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Research Article

Improved salt tolerance in rice (*Oryza sativa*) by co-expression of two vacuolar transporter genes involved in sodium compartmentation and proton gradient generation

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Abstract

Soil salinity is one of the world's most serious environmental problem which reduces crop yield. Sequestration of excess cytosolic Na^+ into vacuole is one of the most important successful strategies towards maintenance of ion homeostasis that averts the deleterious effects of Na^+ in the cytosol by maintaining a higher ratio of K^+/Na^+. In the present study, transgenic rice plants (Var. Vikas) co-expressing *PgNHX1* (Tonoplast Na^+/H^+ antiporter from *Pennisetum glaucum*) and *AtAVP1*(vacuolar H^+-ppase from *Arabidopsis thaliana*) were developed along with single gene transgenics of *PgNHX1* for comparing the performance of single and double transgenics by tissue culture-independent in planta transformation technique. Transgenic rice plants co-expressing *PgNHX1* and *AtAVP1* performed much better under high salt concentration of 300 mM NaCl with extensive root system. Physiological and molecular analysis proved that the co-expression of *PgNHX1* and *AtAVP1* enhanced salt tolerance to much higher level than single transgenics of *PgNHX1* and wild type treated plants.

Keywords: *AtAVP1,* ion homeostasis, *NHX1*, Na^+ compartmentation, salinity, transgenic

1. Introduction

Saline soil has an electric conductivity (EC) of ≥4 dS m^{-1} (USDA-ARS, 2008), equivalent to 40 mM that generates an osmotic pressure of approximately 0.2Pa, which significantly reduces the yield of most crops (Munns and Tester, 2008). The primary cause of ion toxicity in crop plants is Na^+ and hence maintenance of cellular Na^+ concentration is critical for optimal metabolic activity under salt stress (Tester and Davenport, 2003). Osmotic and salt specific effects are the two major factors of salinity with a negative impact on plants resulting in plant growth inhibition. These two effects give rise to a two phase growth response to salinity. The first phase of plant growth response results from the effect of salt in the soil that reduces leaf growth and to a lesser extent root growth. Neither Na^+ nor Cl^- builds up in growing tissues at a toxic concentration. The second phase of growth response is from the toxic effects of salt within the plant resulting from the rapid buildup of salt in the cytoplasm, inhibiting the enzymatic activity (Munns and Tester, 2008).

Transport of Na^+ into the vacuoles is mediated by a Na^+/H^+ antiporter (*NHX1*) driven by the electrochemical gradient of protons generated by the vacuolar H^+- translocating enzymes, H^+- ATPase and H^+-PPase (*AtAVP1*) (Yamaguchi and Blumwald, 2005). Overexpression of *AtNHX1* in *Arabidopsis* resulted in plants exhibiting increased salt tolerance (Apse *et al.*, 1999). In addition to Na^+/H^+ antiporter role, *NHX1* also plays a significant role in intracellular vesicular trafficking and transcription regulation (Sottosanto *et al.*, 2004). *AtNHX1* has been over-expressed in several dicotyledonous plants (Zhang and Blumwald, 2001), (Zhang *et al.*, 2001), (Soliman *et al.,* 2009) and in crop plants (Xue *et al.*, 2004) (Yin *et al.,* 2004). *NHX1* from different plants like *Atriplex gmelini AgNHX1* (Ohta *et al.,* 2002*)*, *Pennisetum glaucum PgNHX1* (Verma *et al.*, 2007*)*, *Suaeda salsa SsNHX*1 (Zhao *et al.*, 2006a)

