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Constitutive expression of *OsNHX1* under the promoter Actin1D can improve the salt tolerance and yield characteristics of Bangladeshi rice Binnatoa

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Abstract

Transgenic rice cv Binnatoa expressing the *Oryza sativa* vacuolar Na^+/H^+ antiporter *OsNHX1* under the constitutive rice Actin1D promoter showed enhanced tolerance and improved yield in NaCl stress. Transgenic status of the plants was assured by PCR and Southern blot analysis from T_0 to T_3 state. Over-expression of *OsNHX1* under Actin1D promoter was confirmed by semi-quantitative RT-PCR and Western Dot-Blot analyses. At 160 mM salt stress, transgenic seedlings grew well and showed minimal reduction in shoot and root length compared to controls. Leaf chlorophyll estimation assay at 160 mM NaCl showed significantly high chlorosis in wild-type in contrast to the transgenic line. After salt stress, lower K^+/Na^+ ratio in transgenic leaf compared to the wild-type indicated the increased Na^+ accumulation in vacuoles. At reproductive stage transgenic plants showed improved yield charateristics compared to the wild-type after exposure to 60 mM NaCl stress.

Keywords : Constitutive expression, Chlorosis, Ion imbalance, transgenic plant

Introduction

Salinity is a major limiting factor to agricultural productivity. High salinity causes ion imbalance and hyperosmotic stress in plants (Boyer 1982, Bartels and Sunkar 2005). One way to increase salt tolerance in plants is to increase the solute concentration in the vacuoles of plant cells (osmotic adjustment). Increasing the vacuolar osmotic potential would favor water movement from apoplast to the cell. Two approaches have been used to increase solute contents in plant vacuoles (Gaxiola et al., 2002). The first approach involves increasing the activity of a vacuolar sodium/proton (Na^+/H^+) antiporter that mediates the exchange of cytosolic Na^+ for vacuolar H^+ (Apse et al., 1999, Fukuda et al., 1999, Yokoi et al., 2002, Xue et al., 2004, Wang et al., 2005, Wu et al., 2005, Lu et al., 2005). The second approach involves increasing the activity of an H^+ pump on the vacuolar membrane to move more H^+ into the vacuoles, therefore generating a higher proton electrochemical gradient ($\Delta\mu H^+$) that can be used to energize secondary transporters including vacuolar Na^+/H^+ antiporters. Both approaches enhance ion accumulation in the vacuoles and reduce the potential of Na^+ toxicity in the cytoplasm, leading to higher salt and drought tolerance in transgenic plants (Wyn Jones and Pollard 1983, Blumwald et al., 2000). However, most of the tolerance data was obtained in laboratory conditions, with the exception of the work on overexpression of the AtNHX1 gene in wheat. Although the yield in the transgenic wheat plants was reduced by 40% compared to 60% in the untransformed control, the field experiments were conducted in cold temperatures of -1.9 ^{0}C to 13^{0}C, which do not support high transpiration rates and therefore will not reflect actual salt tolerance of the plants. Rice is one of the most important crops in the world and its production is severely affected by salinity, it is essential to improve salt tolerance of rice (Akbar and Ponnamperuma 1980). The *Oryza sativa* vacuolar Na^+/H^+ antiporter gene was cloned (GenBank accession No. AB021878) and may play an important role in salt tolerance of rice under

Table 1. Means of different growth parameters of wild-type and transgenic seedlings under control and 160 mM NaCl stress

		Biomass (g)	Shoot length (cm)	Root length (cm)	Leaf number (cm)	Leaf width (cm)
Wild-type	Control	0.281	49.56	16.36	6.40	1.10
	Stress	0.171	40.80	11.24	6.20	0.78
OsNHX1-143	Control	0.199	45.96	14.06	6.20	0.86
	Stress	0.187	42.90	13.30	6.20	0.82
Variety x Treatment		**	**	**	ns	*
$LSD_{0.05}$		0.027	1.107	1.012	0.641	0.192

*, **, ns indicate significance at 5%, 1% and not significant respectively (n=5).

