the necessary turgor essential for cell growth and subsequent adaptation to salt

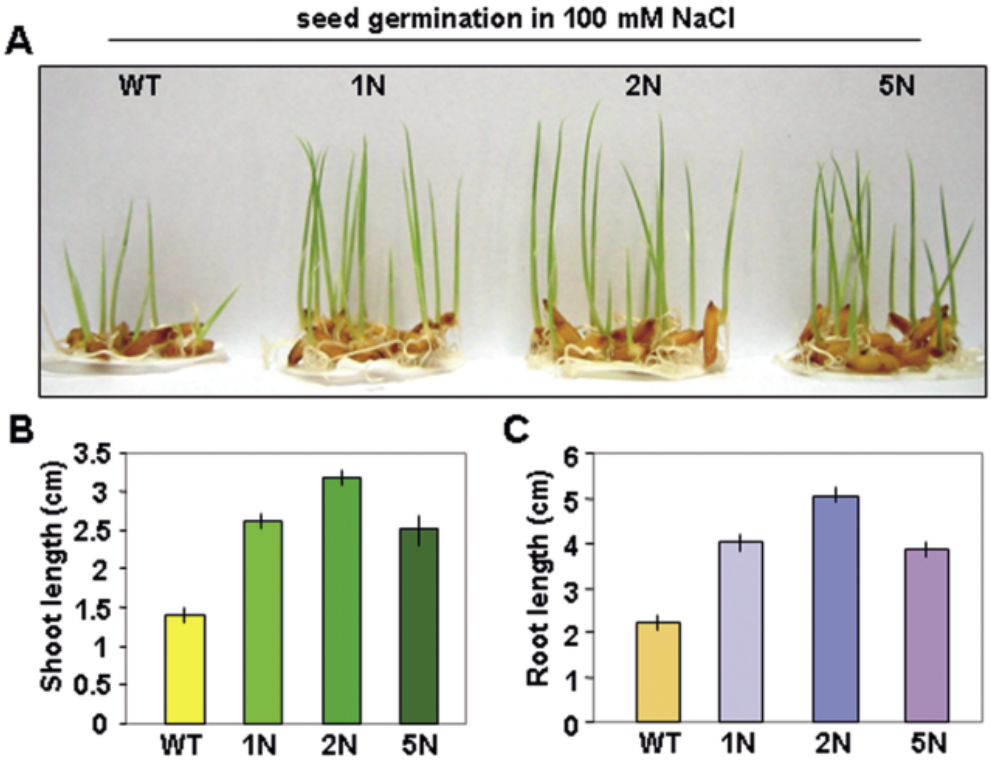

Figure 4. Effect of salt stress on seed germination of PgNHX1 overexpressing lines. (**A**) Extent of seed germination and their relative shoot (**B**) and root growth (**C**) for wild-type and transgenic lines after 10 d of germination on 100 mM NaCl supplemented medium. The data are mean values ± standard error of three independent experiments. Note that the wild-type seeds are able to germinate in the presence of 100 mM NaCl but their growth is compromised.

stress. Our results provide evidence that ectopic expression of *PgNHX1* can confer higher levels of salinity tolerance in transgenic plants. The data also validate *PgNHX1* function towards alleviation of toxic effects of salt stress at all stages of the life cycle from seed germination to the seed set stage.

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