were overexpressed in rice and wheat *TaNHX1* was overexpressed in *Arabidopsis* exhibiting higher salt tolerance (Brini *et al.,* 2007) and normal growth under high saline conditions. Similarly, overexpression of *AtAVP1* in *Arabidopsis* enhanced sequestration of Na^+ into the vacuole, (Gaxiola *et al.*, 2001, Guo *et al.,* 2006, Brini *et al.,* 2007). The H^+-PPase gene was also overexpressed in tobacco (D'yakova *et al.,* 2006), (Gao *et al.*, 2006), in Alfa alfa (Bao *et al.,* 2009) and in other crop plants (Park *et al.,* 2005) to enhance the salt tolerance. There are reports on transporters that have emphasized the importance of *NHX1* and *AtAVP1* in Na^+ sequestration into the vacuole. Unfortunately, the co-expression studies in monocots are few and isolated, with only one study in rice (Zhao *et al.*, 2006b), that reported higher salt tolerance than the respective single transgenics. Similar kind of study was conducted in tomato to improve salt tolerance by co-expressing *AtAVP1* and *PgNHX1,* which resulted in the enhanced performance of co-expressing transgenic lines than the single gene transgenics (Bhaskaran. and Savithramma, 2011). More recently, Fan *et al.* (2019) reported that, co-expression of *SpSOS1* and *SpAHA1* in *Arabidopsis* resulted in better salt tolerance in terms of maintaining higher K^+ and lower MDA levels than the single transgenics of *SpSOS1* and *SpAHA1* and concluded that, coordinated action of these two genes might be a novel and effective method for increasing the salt tolerance of crops. Present study deals with the co-expression of *PgNHX1* and *AtAVP1* in rice and analysis of their salt tolerance capacity in comparison with the single gene transgenics and wild type plants.

2. Materlals and methods

2.1 Plant material

Vikas variety 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.

2.2 Development of double gene vector

The *NHX1* gene from *Pennisetum glaucum* was obtained from Dr. M. K. Reddy, ICGEB, New Delhi. This gene was cloned at *BamHI and XhoI* sites of the pRT-100 vector. Further, gene cassette comprising the 35s promoter, *NHX1* gene and polyadenylation cassette was subcloned into pCAMBIA 1301 binary vector. The second gene *AtAVP1* from *Arabidopsis thaliana* was obtained from Dr. R A Gaxiola, University of Connecticut, Farmington, Connecticut, United States. The gene was cloned at smal site of pRT-103 vector, digested with *HindIII* to get the whole cassette of 2 x 35s promoter, *AtAVP1*gene and Polyadenylation sequence are cloned into pCB302 in the *HindIII* site. For developing double gene construct of *NHX1* and *AtAVP1*, the *AtAVP1* gene cassette was partial digested with *HindIII* from pCB302:*AtAVP1* vector and the entire cassette of 3.3 Kb fragment was inserted into pCAMBIA 1301 at *HindIII* site. (Fig. 1). The single gene construct of *PgNHX1* and double gene construct of *PgNHX1* and *AtAVP1* were mobilized into electro-competent *Agrobacterium tumefaciens* strain EHA105 (Competent cells were prepared according to Shen and Forde, 1989) by applying an electric pulse using a Gene Pulser Ô with pulse controller unit (Bio-Rad) separately and were used for plant transformation.

2.3 Transformation and development of transformants

Transformation of rice and generation of the primary transformants (T_0) 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 Soil rite (vermiculite equivalent; Keltech Energies Ltd, Bengaluru, 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 an optimum temperature of 28 ± 1°C and 85% relative humidity.

2.4 Screening of T_1 generation plants at seed level

Both WT (Wild type) as well as T_1 generation seeds of single and double transgenics 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 200 mM and 300 mM NaCl (Sushma *et al.,* 2011). 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.

2.5 Assessment of salinity tolerance levels of T_1 transgenics under salt stress in outdoor condition

Single and double transgenic seedlings which passed the seed level screening along with the wild type seedlings were then transferred to rectangle plastic trays containing half strength Hoagland's solution (Hoagland and Arnon, 1950) and allowed to grow for 10 days under outdoor condition. Then they were subjected to salt treatments of 0 mM, 200 mM and 300 mM NaCl concentration that were imposed incrementally i.e., by increasing 50 mM for every three days till a final concentration of 200 mM and 300 mM was reached. After two weeks of 200 mM and 300 mM of salt stress, the plants were used for physiological and molecular analysis.