CaMV35S promoter (Fukuda et al., 2004). No yield data were however provided by Fukuda et al., (2004). The rice Actin1D promoter is a strong constitutive promoter and shows very efficient expression in monocots particularly in rice (McElroy et al., 1990). Here we have reported expression of the japonica rice Nipponbare *OsNHX1* gene under the constitutive promoter Actin1D in a Bangladeshi indica rice landrace Binnatoa. In this report we have provided complete yield data showing improved salt tolerance of the transgenic plants compared to the wild-type which documents the competence of Actin1D promoter in the expression and enhanced performance of the *OsNHX1* gene.

Materials and methods

Development of Actin1D-OsNHX1 construct

1904 bp *OsNHX1* cDNA (synthesized using Superscript™ First-Strand System of Invitrogen from total RNA extracted from 7-day-old *Oryza sativa* L. cv. Nipponbare seedlings after 24 h of 100mM NaCl stress using TRIZOL reagent) was reversed transcribed and amplified by PCR with the forward (5′-ACA AAA CCA TGG AGA AGA GAG TTT TGT AGC GA-3′) and reverse (5′-ATA TAA GGT TAC CTC ATC TTC CTC CGT GAC TCT GC-3′) primers using the SuperScript™ system of Invitrogen. An *Nco* I restriction site was incorporated into the forward primer and a *Bst* EII site was incorporated into the reverse primer. In addition, an *Nco* I site was deleted from the reverse primer. Primers were designed from the coding sequence of *OsNHX1* (GenBank accession No. AB021878) using the software OMIGA version 2.0. The PCR conditions were an initial denaturation at 94ºC (5 min), 35 cycles of: denaturation at 94ºC for 1 min, annealing at 56.8ºC for 1 min and extention at 72ºC for 2.30 min and final extension at 72ºC for 7 min. The resulting fragment was cloned into pCAMBIA1305.1 (Fig 1a), obtained from Dr. Richard Jefferson, ISAAA, through the Rockefeller Foundation network, using electro-competent *E.coli* DH5α according to the standard protocol (Sambrook et al., 1989).

To insert the rice Actin1D promoter upstream of the *OsNHX1* cDNA, the CaMV35S promoter and part of the Lac Z alpha gene was cut out of the plasmid CaMV35S-*OsNHX1* with *Pst* I and *Nco* I and 2.2 kb rice Actin1D promoter fragment was cut out of the plasmid pActBt1291 with the same enzymes (Fig 1b) re-synthesized from p*Act*1-D*,* McElroy et al., 1990 and obtained from J. Bennett, IRRI). The actin1-D promoter fragment and pCAMBIA1305.1- *OsNHX1* DNA were ligated overnight using 1 unit of T4 ligase (Invitrogen) and transformed into *E.coli* DH5α competent cells through electroporation using the standard protocol (Sambrook et al., 1989).

Production of transgenic rice

Agrobacterium tumefaciens (LBA 4404) was electroporated with the construct pCAMBIA-1305.1/Actin1D-OsNHX1 using standard protocols (Sambrook et al., 1989). By using the modified Khanna and Raina protocol (Rasul et al., 1997, Khanna and Raina 1999) transformation- responsive calli of Bangladeshi rice Binnatoa were infected and regenerated to produce T_0 plants.

Analysis of transgenic plants

T_0 plants were advanced to T_1 generation and transgenic status was confirmed by PCR and Southern blot analysis. The primers used in PCR for *OsNHX1* were: 5′-GCT GGA TTG CTC AGT GCA TA-3′ (Forward-679) and 5′-AAG GCT CAG AGG TGA CAG GA-3′ (Reverse-679). The PCR conditions were as above except that the annealing temperature was 60ºC for 1 min. After molecular confirmation T_1 plants were advanced to T_3 generation and tested by PCR and Southern blot

