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Agrobacterium-mediated Transformation and Constitutive Expression of *PgNHX1* from *Pennisetum glaucum* L. in *Oryza sativa* L. cv. Binnatoa

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Abstract

In an effort to improve salinity tolerance in rice, the *Pennisetum glaucum* vacuolar Na^+/H^+ antiporter gene (*PgNHX1*) was transformed and expressed in Bangladeshi rice Binnatoa under the constitutive promoter CaMV35S. Transgenic status of the plant was confirmed by PCR and Southern blot hybridization. RT-PCR and Western dot-blot analyses were performed to validate the expression of the transgene at RNA and protein levels, respectively. At 160 mM (ECe of 16 dS/m) NaCl stress the transgenic seedlings showed enhanced tolerance compared to controls.

Introduction

Salt stress is an important environmental factor that severely affects plant growth and productivity. To cope with stress, many naturally occurring salt and drought-tolerant plants have developed a variety of adaptation mechanisms (Bohnert et al. 1995). One of them is the compartmentation of Na^+ into vacuoles, which can be achieved by the action of Na^+/H^+ antiporters on the vacuolar membranes. This process separates Na^+ from the cytosolic enzymes, and also helps balance the high extracellular osmotic potential created by the salt stress. The electrochemical gradient of protons generated by two vacuolar H^+ translocating enzymes H^+-ATPase and H^+-PPase is the driving force for this process (Wyn Jones and Pollard 1983, Blumwald et al. 2000). The ability to compartmentalize Na^+ in the vacuole is likely to be the principal determinant of salt tolerance capacity in most plant species (Blumwald and Poole 1985, Apse et al. 1999, Glenn et al. 1999, Xiong and Zhu 2002, Fukuda et al. 2004).

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Pennisetum glaucum (pearl millet) *is* a glycophyte which has a natural ability to withstand higher level of salinity, drought and heat stress (Rajagopal et al. 2007). The Na^+/H^+ antiporter gene (*PgNHX1*) from *Pennisetum glaucum* has been isolated and its importance has been shown in enhancing salinity tolerance in *Brassica* (Rajagopal et al. 2007). Its role in improving salinity tolerance in rice cv PB1 (Pusa basmati 1) under the ABA (abscisic acid) inducible promoter ABRC (abscisic acid response complex) was also reported (Verma et al. 2007). The authors have performed the *Agrobacterium*-mediated transformation with the same construct having the gene *PgNHX1* under the constitutive promoter CaMV35S in a Bangladeshi rice cultivar Binnatoa, which has been found to be very tissue culture responsive with greater than 4% transformant recovery (Rasul et al. 1997) and confirmed its expression for conferring enhanced salt tolerance.

Materials and Methods

Agrobacterium-mediated transformation through electroporation: Agrobacterium tumefaciens strain LBA4404 was purchased from Netherlands Culture Collection of Bacteria (NCCB) (www.bacterio.cict.fr/collections.html). The pCAMBIA 1301/CaMV35S-PgNHX1 construct was obtained as plasmid DNA from ICGEB (International Centre for Genetic Engineering and Biotechnology), New Delhi, India under an MTA between ICGEB and the University of Dhaka. The plasmid was transformed into *E. coli* strain DH5α, isolated and transformed into *A. tumefaciens* LBA4404 by electroporation. Positive clones were selected by lysate PCR (Sambrook et al. 1989) using forward primer (5'-ATG GCT GTG TTC AGC AGG AC-3') and reverse primer (5'- TCA CCA AAA ACA TGT CTT CAT CTC-3').

Agrobacterium culture and pre-induction: One confirmed clone of LBA4404 containing pCAMBIA 1301/CaMV35S- PgNHX1 construct was streaked on AB medium (Chilton et al. 1974) supplemented with 50 mg/l kanamycin and incubated at 28°C for 48 - 72 h, until colonies appeared. A single colony was transferred to 5 ml AB liquid medium containing the same selective antibiotic and the culture was allowed to grow overnight with shaking at 200 rpm. The overnight culture was transferred to 50 ml Bacterial Preinduction Medium (AB salts, 10 g/l glucose, 75 mM MES Buffer pH 5.6, 2 mM sodium phosphate buffer, acetosyringone (AS) 250 μM, pH 5.6) containing the same selective antibiotic according to modified protocol of Khanna and Raina (1999) (Rasul 2005) and grown overnight under the same conditions as described above. Bacteria were collected by centrifugation in a 50 ml tube at 4000 rpm for 10 minutes. The supernatant was removed and the bacteria washed in 10 ml of bacterial resuspension medium (AB medium with 36 g/l glucose, 68.5 g/l sucrose, AS 250 μM, 50 mg/l kanamycin, pH 5.2), pelleted and resuspended again in 10 ml of

bacterial resuspension medium. The culture was adjusted to 0.005 OD with the same medium and used for infection and cocultivation.