2.5.1 Physiological analysis

2.5.1.1 Chlorophyll estimation

Leaf bits (50 mg) of the WT, single and double gene putative 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 652 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):

$$\text{Total chlorophyll content} = \frac{\text{A. 652}}{34.5} \times \frac{\text{Volume}}{\text{Fresh weight}}$$

$$\text{Reduction over control} = \frac{\text{(Abs. Control-Stressed)}}{\text{Abs.control}} \times 100$$

Chlorophyll stability index = 100-R

2.5.1.2 Cell Membrane stability (CSI)

Percent leakage, which reflects loss of cell membrane integrity, was quantified. The leaf bits (50 mg) of the wild type, treated control, single and double transgenics from the above salt treated plants were incubated in 10 ml of de-ionized water for three hours. Initial leakage was recorded by taking absorbance at 273 nm. The leaf bits were then incubated in hot water bath of 65°C for 15 minutes and final absorbance was recorded at 273 nm using UV visible spectrophotometer. Leakage of solutes from leaf samples were calculated using the formula

Leakage(%) = [Initial absorbance/Final absorbance] x 100

2.5.1.3 Cell Viability Test

Cell viability of wild type, treated control, single and double putative transformants from the above salt treated plants were analyzed using 2,3,5-triphenyl tetrazolium chloride (TTC) reduction by respiratory enzymes, which converts colorless TTC to red color formazon. The red formazon formed in the tissue was extracted with methoxy ethanol and its absorbance was recorded at 485 nm. The absorbance is a direct reflection of cell viability and mitochondrial activity (Kalina and Parmer, 1986).

2.5.1.4 Estimation of Shoot and root K^+/Na^+ ratio

K^+/Na^+ ratio of salt treated control and transgenic plants were determined by using an Inductively Coupled Plasma-Optical Emission Spectrometry (Thermo scientific, USA). Samples were prepared by grinding leaf tissue in pestle and mortar separately. 5ml of concentrated nitric acid was added to 100mg of powdered sample and were incubated in digestion hood overnight. Then 5ml of diacid mixture of nitric acid and perchloric acid (10:4) was added and placed on sand bath till white residue was left in the flask. The volume was made to 25 ml with milli Q water and further dilutions were made if the concentration was high. These diluted solutions were used for calculating potassium to sodium ratio. A total of three replications were maintained and their average was used for calculations. K^+/Na^+ ratio was calculated using the formula

$$K^+/Na^+ = \frac{\text{Potassium content (mmol } g^{-1} \text{ dry weight)}}{\text{Sodium content (mmol } g^{-1} \text{ dry weight)}}$$

2.5.2 Molecular analysis

2.5.2.1Genomic DNA isolation

Leaf tissue from the progeny plants were analyzed for the presence of gene integration. Genomic DNA was isolated following the protocol of Doyle and Doyle (1998) from fresh leaf tissue.

2.5.2.2 PCR analysis

The transgenics and wild type plants were used for PCR analysis. 50 ng of genomic DNA was used as template from wild type, single and double transgenic lines. 35s forward primer (TCC TTCGCAAAGACCCTTC) and *PgNHX1* specific reverse primer (TCCCGCCAGAACTAATCCTA), 2 X 35S forward primer (TCC TTCGCAAAGACCCTTC) and A*tAVP1(*GCGAACAAGGGAGCAAAGACAAG*)* specific reverse primers were used to confirm the gene integration. The PCR reaction was performed in 20µl reaction mixture containing Template DNA - 100 ng, *Taq* buffer - 2X concentration, dNTPs- 0.2 mM, $MgCl_2$- 2.5mM, forward and reverse primers-3 picomoles each and Taq polymerase-1unit at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C (1 minute), annealing at 58°C (45 seconds), extension at 72°C (1minute) and then final extension at 72°C for 10 minutes.

2.5.2.3 RT- PCR (Reverse Transcriptase- Polymerase Chain Reaction) analysis

Total RNA was extracted from control and salt treated transgenic plants according to the protocol described by Datta *et al.* (1989). Then cDNAs were synthesized from 5 µg total RNA (pretreated with DNase 1) from transgenic and non-transgenic plants and was amplified using different sets of primer combinations as mentioned above in PCR analysis.

2.6 Statistical analysis

In this study, each experiment contained three biological replicates. The results are expressed as mean ± SE and differences with a p-value <0.05 were considered statistically significant.

3 Results and discussion

Around 45 T_0 single and double transgenic plants were established in greenhouse, with an average of 13 panicles per plant (Fig. 2) The seeds produced from these plants were selected for analysis in the T_1 generation.

