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Possibility of Improved Salt Tolerance in Rice Transgenics Overexpressing *pgNHX1*

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ABSTRACT

In the present study, transformants in rice were developed with *PgNHX1* gene following a tissue culture-independent *in planta* transformation protocol. Analysis of T_1 plants by a stringent salt screening test at seedling and plant level identified putative transformants. Integration of the transgenes in T_1 generation plants was confirmed at the molecular level by PCR, genomic Southern and RT-PCR analysis. Physiological studies such as chlorophyll estimation and membrane permeability tests revealed that some of the T_1 transformants showed lower percent reduction in chlorophyll content and less membrane leakage compared to WT under salt stress. These results clearly demonstrate that transgenic rice plants overexpressing *PgNHX1*, a vacuolar antiporter have better salt-tolerance. The stable integration and inheritance of the transgene in subsequent T_2 generation was also confirmed by seed germination assay and PCR analysis.

Keywords: *Agrobacterium*, *in planta* transformation, *pgNHX1*, salt stress
Abbreviations: CSI, Chlorophyll Stability Index; **DMSO**, dimethyl sulfoxide; **EC**, electric conductivity; **SDS**, sodium dodecyl sulfate; **SSC**, saline-sodium citrate; **WT**, wild type; **WTT**, wild type treated

INTRODUCTION

Soil salinity is a major constraint to food production because it limits crop yield and restricts use of land previously uncultivated (Yokoi *et al.* 2002). More than 953 million hectares of land throughout the world are salt affected, either by salinity (397 million ha) or the associated condition of sodicity (434 million ha) (FAO 2005; FAO/UNESCO 2009). Reducing the spread of salinization and increasing the salt tolerance of high yielding crops are important global issues. Salinity always results in high concentration of Na^+ in external medium compared to other nutrients such as K^+ and other cations, altering the ionic balance of soil solution (Tester and Davenport 2003). The important mechanisms that contribute Na^+ tolerance of plants, are the processes involved in establishing ionic and osmotic homeostasis. Plant vacuoles which occupy 90% of the cells volume play an important role in the maintenance of turgor pressure and storage of ions and metabolites. Vacuolar membrane of plant cell contains a Na^+/H^+ antiporter. The vacuolar sodium sequestration is mediated by these Na^+/H^+ antiporter at the tonoplast using the proton motive force generated by the vacuolar H^+ ATPases and H^+ inorganic pyrophosphates (Hasegawa *et al.* 2000; Xue *et al.* 2004; Yamaguchi and Blumwald 2005). Manipulating the vacuolar Na^+/H^+ antiporter to improve Na^+ homeostasis is recognized as an attractive strategy in plants. Recently, *AtNHX1* has been over-expressed in several dicotyledonous plants, including *Arabidopsis* (Apse *et al.* 1999; Li *et al.* 2007; Qaio *et al.* 2007; Li *et al.* 2009; Liu *et al.* 2010), tomato (Zhang and Blumwald 2001), *Brassica juncea* (Rajagopal *et al.* 2007) and *Brassica napus* (Zhang *et al.* 2001). These transgenic plants displayed robust salt tolerance and could grow normally and produced fruits and seeds under high saline conditions. More recently, *AtNHX1* also has been introduced into crop plants such as wheat (Xue *et al.* 2004), tobacco (Soliman *et al.* 2009), rice (Chen *et al.* 2007) and *Petunia* (Xu *et al.* 2009) improving salt tolerance.

Rice is an important cereal crop of India. However, its productivity is limited by salinity. If salt tolerance were to be enhanced in rice, so that the reduction in growth and yield is only 30-40% instead of 80%, this would greatly benefit rice productivity in saline areas. To achieve this, the *NHX*1 gene from pearl millet (*Pennisetum glaucum*), a drought tolerant species was introduced into rice using a tissue culture-independent *in planta* transformation protocol developed by our group in several crop species (Arthikala *et al.* 2009). Earlier studies have shown that transferring the *NHX1* gene from stress-tolerant species or even halophytes like the mangrove species, *Sueda* (Zhao *et al.* 2006) instead of a stress susceptible species like *Arabidopsis* (Apse *et al.* 1999) results in *NHX1* transgenics which can tolerate higher levels of salt with better growth. The main objective of the study was to improve the salt tolerance of a rice genotype grown in the coastal areas. We report the possibility of tolerance to higher levels of salt in rice overexpressing the *PgNHX1* gene.

MATERIALS AND METHODS

Plant material

A rice genotype from the coastal area *viz*., 'Vikas' was used for transformation studies. Seeds were soaked overnight in distilled water and were surface sterilized first with 1% Bavistin for 10 min and later with 0.1% $HgCl_2$ for a few seconds. After treatment with each sterilant, the seeds were washed thoroughly with distilled water. They were later put for germination in petriplates (Tarsons, Kolkata, India) at 30°C. Two-day old seedlings were taken as *ex plants* for *Agrobacterium* infection.