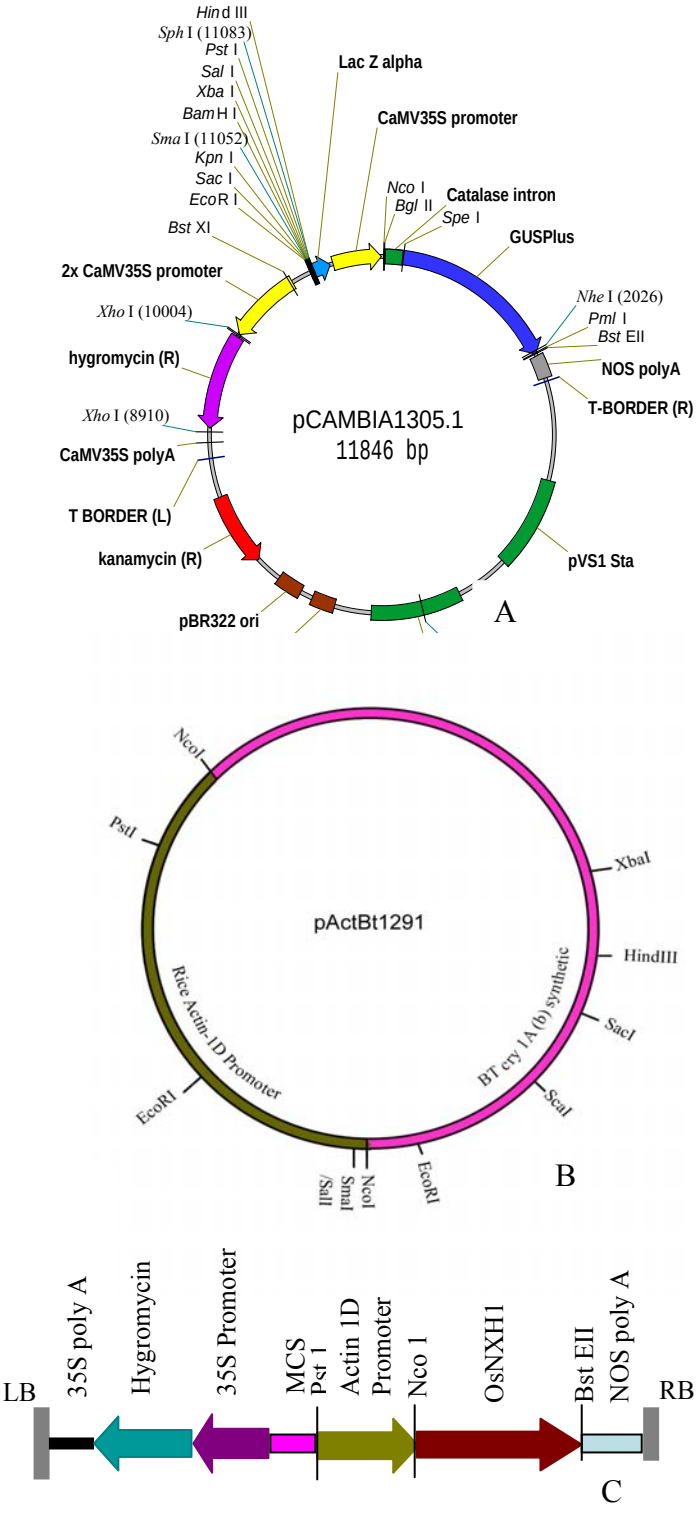

Fig 1. pCAMBIA1305.1 construct (A), plasmid pActBt1291(B) and gene scheme (C)

analysis at each generation. For Southern blot hybridization, 20 μg of genomic DNA from PCR positive rice lines was sequentially digested with *Nco* I and *Bst* EII enzymes (cloning site for *OsNHX1* in pCAMBIA1305.1), blotted onto the nylon membrane and probed using DIG labeled PCR amplified product (679 bp) from *OsNHX1* gene (1.9 Kb) produced by using Forward-679 (5′-GCT GGA TTG CTC AGT GCA TA-3′) and Reverse-679 (5′-AAG GCT CAG AGG TGA CAG GA-3′) primers according to the standard protocol (Roche Diagnostics Inc).