Five single transgenics (S1 – S5) of *PgNHX1* and three double transgenics (D1 – D3) of *PgNHX1* and *AtAVP1* were assessed for their salt tolerance. Six replications were maintained for each of absolute control, treated wild type, five lines of single transgenics and three lines of double transgenic on 0 mM, 200 mM, 300 mM NaCl in 0.6% agar medium (Fig. 3). Salt stress was imposed and the plants were analyzed for salt tolerance at the physiological level after 12 days of stress imposition. Four days after recovery, root and

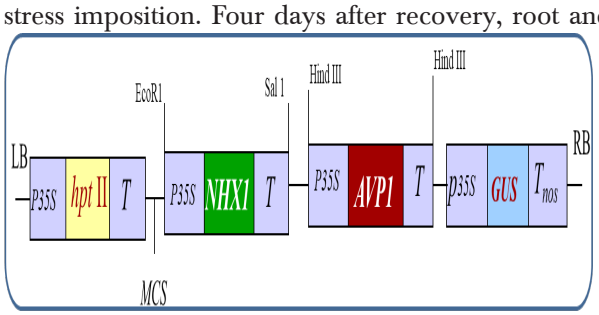

Fig. 1: T-DNA map of binary vector pCAMBIA 1301 harboring *pgNHX1-AtAVP1* as gene of interest , hptII as marker gene and . LB-left border; RB-right border; mcs-multiple cloning site.

shoot length of recovered seedlings was measured (Fig. 4). The seedlings which passed the seedling level screening were further analysed for their salt tolerance capacity by growing them in a hydroponics system where 200 and 300 mM NaCl solution was given along with half strength Hoagland's nutrient solution (Fig. 5). At the end of the stress period sampling was done for physiological and molecular analysis.

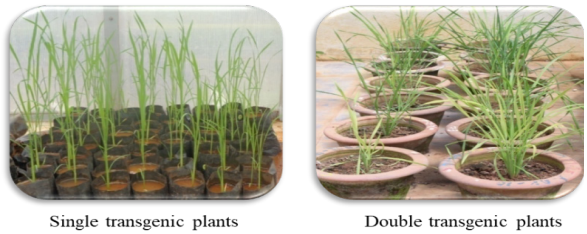

Fig. 2: Photograph showing putative T_0 single and double gene transgenic rice plants in green house

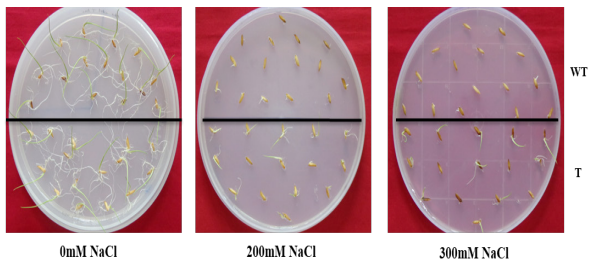

Fig. 3: *In vitro* screening of T_1 seeds on 200 and 300 mM NaCl salt concentration. WT-Wild type, T-putative transgenics

3.2 Plants co-expressing double gene maintained higher Chlorophyll Stability Index (CSI) than single and wild type plants

The CSI was higher in double transgenic lines and single transgenics than wild type treated plants with average CSI of 67.38 ± 10 in D1,65.04 ± 11 in S5 compared to 52.8 ± 6.4 in treated control plants subjected to 200 mM salt. Similarly, in 300 mM salt treated plants, the chlorophyll stability index was 64.24 ± 7.2 in D1,63.44 ± 6.33 in S5 compared to 35.94 ± 11.1 in treated wild type plants. However, the CSI of S1 to S4 lines under 300 mM salt stress was less compared to CSI of double transgenic lines (Fig. 6). Thus, D1 line among double transgenics and S5 line among single transgenics performed much better under salt stress condition than wild type treated plants.

3.3 PgNHX1-AtAVP1 co-expressing plants had higher cell membrane stability under salt stress

Cell membrane stability was studied to assess the primary injury faced by the membrane under salt stress. Electrolyte leakage indicates the extent of damage caused to the cell membrane under salt stress. Overall, the leakage in double transgenics ranged from 13 to 14%, whereas in single transgenics it ranged from 13 to 17% compared to 25% of leakage in wild type treated plants under salt stress of 200 mM. In 300 mM salt concentration, the leakage was 14% in double transgenics, 16 to 25% in single transgenics and 31% in wild type treated plants. There was 10% leakage in absolute control plants. Thus electrolyte leakage of 13.58 ± 2.34% in D1, 13.64 ± 2.7% in