Vector for transformation

The *NHX1* gene construct was obtained under a MTA from Dr. M. K. Reddy, International Centre for Genetic Engineering and Biotechnology, New Delhi. This gene was cloned into the *Pst*I (35S promoter-antiporter-polyadenylation sequence) site of plant trans-

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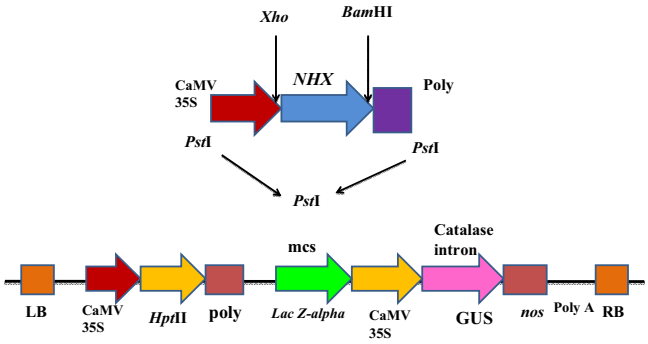

Fig. 1 T-DNA map of the binary vector pCAMBIA1301 harboring *PgNHX1* and *hpt*II as the gene of interest and marker gene respectively. LB: left border; RB: right border; mcs: multiple cloning site.

formation vector pCAMBIA1301 (**Fig. 1**). The vector possesses *hpt*II as a selectable marker.

Transformation and development of transformants

Transformation of rice and generation of the primary transformants was accomplished using a tissue-culture independent *in planta* transformation procedure (Rohini and Rao 2000). The seedlings with just emerging plumule were infected by wounding at the meristem with a sterile needle and subsequently immersed in *Agrobacterium* culture for 1 h. Following infection, the seedlings were washed briefly with sterile water and later transferred to autoclaved Soilrite (vermiculite equivalent; Keltech Energies Ltd, Bangalore, India) moistened with water for germination under aseptic conditions in the growth room in wide mouth capped glass jars of 300 ml capacity, 5 seedlings per jar. The growth chamber was maintained at 28 ± 1°C under a 14 h photoperiod with fluorescent light (FL40S.W, Mitsubishi, Tokyo) of 35 µmol m^{-2} s^{-1} intensity. After 5-6 days, the seedlings were transplanted to 45 × 30 cm diameter earthen pots containing autoclaved red loamy soil (volume ~12 kg) and a dose of 120 N: 80 P: 50 K (Nagarjuna Fertilizers Pvt. Ltd., Hyderabad, India) was applied to the soil. These T_0 plants were shifted to the greenhouse with on optimum temperature of 28 ± 1°C and 85% RH.

Analysis of the transformants

1. Standardization of screening for identification of putative transformants at seed level

Uniform wild type (WT) seeds (var. 'Vikas') were selected and surface sterilized with 0.1% $HgCl_2$ for 10 min and with 0.5% Bavistin for 30 min, washed thoroughly with sterile distilled water, soaked overnight and spread uniformly in Petri dishes, which were drenched with water and placed in an incubator at 30°C for germination. After 24 h, germinated seedlings of uniform size were selected and transferred to Petri plates with 0.6% Agar (Hi Media laboratories, Mumbai, India) media containing NaCl at six different concentrations of 150, 200, 250, 300, 350, and 400 mM (six replicates were maintained per plate) for 12 days. One set of seedlings were maintained without salt treatment served as control. Further, after 4 days of recovery, root and shoot lengths of all the seedlings were measured.

2. Primary screening of T_1 generation plants at the seed level

Both WT as well as T_1 generation seeds were surface sterilized with 0.1% $HgCl_2$ for 10 min and with 0.5% Bavistin for 30 min, washed thoroughly with sterile distilled water and soaked overnight. After 24 h, just germinated seedlings of uniform size were selected and transferred to Petri plates with 0.6% Agar media containing 350 mM NaCl. Salt treatment was continued for 12 days. Then seedlings were transferred to Petri dishes with filter paper rinsed with water for recovery for 4 days. Seedlings that recovered with fresh root and shoots were selected as putative transformants for further analysis.

Six leaf bits of known weight (50 mg) from individual putative transformants and WT were transferred to ¼-strength Hoagland's medium (Hoagland and Arnon 1950) solidified with 0.6% agar and 450 mM NaCl for 72 h under dark conditions. After 72 h, based on scoring the extent of chlorosis symptoms (Eker *et al.* 2006), only those putative lines which remained green were selected for further molecular and physiological analysis.

Physiological analysis

1. Chlorophyll estimation

Leaf bits (50 mg) of the WT and putative *NHX1* transformants which had undergone secondary screening were incubated in 8 ml acetone: DMSO (1:1) solution for overnight. The extract was taken and absorbance was recorded at 645, 652 and 663 nm, using UV-Vis spectrophotometer (UV 2450, Shimadzu, Japan). Chlorophyll (Chl) content was estimated by substituting the absorbance values in the formulae given below. Total Chl was expressed as mg g^{-1} fresh weight (Arnon 1949):

$$\text{Chl } a = 12.7\ (A_{663\ nm}) - 2.69\ (A_{645\ nm})\ \text{V/weight} \times 1000$$

$$\text{Chl } b = 22.9\ (A_{645\ nm}) - 4.68\ (A_{663\ nm})\ \text{V/weight} \times 1000$$

$$\text{Total Chl (mg g}^{-1}\text{ FW)} = (\text{Chl } a + \text{Chl } b)$$

$$\text{Chl Stability Index (CSI)} = 100 - R$$

where R = percent reduction in total Chl content, V = volume of aliquot (5 ml), A = absorbance and FW = fresh weight of leaf tissue (50 mg).