Semi-quantitative RT-PCR and Western Dot-Blot analysis

Total RNA was extracted from both shoot and root of 10-day-old seedlings of transgenic and nontransgenic Binnatoa according to the TRIzol® Reagent manufacturer's instructions. Then, cDNAs were synthesized from 1 µg total RNA (pre-treated by DNase I) of transgenic and nontransgenic root and shoot as explained above. The PCR reaction was performed for 25 cycles by using F- and R-679 primer pair as above. Overexpression of transgene at protein level was confirmed by Western Dot-Blot analysis according to the standard protocol of Western Breeze® (Invitrogen™). To do that poly-clonal antibody were raised in rabbit against a synthetic peptide derived from the C-terminal region of the *OsNHX1* amino acid sequence (Val-Pro-Phe-Ser-Pro-Gly-Ser-Pro-Thr-Glu-Gln-Ser-His-Gly-Gly-Arg) (Fukuda et al., 2004). Total protein from both transgenic and non-transgenic plants was equally quantitated (166 ng per dot) by modified Lowry method (Lowry et al., 1951).

Analysis of effects of salt stress at seedling stage

T_3 seeds of both transgenic and wild-type plants were germinated in wet filter paper at 37ºC and grown hydroponically in Yoshida solution (Yoshida et al., 1976). After 8 days 80 mM NaCl stress was applied to the Yoshida solution. The stress was gradually elevated up to 160 mM within 8 days and finally the plants were retained in 160 mM stress for the next 8 days in the net house. All plants were kept in the solution using a large single cork-floater in a PVC tray. The tray contained 10 Litres of solution and 11 biological replicates of each of the transgenic and non-transgenic lines. The tray was kept in a net house where the average temperature and humidity were 32ºC and 75% respectively. A same set of plants were transferred to water control (only Yoshida solution) without any stress. After 16 days, when the wild-type plants in stress were dead, tolerance related traits

Table 2. Response of wild-type and transgenic line to 60 mM salt stress depending on grain yield and yield related parameters

		Tiller number/plant	Panicle number/plant	Panicle length (cm)	Spikelet number/plant	Spikelet fertility (%)	Grain yield (g/plant)	100-grain wt (g)
Wild-type	Control	7.67	4.67	19.53	272.33	88.88	5.61	2.32
	Stress	4.33	3.67	16.23	140.33	79.09	1.29	1.17
OsNHX1-143	Control	7.67	4.33	19.37	267.67	89.13	6.07	2.55
	Stress	4.33	3.67	17.43	172.67	86.84	1.79	1.19
Variety		ns	ns	ns	**	ns	**	ns
Treatment		**	ns	**	**	ns	**	**
V x T		ns	ns	ns	**	ns	ns	ns
$LSD_{0.05}$		1.200	1.289	1.351	12.383	9.258	0.389	0.327

*, **, ns indicate significance at 5%, 1% and not significant respectively (n=3).

(biomass, root length, shoot length and leaf width) of all stressed plants were measured.

Measurement of chlorophyll and Na^+ and K^+ concentration

After 16 days of hydroponic treatment leaf chlorophyll concentration of each transgenic and wild-type plant was determined (Vernon 1969). After sun drying for 7 days, leaves were placed in an oven at 70ºC for 3 days, Na^+ and K^+ content was determined using the Flame Photometer 410 (Sherwood, UK) and K^+/Na^+ ratio was calculated.

Analysis of salinity tolerance and yield performance at reproductive stage

After 16 days in hydroponic system in Yoshida, 24 day-old water control plants were transferred to individual pots containing soil in a net house. The pots were perforated and the soil was contained in porous cloth sacks to prevent leakage through the holes of pots. After one week the pots were transferred to large bowls filled with 60 mM NaCl solution in three biological replicates. The salinity was maintained at the same level throughout the experiment till completion of the life cycle of the plants. One plant from each of the wild-type and transgenic line were grown in water control without any stress. The average temperature and humidity of the net house were 28°C and 87% respectively. At the end of reproductive stage seeds were collected form transgenic and non transgenic plants and weighed for determining the grain yield. Other yield related traits (tiller number, panicle number, panicle length , spikelet number, spikelet fertility and 100-grain weight) were also measured.