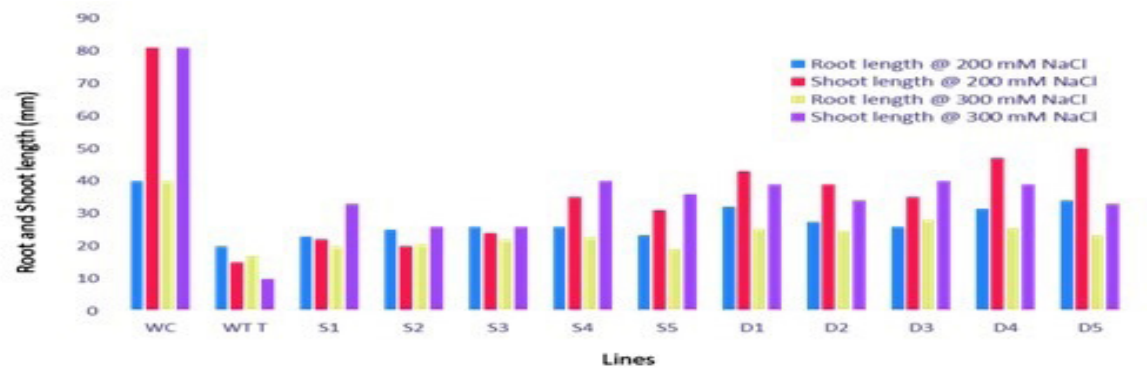

Fig. 4: Variation in shoot and root length of salt stressed seeds of single and double T_1 transgenics compared to absolute control and wild type treated seedsafter three days of recovery. (WC-wild type control, WT T- wild type Treated,S1to S5 single (*NHX1*) transgenics& D1 to D5-double(*NHX1-AtAVP1*) double transgenics)

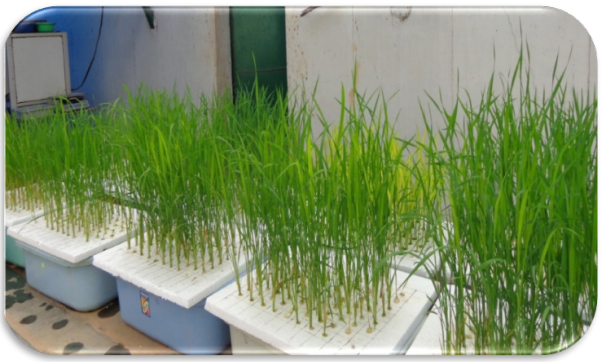

Fig. 5: Hydroponics experimental set up used for salt screening

S5 and 25.27 ± 4.7% in treated control under 200 mM salt stress was observed. In 300 mM salt stress it was about 14.74 ± 4.5% in D1, 16.7 ± 3.8 % in S5 and 31.61 ± 3.9% in treated wild type plants (Fig. 7). This indicated improved salt tolerance in the double transgenics compared to single transgenics.

3.4 Co-expressing plants maintained higher cell viability under salt stress

Cell viability of treated wild type and transgenic lines was assessed by TTC reduction test. Absorbance at 485nm is a direct reflection of cell viability and mitochondrial activity. Under salt stress the cell viability was found to be reduced in all treated plants than absolute control (Fig. 8). In double transgenic plants the A_{485} ranged from 1.56 ± 0.057 to 1.72 ± 0.11, whereas in single transgenics it ranged from 1.11 ± 0.13 to 1.3 ± 0.15 compared to 0.99 ± 0.06 in treated control plants under salt stress of 200 mM. Similarly, in 300 mM salt stress condition, A_{485} ranged from 0.93 ± 0.1 to 1.38 ± 0.15 in double transgenics, 0.5 ± 0.08 to 0.98 ± 0.17 in single transgenics compared to 0.33 ± 0.031 in treated wild type plants. The A_{485} of absolute wild type plants was 1.91 ± 0.034.