Cell membrane stability

Percent leakage, which reflects loss of membrane integrity, was quantified. The leaf bits (50 mg) of the WT and *NHX1* putative transformants after secondary screening were incubated in 10 ml of distilled water with continuous shaking on a magnetic stirrer for 10 min. Initial electric conductivity (EC) was taken using EC-TDS analyzer (ELICO-CM183). Then the leaf bits were put in fresh test tubes containing 10 ml of distilled water, boiled for 10 min at 65°C and the final EC was taken. Cell leakage was computed using the formula:

$$\text{PercentLeakage} = \frac{\text{FinalEC- InitialEC}}{\text{FinalEC}} \times 100$$

Molecular analysis

Leaf tissues from the progeny plants were analyzed for the presence of the introduced gene. Genomic DNA was isolated following the procedure of Dellaporta *et al.* (1983) from fresh leaf tissue of greenhouse-grown plants.

1. PCR analysis

The DNA amplification was carried out using two different sets of primers; marker specific (*hpt*II) forward (5′-AGCTCGGCCGATGGTTTCTACAA-3′) and reverse (5′-ATCGCCTCGCTCCAGTCAATG-3′) primers; 35-S (promoter specific) forward (5′- TCCTTCGCAAAGACCCTTC-3′) and *NHX1* gene specific reverse (5′- TCCCGCCAGAACTAATCCTA-3′) primers were also used in order to avoid the interference of endogenous *NHX1*gene. All the above primers were synthesized in house (Department of Crop Physiology, UAS, GKVK) and yielded 500-bp and 1-kb fragments, respectively. PCR of both sets of primers was initiated by a hot start at 94°C for 4 min followed by 25 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min with a final extension of 10 min.

2. Southern analysis

In order to analyze the total genomic DNA for transgene integration, 15 µg of total genomic DNA was digested overnight with *Bam*HI. The digested DNA samples were electrophoresed on a

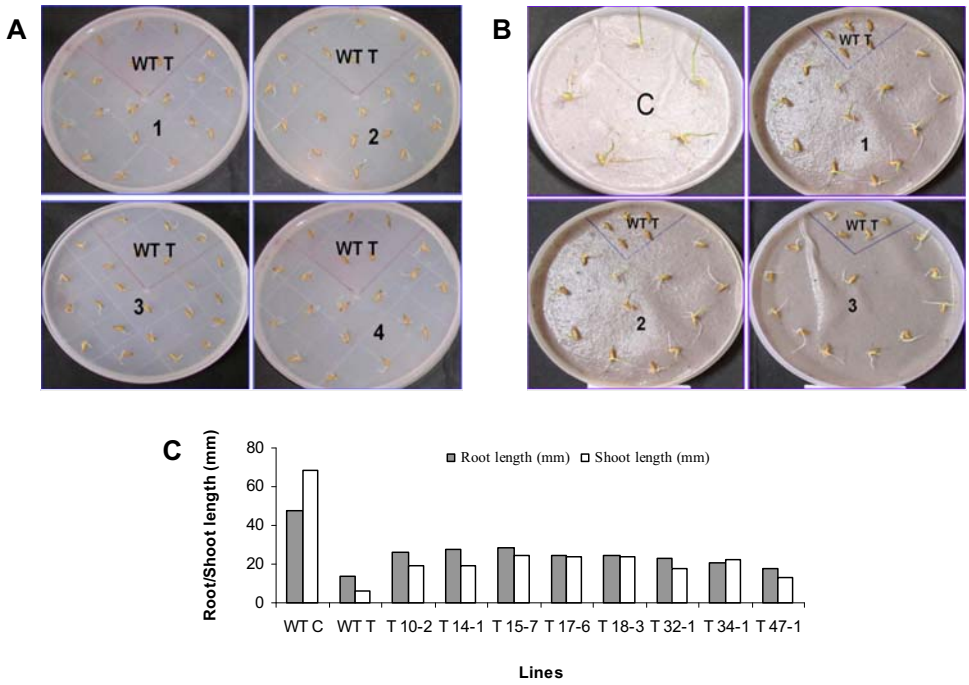

Fig. 2 Screening of T_1 generation seeds by germination assay on salt for the selection of putative transformants. (**A**) T_1 generation and WT seeds were screened for tolerance on 0.6% agar containing 350 mM NaCl. (**B**) Recovery of the seeds following 9 days of salt stress. WTT: WTT; 1-4: seeds from different T_0 plants. (**C**) Graphical representation of the root and shoot lengths of the T_1 generation and WT seedlings following salt stress.

0.8% agarose gel. The separated fragments were transferred onto a positively charged nylon membrane (Pall Pharmalab Filtration Pvt. Ltd., Mumbai, India). The membrane was hybridized with α-^{32}P-radioactive probe which was made from purified 450-bp *uid*A gene-specific fragment labeled by random prime labeling. Hybridization was carried out at 65°C in Church buffer (0.25 M sodium phosphate buffer, pH 7.2; 1% BSA; 1 mM EDTA; 7% SDS, Sigma Chemicals, USA; Church and Gilbert 1984) for 18 h. Membranes were washed for 30 min each in 3X SSC/0.1% SDS; 0.3X SSC/0.1% SDS; 0.1XSSC/0.1% SDS; 2X SSC/0.1% SDS at 65°C (Sambrook *et al*. 1989). Membrane was wrapped, placed overnight on FUJI Image Plate (IP) and IP was read using phosphor imager (FUJI FILM FLA-5100, Fuji Photo Film Co. Ltd., Tokyo, Japan).