Callus induction, pre-incubation and co-cultivation: Dehusked mature seeds of Binnatoa collected from BRRI (Bangladesh Rice Research Institute) were surface sterilized first with 99% ethanol for 2 - 3 min, then in 2% sodium hypochlorite supplemented with one - two drops of Tween-20 for 30 min. After three rinses with sterile water, mature seeds were plated directly on MS callus induction medium (MS salts, organic elements, 30 g/l sucrose, 500 mg/l casein hydrolysate, 100 mg/l myo-inositol, 1 mg/l thiamine hydrochloride, 2 mg/l 2,4-D, 3 g/l phytagel, pH 5.8) and incubated in dark for 21 - 30 days at 25 - 27°C. Pieces of actively proliferating embryogenic calluses (2 - 3 mm diameter) were isolated and subcultured on fresh callus induction medium four days prior to pre-incubation. Calli were pre-incubated for 72 h in semisolid explant pre-incubation medium (half strength MS salts, organic elements, 30 g/l sucrose, 500 mg/l casein hydrolysate, 100 mg/l myo-inositol, 0.5 mg/l BAP, 0.5 mg/l NAA, 12.5 mM phosphate buffer (K_2HPO_4-NaH_2PO_4, pH 5.6) 1 mg/l thiamine hydrochloride, 3 g/l phytagel) (Khanna and Raina 1999).

The calli were immersed in bacterial suspension for 10 min. Excess bacterial suspension was removed by placing them on sterile filter paper before transferring to semisolid co-cultivation medium (Explant pre-incubation medium, AS 500 μM, 3 g/l phytagel, pH 5.8). The cultures were incubated at 25°C for three days in the dark. After three days the calli were removed and transferred to callus selection media (callus induction medium, 50-100 mg/l hygromycin, 500 mg/l carbenicillin, 250-500 mg/l cefotaxime).

Regeneration of Binnatoa transformants: The calli after co-cultivation were incubated in the selection media under dark at 25°C and transferred to fresh selection media with sequentially lowered antibiotic for three to five times until no bacterial growth was observed on selection plates. After four weeks, proliferating hygromycin-resistant microcalli were subcultured on plant regeneration medium (MS salts, organic elements, 30 g/l sucrose, 2 mg/l BAP, 0.2 mg/l NAA, 100 mg/l myo-inositol, 3 g/l phytagel, 50-100 mg/l carbenicillin, 50-100 mg/l cefotaxime and 1 mg/l thiamine hydrochloride, pH 5.8). Regenerants were transferred to rooting medium (one fourth strength MS salts (full strength Fe_2EDTA), 2 mg/l BAP, 0.2 mg/l NAA, 2 mg/l IBA, 500 mg/l casein hydrolysate, 100 mg/l myo-inositol, 30 g/l sucrose, 50-100 mg/l carbenicillin, 1 mg/l thiamine hydrochloride, 3 g/l phytagel, pH 5.8). Thereafter, they were transferred to a hydroponic system of Yoshida solution (Yoshida et al. 1976) for hardening and finally transferred to soil. The transgenic plants were kept at a confined area in a nethouse.

Molecular confirmation of transgenic plants: In five individual experiments ~120 calli were infected and successfully transformed T_0 plants were advanced to T_3 generation by germinating T_1 and T_2 seeds in hygromycin (20 mg/l) and selecting plants at T_1 which were more tolerant than the untransformed controls in hydroponic screening tests at the seedling stage followed at IRRI (Gregorio et al. 1997). Transgenic plants were selected by PCR (forward primer: 5'-ATG GCT GTG TTC AGC AGG AC-3'; reverse primer: 5'- TCA CCA AAA ACA TGT CTT CAT CTC-3'). For Southern blot hybridization, 10 μg of genomic DNA from PCR positive rice lines was digested with *Xho*I and *Bam*HI enzymes (cloning site for PgNHX1 in pCAMBIA1301), blotted onto the nylon membrane and probed using DIG labeled PCR amplified product (478 bp) from *PgNHX1* gene (1413 bp) produced by using forward primer (5'-GTC GAT TGC TCT TGC TTA C-3') and reverse primer (5'- TCA CCA AAA ACA TGT CTT CAT CTC-3') according to the standard protocol (Roche Diagnostics Inc). Expression of the transgene was confirmed by RT-PCR. Total RNA was extracted from both shoots and roots of ten-day-old seedlings of transgenic and non-transgenic Binnatoa using TRIzol® reagent (Invitrogen) and cDNA was synthesized from 1 μg total RNA (pre-treated by DNase I) following manufacturer's protocol. The cDNA was used to perform PCR using forward (5'-GTC CAT TGC TCT TGC TTA C-3') and reverse (5'- TCA CCA AAA ACA TGT CTT CAT CTC-3') primers. The PCR reaction was performed at 95°C, 5 min, and followed by 30 cycles of 30 s at 94°C, 40 s at 59°C and 1.5 min at 72°C, then 7 min at 72°C. Expression of the transgene at protein level was confirmed by Western dot-blot analysis according to the standard protocol of WesternBreeze® (Invitrogen™). To do that, polyclonal 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. 1999) synthesized by invitrogen™. This C-terminal sequence is conserved for at least 8 amino acids in the *PgNHX1*. Therefore it was used as antigen for *PgNHX1*. Total protein from both transgenic and non-transgenic plants was equally quantitated (166 ng per dot) by modified Lowry method (Lowry et al. 1951). The raised antibodies were found to cross-react with the *PgNHX1* protein.