3.5 Estimation of shoot and root K^+/Na^+ ratio

Shoot and root K^+/Na^+ ratio was assessed using ICP-OES in the transgenic and wild type plants treated

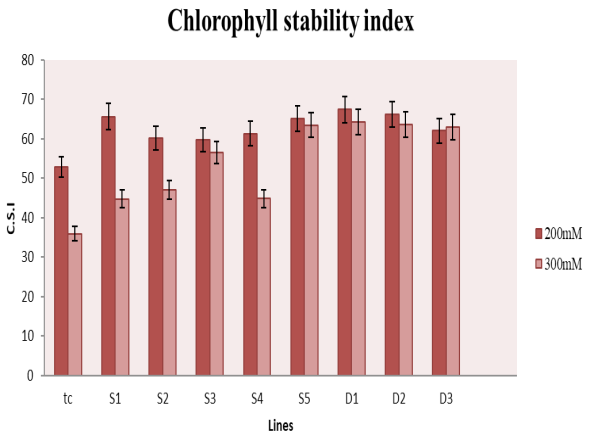

Fig. 6: Chlorophyll stability index of T_1 transgenic plants and wild type plants under salt stress. (tc-treated control, S1-S5-single transgenics, D1-D3-double transgenics)

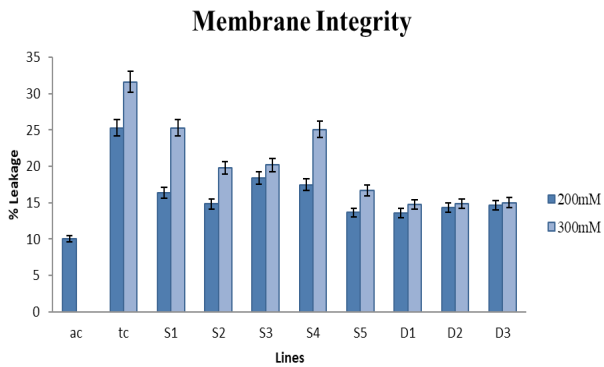

Fig. 7: Membrane stability of T_1 transgenic plants and wild type plants under salt stress. (ac-absolute control, tc-treated control, S1-S5-single transgenics, D1-D3-double transgenics)

with 200 mM and 300 mM salt. In shoots, the K^+/Na^+ ratio was higher in double transgenic and single transgenic plants compared to wild type treated plants in both 200 and 300 mM salt treated condition. In double transgenics the shoot K^+/Na^+ ratio was 1.3 ± 0.36 and 0.8 ± 0.33 in 200 and 300 mM, respectively. In single transgenics it was about 1.2 ± 0.47 and 0.6

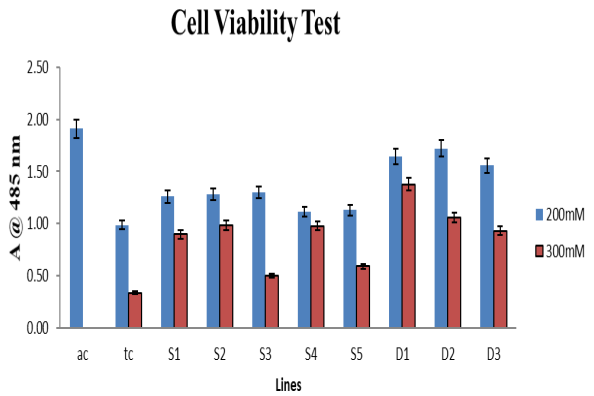

Fig. 8: Cell viability Test of T1 transgenic plants and wild type plants under salt stress. (ac-absolute control, tc-treated control, S1 to S5-single transgenics, D1to D3-double transgenics)

± 0.16 compared to 1.08 ± 0.16 and 0.3 ± 0.1 in wild type under 200 and 300 mM salt stress respectively (Fig. 9a). In roots the K^+/Na^+ ratio was higher in 200 mM treated wild type plants than single and double transgenics as shown in Fig. 9b. In 300 mM salt stress condition no significant difference in K^+/Na^+ ratio could be seen in the roots of wild type and transgenic plants. Thus co-expression of *pgNHX1* and *AtAVP1* in double transgenics resulted in increased salt tolerance compared to single transgenics of *PgNHX1*. Both the single and double transgenics showed higher K^+/Na^+ in their shoots compared to wild type under salt stress condition of 200 mM and 300 mM. It was also observed that in roots of wild type plants treated with 200 mM NaCl, a higher K^+/Na^+ was exhibited than transgenic plants which implicates that wild type treated plants were unable to maintain a higher K^+/Na^+ in their shoots. Although K^+/Na^+ was almost similar in all wild type and transgenic plants when exposed to 300 mM salt stress condition. Thus these results indicate that the transgenic lines exhibited higher K^+/Na^+ratio in shoots due to increased ion homeostasis.