3. Semi-quantitative RT-PCR analysis

Total RNA was extracted following the phenol-SDS method (Datta *et al.* 1989). About 5 µg of total RNA was reverse transcribed to single stranded cDNA in a 30 µl reaction mix consisting of 25 units M-MuLV reverse transcriptase (MBI Fermentas, USA), 5X MMuLV- RT buffer, 40 ρM oligo dT primer and 10 mM dNTP mix. Reverse transcription was performed at 42°C for 1 h. To study the expression pattern of *NHX1* gene (primers – forward 5′-AATGCG CTCCAGAACTTTGA-3′; reverse 5′-TCCCGCCAG AACTAA TCCTA-3′; University of Agricultural Sciences, GKVK, Bangalore, India), 1 µl of the cDNA mix was used as template for PCR amplification of a 500-bp fragment. Actin was used as an internal control (primers: forward 5′-TCCATAATGAAGTGTGAT GT-3′; reverse 5′-GGACCTGACTCGTCATACTC-3′; University of Agricultural Sciences, GKVK, Bangalore, India) to amplify a 250-bp fragment. The PCR was carried out in 20 µl of reaction mixture containing 1 U of *Taq* Polymerase (Bangalore Genei, India) in 1X reaction buffer, 25 mM $MgCl_2$, 2 mM dNTP mix, 3 ρmol of primers and amplified products were separated on agarose (Sigma Chemicals, USA) (1.2%) gel stained with ethidium bromide and documented. PCR was carried out for the transgenes, *PgNHX1*, *hpt*II and actin as internal control.

RESULTS

Development of transgenic rice overexpressing *NHX1*

To develop transgenic rice plants overexpressing *NHX1* standardized *in planta* transformation protocol (Hanjagi *et al*. 2011) was followed. A total of 100 rice seeds were surface sterilized and allowed to germinate for 24 h, pricked randomly on embryo axis with a 28 gauge-sewing needle. Wounded seeds were infected with *Agrobacterium*, cultured in the AB minimal medium. After 45 min of infection, seeds were placed on sterile Soilrite and allowed to grow for 4 days in a growth chamber. After establishment in the growth chamber, seedlings were hardened in green house before shifting them to pots. Around 60 T_0 plants were established in greenhouse, with an average of 16 panicles in each plant. The seeds produced by these plants were selected for analysis in the T_1 generation.

Standardization of a stringent salt screening test for salt tolerance

In planta transformation protocol gives rise to a large number of T_1 seeds and a high throughput screening protocol to select high expressing lines is required to screen such a large number of plants. In this direction, a standardized stringent salt screening test was followed for T_1 transformants. Growth during salt stress at different NaCl concentrations and after recovery was analyzed primarily in the WT (data not shown). At 350 mM NaCl there was significant reduction in growth. In further screening experiments, 350 mM was selected to screen the transgenic seeds.

Stringent screening test for salt tolerance for analysis of putative transformants in the T_1 generation

To examine whether the overexpression of the vacuolar Na^+/H^+ antiporter conferred salt tolerance to the plants, the putative T_1 seeds were screened at two levels, *viz*., at seed level (primary screening) and at plant level (secondary screening).

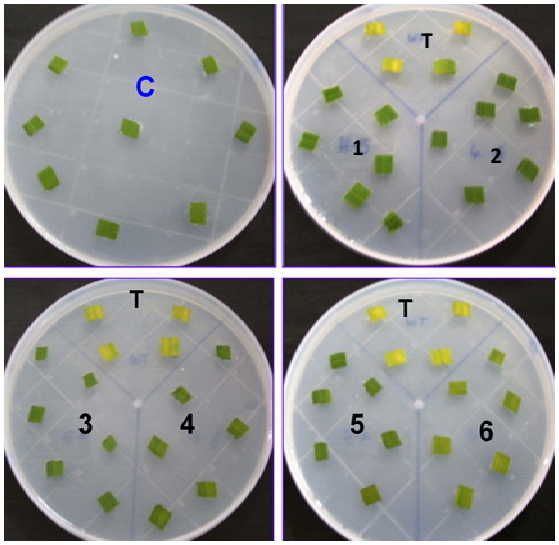

Fig. 3 Leaf bioassay for stress-induced senescence in T_1 generation plants. Resistance to salt-induced senescence in detached leaves of transgenic rice plants compared to the WT. C: absolute control; WTT: wild type treated; 1-6: putative transformants.

1. Primary screening (screening at seed level)

Approximately 3000 putative T_1 seeds from 60 T_0 plants were screened. The growth of both WT as well as transgenics was inhibited in agar media. However the inhibition of growth was more in WT seedlings compared to T_1 seedlings (**Fig. 2A, 2B**). Four days after recovery, around 500 seedlings of the putative T_1 transformants recovered. Some of the putative transgenics showed significantly higher root and shoot growth compared to WT (**Fig. 2C**). Out of 3000 T_1 seedlings 116 seedlings were selected on salt media (seed level/primary screening) and transferred to pots in greenhouse for further analysis.

2. Secondary screening (plant level screening) by leaf senescence bioassay

Plant level screening was carried out by leaf senescence assay on all the 116 putative transformants following the seed level screening. At the end of 72 h, selection of plants was based on the extent of chlorosis. Accordingly, 25 plants were found to be tolerant .i.e., remained greener compared to the treated WT which showed chlorosis (**Fig. 3**).

