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Vacuolar Na^+/H^+ Antiporter Expression and Salt Tolerance Conferred by Actin1D and CaMV35S are Similar in Transgenic Binnatoa Rice

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In this study, the performance of *Oryza sativa* vacuolar Na^+/H^+ antiporter (*OsNHX1)* was shown to be similar under the two constitutive promoters Actin1D and CaMV35S. Over-expression of the gene under both promoters was confirmed by semi-quantitative RT-PCR. Results of the phenotypic assessment for the level of salt tolerance at seedling and reproductive stages were not significantly different between the two transgenic rice genotypes. Expression of the antiporter gene with the two different promoters resulted in lower K^+/Na^+ ratios in both the transgenic lines compared to controls. The K^+/Na^+ ratios were compatible with the degree of tolerance shown by the seedlings.

Crop production is severely affected by excessive soil salinity. The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt stressed (Flowers and Yeo 1995). High salinity causes ion imbalance and hyper osmotic stress in plants which leads to perturbation of crucial metabolic reactions inside the cells. To cope with salt stress, plants have developed a variety of adaptation mechanisms. One of them is the compartmentation of Na^+ into vacuoles, which can be achieved by the action of Na^+/H^+ antiporters (*NHX1)* on the vacuolar membranes (Wyn Jones and Pollard 1983, Blumwald et al. 2000).

Rice is one of the most important crops in the world whose production is greatly affected by salinity (Akbar and Ponnamperuma 1980). Therefore, it is of agricultural importance to improve salt tolerance in rice. In an effort to improve salt tolerance in rice, a Bangladeshi rice variety Binnatoa was transformed with the Na^+/H^+ antiporter gene, *OsNHX1*, isolated from Nipponbare rice cDNA under the constitutive promoter CaMV35S (Rasul 2005). Recently, the same gene has been reported to be transformed and expressed under the rice promoter Actin1D in Binnatoa (Islam et al. 2009). The rice Actin1D promoter is a strong

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constitutive promoter and shows very efficient expression in monocots particularly in rice (McElroy et al. 1990). In this report, expression of the *OsNHX1* gene under the Actin1D and CaMV35S promoters was compared and the degree of salt tolerance provided was evaluated.

In previous studies, transformation and enhanced expression of the 1.9 kb *OsNHX1* gene under the two different promoters was done and later the transgenic lines were advanced from T_0 to T_4 generation by using hygromycin selection at 20 mg/l (Rasul 2005, Islam et al. 2009). In this study, two different transgenic rice cv. Binnatoa lines, CaMV35S-*OsNHX1*-70 and Actin1D- *OsNHX1*-143, were used.

Total RNA was extracted from the shoot of ten-day-old seedlings of transgenic and nontransgenic Binnatoa according to the TRIzol® Reagent (Invitrogen™) 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 specific primers: 5′-GCT GGA TTG CTC AGT GCA TA-3′ (Forward) and 5′-AAG GCT CAG AGG TGA CAG GA-3′ (Reverse).

To compare the performance of the two transgenic lines salinity tolerance assessment was done at seedling and reproductive stages. At first T_4 seeds from T_3 plants CaMV35S-*OsNHX1*-70 and Actin1D- *OsNHX1*-143 were germinated in hygromycin (20 mg/l) and then ten-day-old seedlings were transferred to hydroponics containing Yoshida solution (Yoshida et al. 1976). After four days 80 NaCl mM stress was provided to the hydroponics and the stress was gradually increased to 160 NaCl mM within the next eight days. A parallel control was maintained where there was no stress. After several days, when the non-transgenic Binnatoa was nearly dead, values of growth related parameters were estimated from both stress and control plants.