Results and Discussion

Electroporation of Agrobacterium tumefaciens: Forty four colonies of *Agrobacterium* were found from the transformation attempt of which five provided faint bands by lysate PCR using the *PgNHX1*-F and *PgNHX1*-R primer pair that amplifies a 1413 bp fragment from *PgNHX1* CDS. Plasmids were isolated from these five clones and tested by PCR using the same primer pair. Clear bands of the correct size could be amplified from plasmids of two clones (Fig. 1).

Agrobacterium mediated transformation of an indica rice cultivar Binnatoa: In each of the five individual experiments 120 calli were infected and a total of forty plants were regenerated. Percent regeneration achieved was greater than 6% (Table 1). The different stages of plant regeneration from transformed calli are shown in Fig. 2. The plants were advanced up to T_2 by selection in 20 mg/l

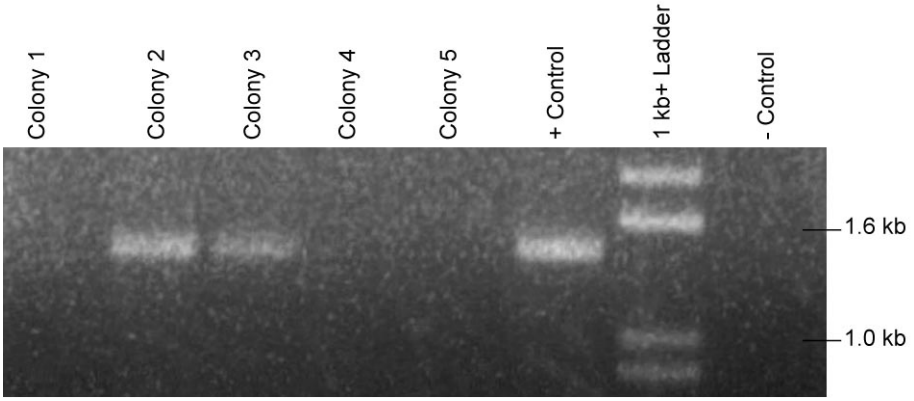


Fig. 1. PCR analysis of CaMV35S- *PgNHX1* transformants.

hygromycin. 8 days-old T_2 seedlings from 10 of the T_0 transformants at were transferred to Yoshida solution (Yoshida et al. 1976) salinized with NaCl at 16 dS/m (Gregorio et al. 1997) out of which progenies from four of the original transformants showed better tolerance than wild type (data not shown). These four T_0 tolerant plants whose progenies were more tolerant than untransformed

Table 1. Results of the transformation of rice embryo calli with CaMV35S-PgNHX1 construct.

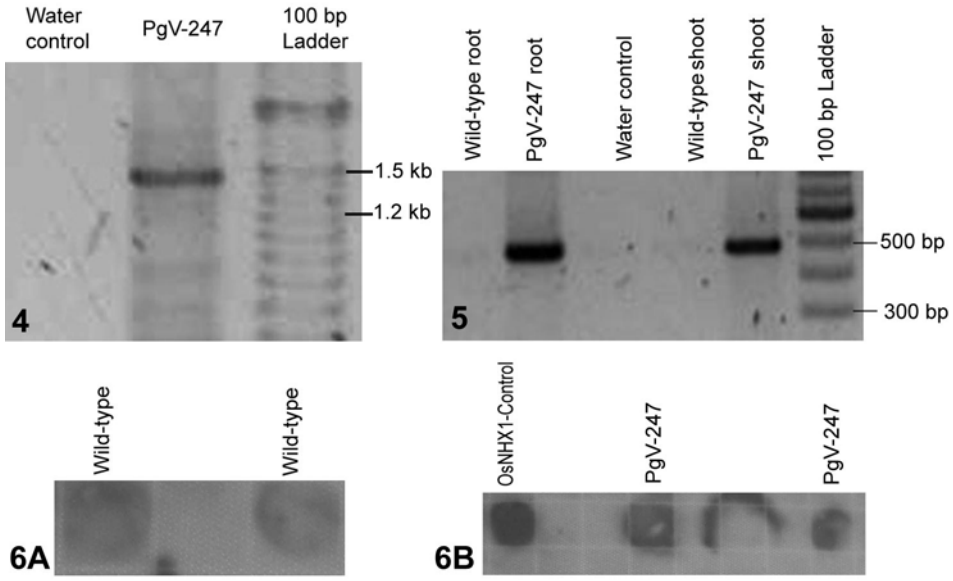
Experiment number	Number of infected calli in each experiment	Number of regenerated plants (T_0)	Total number of T_0 plants whose T_2 progenies were tested for tolerance	Number of T_0 plants whose progenies were tolerant
1		1		
2		21		
3	~120	10	10	4
4		3		
5		5		