Physiological analysis of T_1 transformants

Following leaf senescence bioassay, 25 putative transgenic lines were used to study tolerance of plants to salinity. Leaf samples of putative transformants were exposed to 450 mM NaCl for 72 h and tolerance was assessed in terms of percent reduction in total Chl and Chl stability index over non-stressed control after stress treatment. Membrane integrity as a parameter for stress tolerance was also assessed.

Chl content and stability

There was significant difference in percent reduction of total Chl among putative transgenics and WT with respect to their non-stressed control. Compared to WT, transgenics showed less reduction in Chl ranging from 28-52% (**Fig. 4A**), whereas, the WT showed 61% reduction. All the selected plants of the T_1 generation showed less reduction in Chl when compared to WT, some of the plants showed high performance with 28-30% reduction in Chl. The putative transgenic lines showed Chl stability index (CSI) significantly higher than the WTT with an average stability index of 59.0 ± 6.15 compared to WTT 37.59 ± 8.22 (**Fig. 4B**). Some of the selected T_1 generation plants showed highest

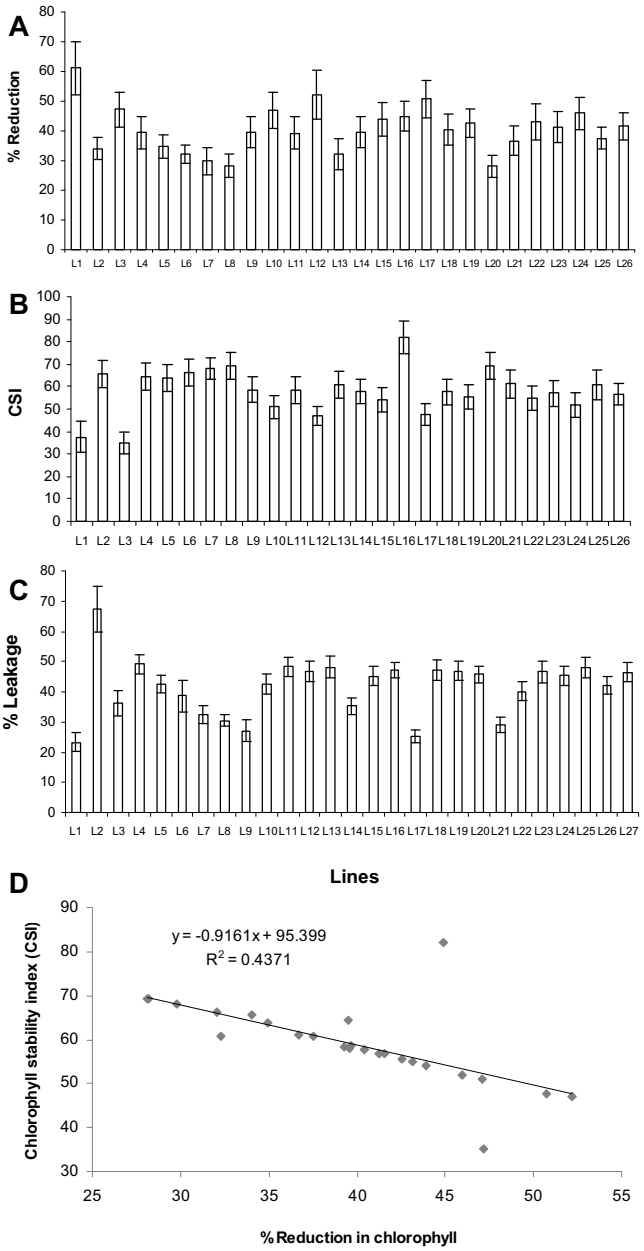

Fig. 4 Physiological analysis of the transformants. (**A**) Percent reduction in the chlorophyll content in transgenics when compared to the WT following stress. (**B**) Chlorophyll stability in the NaCl treated leaf bits compared to the treated and untreated WT. (L1-WT-T, L2-T 9-2, L3-T 9-3, L4-T 10-1, L5-T 10-2, L6-T 12-1, L7-T 14-1, L8-T 15-7, L9-T 17-1, L10-T 17-2, L11-T 17-5, L12-T 17-6, L13-T 18-3, L14-T 21-1, L15-T 23-4, L16-T 23-5, L17-T 25-4, L18-T 27-1, L19-T 31-2, L20-T 32-1, L21-T 32-3, L22-T 34-1, L23-T 34-4, L24-T 38-1, L25-T 47-1, L26-T 49-1). (**C**) Membrane integrity of the salt treated leaves in transgenics when compared to the WT. (the first two bars are WT control (WTC) and WTT) (L1-WTC, L2- WT-T, L3- T 9-2, L4-T 9-3, L5-T 10-1, L6-T 10-2, L7-T 12-1, L8-T 14-1, L9-T 15-7, L10-T 17-1, L11-T 17-2, L12-T 17-5, L13-T 17-6, L14-T 18-3, L15-T 21-1, L16-T 23-4, L17-T 23-5, L18-T 25-4, L19-T 27-1, L20-T 31-2, L21-T 32-1, L22-T 32-3, L23-T 34-1, L24-T 34-4, L25-T 38-1, L26-T 47-1, L27-T 49-1). (**D**) Relation between the % reduction in chlorophyll and the chlorophyll stability index. NOTE: In **A** and **B**, percent reduction in chlorophyll and CSI, the calculations are carried out with respect to the WT whereas in **C**, membrane integrity was analysed in WT also, hence the difference in the number of lines.

CSI of 70%.