After the completion of tolerance assessment at seedling stage, extra transgenic and non-transgenic plants from control system were transferred to pot containing soil for tolerance assessment at reproductive stage. The pot was kept in large bowl completely submerged with 60 mM NaCl water. This lower stress level ensured the proper comparison in yield parameters between the wild-type and transgenic lines. When the plants completed their reproductive cycle, yield data was collected. A control system without salt stress was always maintained. Na^+ and K^+ content of the dry leaves were measured using Flame Photometer 410 (Sherwood, UK) and K^+/Na^+ ratio was analyzed according to the procedure described in Islam et al. 2009.

Following semi-quantitative RT-PCR, both of the transgenic plants exhibited *OsNHX1* specific precise bands of 679 bp (Fig. 1). Wild-type plants produced a faint band confirming the amplification of the endogenous expression. After the

completion of salinity tolerance assessment at 160 mM NaCl, transgenic seedlings of both types showed better physiological status compared to the wild-type. There was no significant difference in growth parameters between the transgenic and wild-type plants in stress free control (Table 1). However, transgenic varieties showed significantly better performance in two parameters (fresh and dry weight) compared to the wild-type. But there was no significant difference between the two transgenic lines containing the two different promoters, except for the dry weight where CaMV35S-70 showed significantly higher value compared to Actin-143, which demonstrates the similar performance of the two promoters CaMV35S and Actin1D to drive the same gene *OsNHX1* for imparting salt tolerance in rice.

Salinity tolerance assessment at reproductive stage provided data of yield performance of transgenic varieties over the wild-type. At stress free control, no significant difference in yield parameters was found between the transgenic varieties and the wild-type (Table 2). But in stress, both the transgenic lines provided significantly higher value in the different yield parameters compared to the wild-type. Interestingly, no significant difference in major yield parameters (spikelet fertility rate, yield per plant and 100-grain weight) between

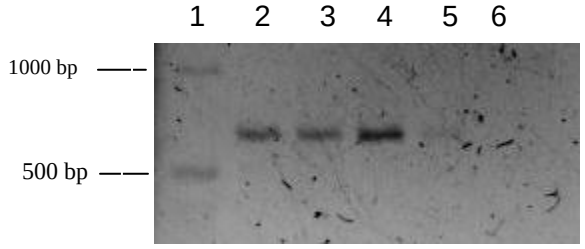

Fig. 1. Semi-quantitative RT-PCR (25 cycles) provides clear bands (679 bp) for transgenic lines (T_4 generation) compared to the wild-type. No distinguishable difference in band intensity is found between the transgenic lines with the promoter CaMV35S (lane 2) and Actin1D (lane 3). Faint band for the wild-type (lane 5) is attributed to the endogenous expression. Lane 1, 4 and 6 refer to the 1 kb bp ladder, positive control and water control, respectively.

the two different transgenic plants was found. This is a clear indication of the similar performance of Actin1D promoter like that of CaMV35S in driving *OsNHX1* to provide salt tolerance in rice. After the analysis of K^+-Na^+ content, both of the transgenic lines showed significantly higher values of K^+/Na^+ ratio compared to the wild-type in stress free control (Fig. 2). But in stress, this ratio was significantly reduced in transgenic lines compared to the non-transgenic control. This is a strong indication of induced ion homeostasis in the transgenic plants (Gao Ji-Ping et al. 2007). There was no significant difference in K^+/Na^+ ratios between the two transgenic lines in both control and stress.

Table 1. Different growth parameters showing the physiological status of wild-type and transgenic seedlings in control and 160 mM stress.