3.6 PCR analysis of putative transformants

In order to identify the presence of transgene in the putative transformants PCR was performed with 35S promoter forward and *NHX1* reverse (Fig. 10a) and 35S promoter forward andgene specific reverse primers (*AtAVP1* and *NHX1,* respectively) (Fig. 10b). Amplification of the expected fragments in the primers used confirmed the transgenic nature and there was no amplification in WT plants.

3.7 Semi-quantitative RT-PCR analysis

Some of the PCR positive lines were analyzed for transcript accumulation. There was difference in the level of expression of *PgNHX1* gene (Fig. 11b). It was confirmed that the variation was because of the transgene as all the samples showed accumulation of hptII transcript, which was absent in WT (Fig. 11a). The house keeping gene Actin was amplified with same cDNA pool and used as internal control (Fig. 11d).

Among the mechanisms conferring salt tolerance at cellular and molecular level, restoring ion homeostasis under salt stress is crucial. In turn there are three aspects of ion homeostasis (Narinder and Gupta, 2005); i. Exclusion [*LCT1* and *HKT* family of genes involved in entry of Na^+ into the roots], ii. Efflux [*SOS1/ NHX7* gene involved in extrusion of Na^+] and iii. Compartmentation [*NHX1*, *AtAVP1*- genes involved in vacuolar compartmentation of Na^+]. Among the mechanisms conferring salt tolerance at cellular and

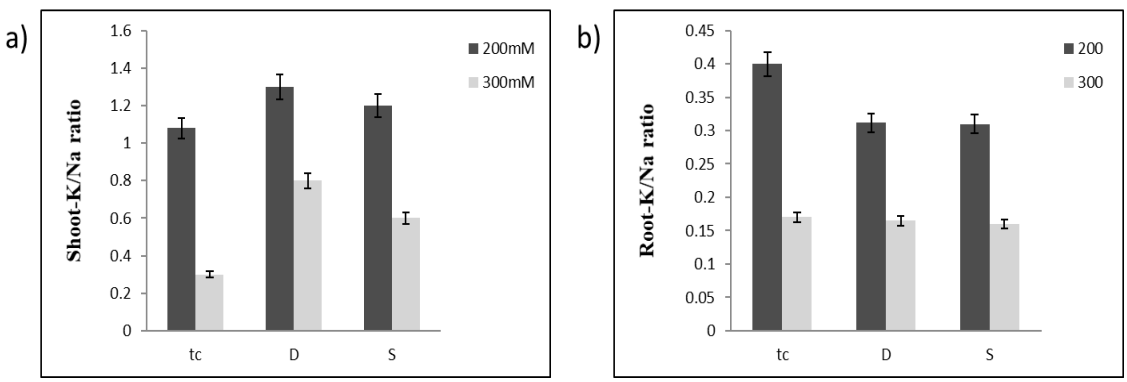

Fig. 9: Shoot and root K^+/Na^+ ratio of T_1 transgenic plants and wild type treated plants. (tc-treated control, D-double transgenics, S-single transgenics)

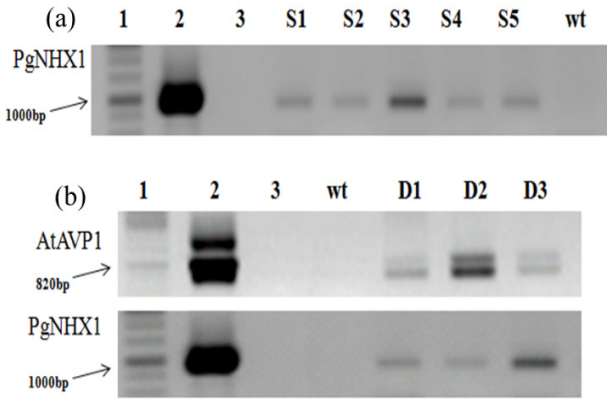

Fig. 10: PCR Analysis of T_1 (a) single transgenics of *PgNHX1*, specific fragment of length 1000 bp was detected with 35S promoter forward and gene specific reverse primer (lanes S1 to S5), (b) double transgenics of *PgNHX1* and *AtAVP1*, specific fragment of length 1000bp and 820bp, respectively (lanes D1 to D3). Lane 1:1 kb ladder, Lane 2: positive control, Lane 3: negative control, wt: genomic DNA from wild type.