Cell membrane leakage

The typical objective of electrolyte leakage studies was to assess injury, presumably at the membrane level, resulting from salt stress. The electrical conductivity of the cell

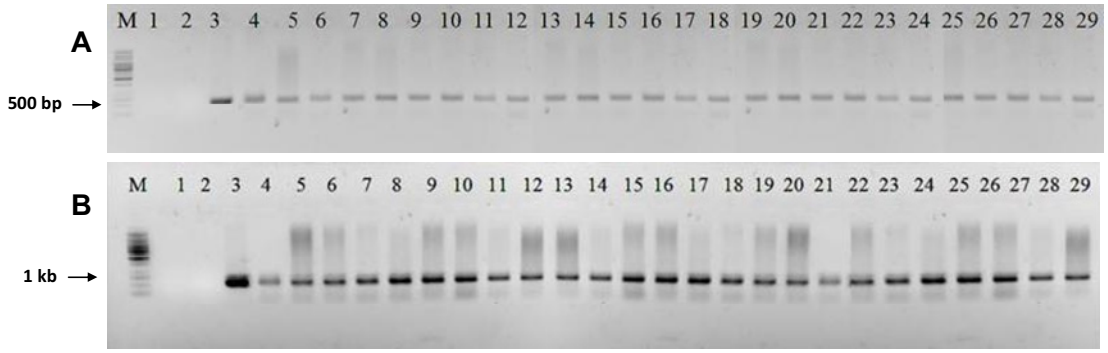

Fig. 5 PCR analysis of T_1 generation plants using (**A**) *hpt*II gene specific primers; (**B**) 35S promoter-*PgNHX1* gene specific primers. Lane M: 1 kb ladder; Lane 1: positive control (plasmid DNA); Lane 2: DNA of the WT plant; Lanes 3-29: DNA from the putative transformants.

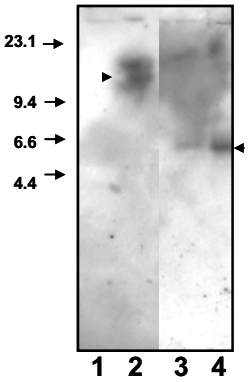

Fig. 6 Genomic Southern analysis. 15 µg of genomic DNA was digested with *Bam*HI and probed with radiolabelled *uid*A gene fragment. Lane 1: untransformed WT plant. Lane 2, 3 and 4: transgenic plants (31-2, 34-1 and 49-1 respectively).

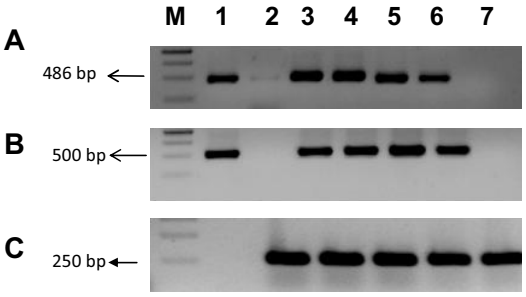

Fig. 7 RT-PCR analysis. Agarose (1.2%) gel stained with ethidium bromide showing amplified product of semi quantitative PCR for *NHX1* gene using cDNA from putative transgenics and WT. (**A**) cDNA amplified with *NHX1* gene specific forward and reverse primers; (**B**) cDNA amplified with *hpt* II forward and reverse primers a and b. Lane M: marker; Lane 1: positive control (plasmid DNA); Lane 2: Blank; Lanes 3-6: transgenic plant samples; Lane 7: WT. (**C**) cDNA amplified with Actin forward and reverse primers as an internal control. Lane M: marker; Lane 1: Blank; Lanes 2-5: transgenic plant samples; Lane 6: WT.

leachates indicates the extent of damage caused to the cell membrane during stress condition. Compared to WTT with NaCl, the leakage in putative transformants was significantly less ranging from 27-48%, whereas, WTT had 67% leakage (**Fig. 4C**).

There was a strong negative correlation observed (**Fig. 4D**) between the two parameters, % reduction in Chl and CSI further confirming the efficacy of the *NHX*1 gene.

PCR analysis of putative transformants

The selected plants following seed and plant level screening were analyzed further at molecular level to identify the presence of transgene. In this direction, PCR was performed with *hpt*II (marker-specific) primers (**Fig. 5A**) and 35s promoter forward and *NHX1*gene-specific reverse primers (**Fig. 5B**). Amplification of the expected fragment in both the primers confirmed the transgenic nature. However, the amplification was not observed in WT.

Genomic Southern analysis

Genomic southern analysis was carried out to identify the T-DNA copy number. For this, *Bam*HI was used as it cuts once in the T-DNA. Three *NHX1* putative transformants (31-2, 34-1 and 49-1) along with WT were taken for southern analysis and then probed with *uid*A gene-specific probe (**Fig. 6**). Variation in the hybridization pattern in all the plants indicated the single copy integration of the T-DNA and their independent transgenic nature.

Semi-quantitative RT-PCR analysis

Some of the PCR positives were further analysed for transcript accumulation. It was evident that there was difference in the level of expression of *NHX1* gene (**Fig. 7A**). Further it was confirmed that the variation was because of the transgene as all the transgenic samples showed accumulation of *hpt*II transcript, which was absent in WT (**Fig. 7B**). The house keeping gene actin was amplified with the same cDNA pool and treated as an internal control (**Fig. 7C**).

Based on the above analysis, five T_1 generation plants (31-2, 32-1, 32-3, 34-1 and 49-1) were selected for further analysis in T_2 generation.