Statistical analyses

Analysis of variance and *t*-test was used to determine the existence of significant effects due to the transgene *OsNHX1*. Analysis of variance was performed by using the software CROPSTAT 7.2, IRRI Bioscience Unit and *t*-test was performed by using Microsoft Office Excel 2000.

Results and discussion

Production and molecular characterization of OsNHX1-expressing transgenic rice pCAMBIA-1305.1/Actin1D-OsNHX1construct (Fig 1c) was produced, cloned in *E.coli* DH5α, confirmed and then transformed into *Agrobacterium tumefaciens* (LBA 4404) through electroporation. By using *Agrobacterium*-mediated transformation three independent T_0 transgenic lines were produced from which Actin 1D-OsNHX1-143 was found to be the most prominent event after molecular and stress tolerance analysis. Therefore in all subsequent studies, the analysis of this transgenic line is shown. Transgenic status was confirmed by PCR and Southern blot analysis at T_0 state and then the T_0 plant was gradually advanced to T_3 generation by selection in hygromycin for further molecular analysis. After PCR and Southern blot analysis precise bands of 679 bp and 1.9 kb respectively were found only in the transgenic sample (Fig 2). Semi-quantitative RT-PCR was conducted to confirm the overexpression of the transgene at RNA level in root and shoot (Fig 3). Here a faint band was found for the wild-type which indicated the endogenous expression. Dark spot of transgenic sample compared to that of wild-type after Western Dot-Blot analysis revealed the overexpression of the gene at protein level (Fig 4).

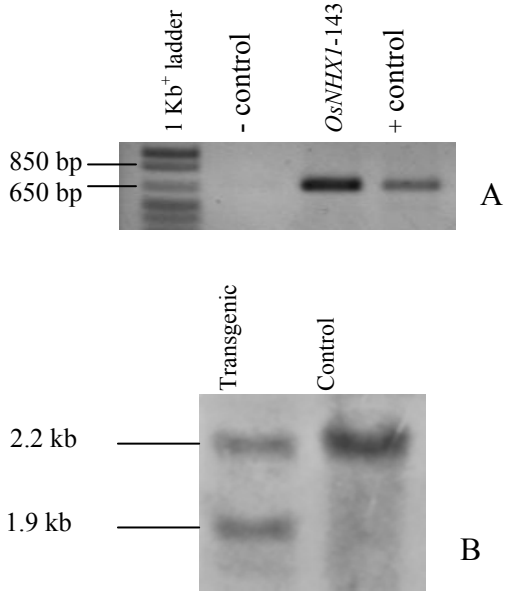

Fig 2. PCR amplification (A) and Southern blot analysis (B) of *OsNHX1* transformed in OsNHX1-143

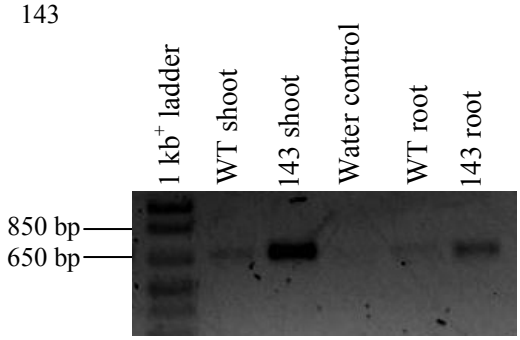

Fig 3. RT-PCR of *OsNHX1* cDNA. Broad bands of amplified product (679 bp) of *OsNHX1* cDNA was found only in *OsNHX1*-143 shoot and root

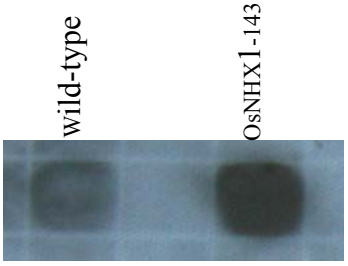

Fig 4. Western Dot-Blot analysis showing faint spots for wild-type Binnatoa and dark spots for *OsNHX1* overexpessing transgenic Binnatoa