molecular level, restoring ion homeostasis under salt stress is crucial, of which, vacuolar compartmentation of Na^+ is the most important one. This function is carried out by the *NHX1* gene, encoding Na^+/H^+ antiporter on the vacuolar membrane. The efficiency of this transporter protein encoded by *NHX1* gene depends primarily on the development of proton gradient across the tonoplast membrane. This function is carried out by a group of *AtAVP1* (vacuolar H^+-pyrophosphatases) transporters. During the last decade, the transgenic approach to enhance salt tolerance has involved targeting the genes encoding one of these vacuolar transporter proteins but not both of them. Logically the co-expression of both these genes should result in the most efficient transgenics with a higher level of salt tolerance. But surprisingly, till date, majority of the

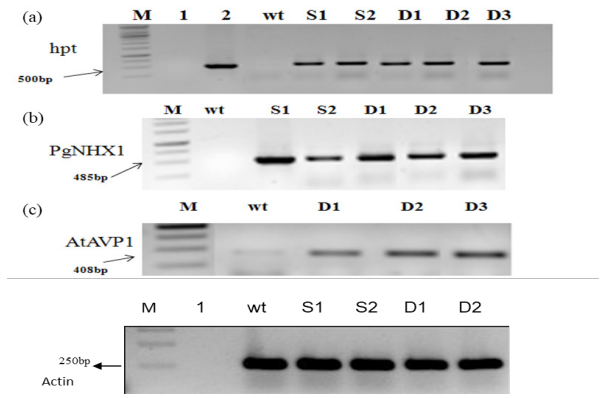

Fig. 11(d): RT- PCR analysis of T_1 single transgenics of *PgNHX1* and double transgenics of *PgNHX1* and *AtAVP1*. Amplification of cDNA using (a) hpt (b) *PgNHX1* and (c) *AtAVP1* and (d) Actin (as an internal control) gene specific forward and reverse primers. Lane M: 1Kb ladder, Lane 1: negative control, Lane 2: positive control, wt: wilt type, S1 and S2: single transgenics, D1 to D3: double transgenics.

studies have been on single transgenics of either *NHX1* or *AtAVP1*. In the present study we have co-expressed both *PgNHX1* and *AtAVP1* in rice var. Vikas. Similar work using *Suaeda salsa NHX1* and *Arabidopsis AtAVP1* has been carried out in rice by Zhao *et al.* (2006). Fan *et al.* (2019) reported that co-expression of *SpSOS1* and *SpAHA1* in *Arabidopsis* resulted in better salt tolerance in terms of maintaining higher K^+ and lower MDA levels than the single gene transgenics of *SpSOS1* and *SpAHA1* and concluded that, coordinated action of these two genes might be a novel and effective method for increasing the salt tolerance of crops.

In our study, co-expression of *PgNHX1* and *AtAVP1* enhanced salinity tolerance compared to single transgenics of *PgNHX1* and treated wild type plants. The above data and figures depicts the *in-vitro* growth of double transgenic seedlings after recovering from 300 mM salt concentration which was not observed in wild type treated seedlings. Transgenic plants subjected to salt stress under outdoor condition exhibited salinity tolerance at physiological level with higher chlorophyll stability index, membrane integrity and cell viability. Double transgenics performed much better than single transgenics while single transgenics performed better than treated wild type plants. The transgenic plants maintained high shoot K^+/Na^+ ratio which is one of the key determinants in plant salt tolerance. Maintenance of higher K^+/Na^+ in transgenic lines under salt stress can be attributed to induced homeostasis caused by the overexpression of vacuolar antiporters (Gao *et al.*, 2004; Zhao *et al.*, 2006a; Ren *et al.*, 2005). Molecular analysis using PCR and RT-PCR were carried out which confirmed the transgenic nature of plants. Thus the present study reveals that co-expression of *PgNHX1* and *AtAVP1* results in higher salt tolerance than that of single transgenics of *PgNHX1* and wild type plants.

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