Analysis of T_2 generation plants

The stability of the *PgNHX1* gene in T_2 generation plants was checked by germinating randomly selected seeds of five T_1 generation plants that were PCR and RT-PCR positive. Seed germination assay provided explicit evidence for the stable inheritance of the transgene as seen in **Fig. 8** (figure showing germination assay in two T_1 lines). There was a conspicuous difference in the root and shoot lengths of transgenics when compared to the treated WT (**Fig. 9**). Further, PCR analysis of the DNA from the representative T_2 plants from all the five T_1 plants was carried out. Amplification of the desired amplicon using primers for *hpt*II (**Fig. 10A**) and 35S forward-*PgNHX1* reverse (**Fig. 10B**) confirmed the stable integration and inheritance of the transgenes in all the selected T_2 generation plants.

DISCUSSION

Agricultural productivity is severely affected by soil salinity because salt levels that are harmful to plant growth affect large terrestrial areas of the world. The development and use of crops that can tolerate the high levels of salinity in the soils would be a practical contribution towards addressing the problem. The existence of salt-tolerant plants

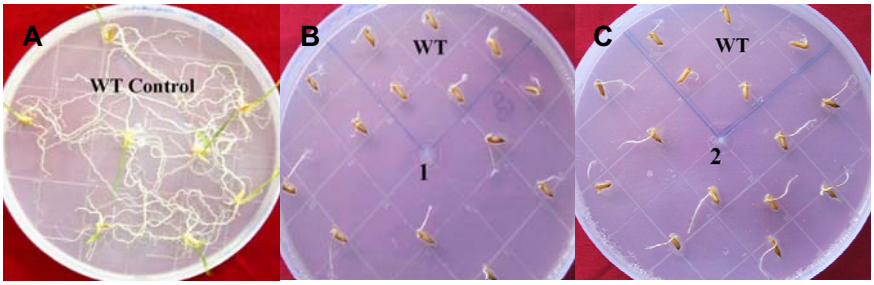

Fig. 8 Screening of T_2 generation seeds by germination assay on salt for tolerance on 0.6% agar containing 350 mM NaCl. (**A**) Absolute control; (**B, C**) Seeds from two transgenics lines, 34-1 and 49-1.

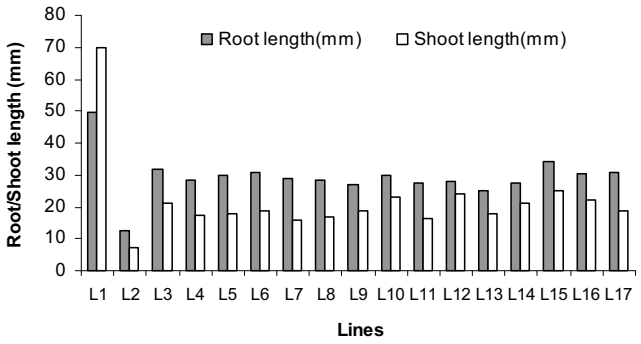

Fig. 9 Graphical representation of the root and shoot lengths of the T_2 generation and WT seedlings following salt stress. L1-WTC, L2-WTT, L3-T 31-2-1, L4-T 31-2-2, L5-T 31-2-3, L6-T 32-1-1, L7-T 32-1-2, L8-T 32-1-3, L9-T 32-1-4, L10-T 32-3-1, L11-T 32-3-2, L12-T 34-1-1, L13-T 34-1-2, L14-T 34-1-3, L15-T 49-1-1, L16-T 49-1-2, L17-T 49-1-3. NOTE: The T_1 generation analysis identified 25 lines. Among them, 5 lines were progressed into the T_2 generation. This figure is the analysis of 17 plants from 5 T_1 lines.

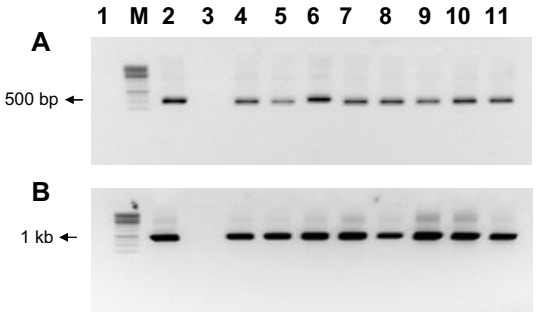

Fig. 10 PCR analysis of T_2 plants. PCR analysis of T_2 generation plants using (**A**) *hpt*II gene specific primers (**B**) 35S promoter-*PgNHX1* gene specific primers. Lane M: 1kb ladder; lane 1: Blank; Lane 2: positive control (plasmid DNA); Lane 3: DNA of the WT plant. Lanes 4-11: DNA from the putative transformants.