Enhanced tolerance of transgenic plants

After 16 days in salt stress, the 24 day-old seedlings of the transgenic line Actin 1D-OsNHX1 showed better physiological status compared to the wild-type (Fig 5). The wild-type seedlings showed significantly higher biomass, root length, shoot length and leaf width compared to the transgenic seedlings in non-stress control (Table. 1). However, in salt stress, the transgenic seedlings showed better performance and significantly higher values for parameters known to be affected compared to wild-type (Table 1). Short stature of transgenic plants in control has been reported before (Chen et al., 2005; Kasuga et al., 1999). Chlorophyll content assay revealed the enhanced retention of leaf chlorophyll in the transgenic line compared to the wild-type (Fig 6). This is a strong indication of damage of the photosynthetic apparatus in wild-type plants by salt stress (Verma et al., 2007). After 16 days stress in 160 mM NaCl transgenic plants showed lower K^+/Na^+ ratio compared to wild-type of the same age (Fig 7). This indicates the increased accumulation of Na^+ in transgenic lines through the over-expression of *OsNHX1*. The increased accumulation of Na^+ ion in the vacuoles may be responsible for alleviating the toxic effects of excessive Na^+ ion in the cytosol.

Fig 5. Twenty-four-days *OsNHX1*-143 transgenic rice showing better physiological status compared to wild-type Binnatoa

Higher yield in transgenic plants compared to Wild-type in salt stress

Yield performance at reproductive stage was assessed at 60 mM NaCl stress. This stress level ensured a proper comparison in yield parameters between the wild-type and transgenic lines. Grain yield, in terms of total weight of seeds per plant, was used as the salient parameter for comparing the salinity tolerance and yield performance of transgenic plants at reproductive stage. Other yield related traits (tiller number, panicle number, panicle length, spikelet number, spikelet fertility and 100-grain weight) are

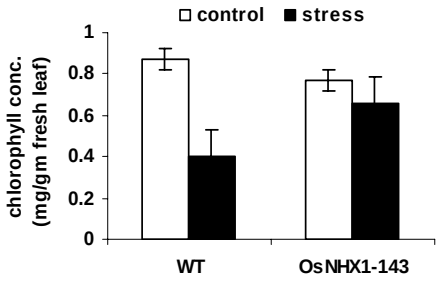

Fig 6. Chlorophyll concentration (mg g^{-1}FW) in the leaves of wild-type and transgenic rice plants after subjecting to control and 160 Mm NaCl stress. Each bar represents the mean ± SE (n=5)

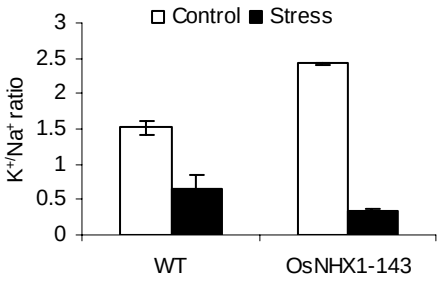

Fig 7. K^+/Na^+ ratio of K^+ and Na^+ concentration (mg g^{-1} DW) in the leaves of wild-type and transgenic rice plants after subjecting to control and 160 mM NaCl stress. Each bar represents the mean ± SE (n=5)

provided as support of better performance of the transgenic line under stress conditions (Table 2). In the case of variety, the transgenic line showed significantly higher spikelet number and yield compared to the wild-type. In the case of Variety x Treatment, only spikelet number was found to be significantly higher in transgenic plants compared to the wild-type.

Conclusion

Our results provide evidence that expression of *OsNHX1* under the constitutive promoter Actin1D can confer enhanced salinity tolerance in transgenic rice plants. The data also validate *OsNHX1* function toward alleviation of toxic effects of salt stress at all stages of the life cycle from seedling to maturity.

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