controls were named PgV-186, PgV-241, PgV-242 and PgV-247. It was observed that the shoot length of all transformants were 1 - 10% shorter than wild type. PgV-247 was selected for further tests because its progenies performed the best during the screening tests; however it showed the greatest decrease of 10% in shoot length compared to wild type. Performance of T_2 seedlings of PgV-247 when tested in hydroponics was much better than the untransformed controls as shown 16 days after exposure to 16 dS/m salt stress (Fig. 3). In the fig. wild-type

Binnatoa is almost dead, although its shoot length is similar to the transformants, because the latter seedlings were 10% shorter at the start of the experiment. Besides, salt stress at 16 dS/m is considered high for rice seedlings (Gregorio et al. 1997), which resulted in the near death of the untransformed plants 16 days after salt stress application. The untransformed controls registered only 0.5 - 0.7 cm increase in length up to eight days after application of salt stress.

Figs. 2-3: 2. Different stages of plant regeneration from transformed calli: in regeneration media (a), rooting media (b), hydroponic system (c) and in soil (d). 3. Twenty four-days-old PgV-247 showing more vigor and verdant status compared to wild-type Binnatoa.

Molecular analysis of transgenic plant: Transgenic state of the plants was confirmed by PCR. Fig. 4 shows the desired band of 1413 bp produced by PCR amplification of the *PgNHX1* transformed in PgV-247. Southern Hybridization was also performed and single-site integration was confirmed (results not shown). Other transgenic lines also showed the same results. RT-PCR confirmed



Figs. 4-6. 4. PCR amplification of *PgNHX1* transformed in PgV-247. 5. RT-PCR of *PgNHX1* cDNA. Amplified product (478 bp) of *PgNHX1* cDNA is found from shoot and root of PgV-247 which is absent in wild-type shoot and root. 6. Western dot-blot analysis showing faint spots for wild-type Binnatoa (a) and dark spots for *PgNHX1* expressing transgenic Binnatoa (b).

the ectopic expression of the transgene. Fig. 5 shows the result of RT-PCR. In this case 478 bp bands from *PgNHX1* cDNA amplification were found in shoot and root of the transgenic plant. By Western Dot-Blot analysis expression of the gene at protein level was confirmed. Fig. 6 shows the faint spots for non-transgenic wild-type Binnatoa (a) and distinctly dark spots for *PgNHX1* expressing transgenic Binnatoa and (b). Faint spots were found for wild-type Binnatoa because of the expression of the endogenous *OsNHX1* gene, which shares 80% homology with *PgNHX1* at the nucleotide level (Rajagopal et al. 2007). The spots were found to be lighter compared to transgenics expressing the *OsNHX1* gene, because of partial cross-reaction. The authors used the antibodies raised against 16 amino acid residues of the conserved C-terminal *OsNHX1* (Fukuda et al. 1999) for the blots, since it shared 50% homology with *PgNHX1* peptide. In the figures of the results only PgV-247 line is highlighted. Other transgenic lines (PgV-186, PgV-241, PgV-242) also provided similar results.

Expression of the gene under the constitutive promoter CaMV35S shows slightly better tolerance compared to the inducible promoter ABRC reported by Rajagopal et al. 2007. These workers reported seed set at 100 mM NaCl without providing any details of reproductive stage tolerance compared to tolerant or sensitive controls. Detailed studies of the tolerance of the transgenic plants at reproductive stage under the constitutive promoter CaMV35S will be reported elsewhere. The transgenic lines have been advanced to T_3 generation by selfing and will be advanced further with selection to achieve homozygosity of the transgene. These stable plants will be crossed with popular varieties to obtain the transgene in the background of a modern, agronomical superior variety so that high-yielding salt tolerant improved varieties can be provided to farmers of Bangladesh.

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