(halophytes) and differences in salt tolerance between genotypes within salt-sensitive plant species (glycophytes) indicates that there is a genetic basis to salt response. Two basic genetic approaches that are currently being used to improve stress tolerance include: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci (QTLs – regions of a genome that are associated with the variation of a quantitative trait of interest) and subsequent marker-assisted selection, and (ii) generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance. Transgenic approach to engineer salt tolerance can be through various mechanisms. One mechanism involves removal of Na^+ from the cytoplasm by transporting it into the vacuole via Na^+/H^+ exchangers driven by the electrochemical gradient of protons (H^+) generated by the tonoplast H^+ - ATPase (V-ATPase) and H^+ pyrophosphatase (V-PPase) (Niu *et al.* 1995; Qiu *et al.* 2004). In plants, Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across membrane and have a variety of functions, including maintenance of cellular ion homeostasis and regulation of cytoplasmic pH and cell turgour (Horie and Schroeder 2004). Increasing evidence has demonstrated that vacuolar Na^+/H^+ antiporters play a crucial role in plant salt tolerance. Improvement in salt tolerance evoked by overexpression of *AtNHX1* was observed in *Arabidopsis*, tomato, brassica, wheat and tobacco (Apse *et al.* 1999; Zhang and Blumwald 2001; Zhang *et al.* 2001; Xue *et al.* 2004; Li *et al.* 2009; Soliman *et al.* 2009; Xu *et al.* 2009; Liu *et al.* 2010). Increased salt tolerance was also observed in transgenic rice carrying the *OsNHX1* and *AgNHX1* (Ohta *et al.* 2002; Fukuda *et al.* 2004). These results indicate that expression of a single Na^+/H^+ antiporter gene in plants can be effective in reducing Na^+ toxicity. In the present study, it was envisaged to transfer the *Pennisetum glaucum NHX1* (*PgNHX1*) into rice with the aim of improving the salt tolerance of a coastal rice var. 'Vikas'.

Though transgenics in rice have been developed globally using *in vitro* regeneration, the response of rice seems to be genotype dependent. This problem can be overcome by using tissue culture independent transformation strategies that avoid tissue culture steps. This '*in planta*' transformation strategy was first standardized in *Arabidopsis* (Feldmann and Marks 1987). The methodology has also been extended to other crops that are not amenable to tissue culture like rice (Supartana *et al.* 2005), soybean (Chee *et al.* 1989), etc. In our laboratory, we have developed one such *in planta* strategy where *Agrobacterium* is targeted to the apical meristem and the transformants allowed to grow in many of the recalcitrant species like cotton, groundnut (Rohini and Rao 2000), etc. This technique is advantageous because it does not involve regeneration procedures and therefore the tissue culture-induced somaclonal variations are avoided. In the present study, this transformation strategy was extended to rice and primary transformants developed with *PgNHX1* gene. The T_0 transformants were allowed to set seed and T_1 seeds were collected. Since the *in planta* transformation produces chimeras in the T_0 generation, the analysis of the transformants has to be carried out in the T_1 generation. Therefore, it requires standardization of stringent screening techniques to identify the putative transformants.

In the present study, selection of putative transformants was based on two screening strategies, one at seedling level and the other at plant level at a stringent NaCl concentration of 450 mM. These strategies helped in the identification of 25 putative T_1 plants out of the approximately 3000 seeds screened. The transgenic nature and efficacy of these selected plants against salt stress was confirmed both at molecular and physiological level. Physiological analysis of the selected plants supported the salt tolerance exhibited by the selected plants. Integration of the transgenes was confirmed by PCR analysis for both *PgNHX1* and *hpt*II genes. Further, genomic Southern analysis confirmed that the transgene was integrated as a single copy in the selected transgenics. There was an increase in the accumulation of *NHX1* and

*hpt*II transcripts in the transgenic plants. Under stress, the transgenics exhibited better physiological responses as seen in two parameters, CSI and membrane integrity. Further, stability of the transgenes in T_2 generation was also proved by seed germination assay and PCR analysis.

In this method, *Agrobacterium* is targeted to the wounded apical meristem of the differentiated seed embryo. Therefore, *Agrobacterium tumefaciens* transfers the gene into the genome of diverse cells which are already destined to develop into specific organs and the meristematic cells still to be differentiated. This results in the primary transformants (T_0) being chimeric in nature. This is the reason for the analysis of the transgenic plants to be carried out in the T_1 generation. The chimeric plants producing the stable transformants in the T_1 generation depends on the type of cells that were transformed in the T_0 plants. If the transgene is integrated into undifferentiated meristematic cells which are destined to develop into branches, seeds obtained from the reproductive structures of these branches would produce stable transformants in T_1. Though transformation is a random event, the conditions standardized in the procedure allow generation of transformants with varied efficiency. The efficiency of transformation in any crop using *in planta* transformation depends on a number of factors and standard percentage efficiency cannot be set for any crop or experiment. This is because there are many factors that influence the transformability using this protocol. Firstly, it depends on the number of chimeras arising from the total number of T_0 plants. The number of chimeras depends on the number and type of cells that integrate the transgene. Secondly, the number of plants in the T_1 generation that are stable transformants can vary between the chimeric T_0 plants as it depends on how many of the transformed cells develop into germ cells. However, the high transformation efficiency can be attributed to the targeting of the T-DNA by wounding the apical meristem and conditions for the induction of virulence genes. In the present study, the transformation efficiency was 4.68%. Similar transformation efficiencies were also obtained in other *in planta* transformation approaches (Trieu *et al*. 2000; Supartana *et al*. 2005; Zale *et al.* 2009; Mamontova *et al.* 2010). However, further attempt to increase the transformation efficiency is possible with the present transformation protocol.

The present study therefore, demonstrates the feasibility of *in planta* transformation to develop stable transformants in rice. Further, the efficacy of the *PgNHX1* gene in improving the salt tolerance is also unequivocally demonstrated. This study showed tolerance to salt up to 450 mM vs 200 mM shown in various other studies (Apse *et al.* 1999; Zhang and Blumwald 2001). Incorporation of such methodologies would therefore benefit crop improvement programmes in rice.

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