		Leaf number	Leaf length (cm)	Leaf width (cm)	Root number	Root length (cm)	Fresh weight (gm)	Dry weight (gm)
Wild-type	Control	7.00 ± 0.45	49.00 ± 1.92	0.78 ± 0.04	30.00 ± 1.05	10.80 ± 0.86	1.46 ± 0.15	0.58 ± 0.06
Binnatoa	Stress	6.00 ± 0.32	48.58 ± 2.53	0.48 ± 0.04	25.40 ± 1.34	10.14 ± 0.86	1.08 ± 0.05	0.31 ± 0.04
Transgenic variety with promoter CaMV-70	Control	7.20 ± 0.20	48.00 ± 2.16	0.78 ± 0.04	29.40 ± 1.86	11.00 ± 0.59	1.96 ± 0.12	0.64 ± 0.02
	Stress	6.00 ± 0.45	49.40 ± 2.92	0.52 ± 0.05	25.40 ± 2.46	11.00 ± 0.59	1.48 ± 0.05[a]	0.51 ± 0.06[ab]
Transgenic variety with promoter Actin-143	Control	6.80 ± 0.49	47.40 ± 0.87	0.70 ± 0.03	29.20 ± 0.97	10.30 ± 0.54	1.86 ± 0.11	0.60 ± 0.03
	Stress	6.40 ± 0.40	48.88 ± 1.61	0.52 ± 0.04	26.00 ± 1.61	9.30 ± 0.56	1.34 ± 0.06[a]	0.41 ± 0.04[a]

(Values are the mean ± SE, n = 5). [a] indicates the significant difference between the individual transgenic variety and wild-type at $p \leq 0.05$.
[b] indicates the significant difference between the two transgenic varieties at $p \leq 0.05$.

Table 2. Complete yield data of wild-type and transgenic plants in control and 60 mM stress.

		Tiller number	Panicle number	Panicle length (cm)	Spikelet number	Spikelet fertility rate (%)	Yield (gm/plant)	100-grain wt. (gm)
Wild-type	Control	5.33 ± 0.33	6.67 ± 0.33	20.17 ± 0.44	297.00 ± 10.02	82.44 ± 2.54	4.61 ± 0.28	1.51 ± 0.10
Binnatoa	Stress	3.66 ± 0.33	3.00 ± 0.57	14.83 ± 1.48	156.67 ± 8.58	61.40 ± 0.97	1.42 ± 0.06	0.91 ± 0.07
Transgenic variety with promoter CaMV-70	Control	5.33 ± 0.67	7.67 ± 0.33	20.67 ± 0.67	374.67 ± 35.24	86.02 ± 1.43	4.71 ± 0.35	1.68 ± 0.06
	Stress	4.33 ± 0.33	4.33 ± 0.67	17.83 ± 0.33[a]	226.00± 8.51[ab]	77.21 ± 2.36[a]	2.76 ± 0.04[a]	1.28 ± 0.08[a]
Transgenic variety with promoter Actin-143	Control	6.33 ± 0.88	7.00 ± 0.58	19.67 ± 0.88	291.67 ± 17.02	83.38 ± 4.16	4.98 ± 0.36	1.63 ± 0.12
	Stress	5.66 ± 0.33[a]	6.33 ± 0.33[a]	18.67 ± 0.73[a]	188.00 ± 32.66[a]	75.45 ± 1.34[a]	2.79 ± 0.03[a]	1.34 ± 0.05[a]

(Values are the mean ± SE, n = 3). [a] indicates the significant difference between the individual transgenic variety and wild-type at $p \leq 0.05$.
[b] indicates the significant difference between the two transgenic varieties at $p \leq 0.05$.

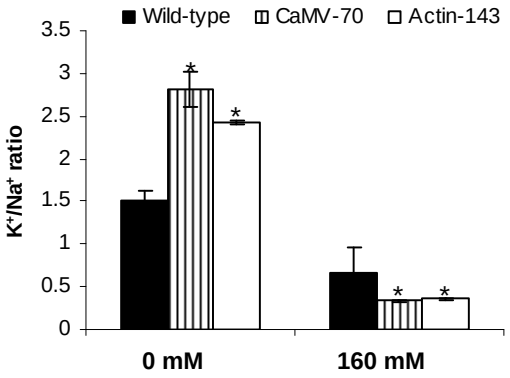

Fig. 2. K^+/Na^+ ratio at seedling stage shows no significant difference between the two transgenic lines. Each bar represents the mean ± SE (n = 3). *indicates the significant difference between transgenic lines and wild-type at the probability of $p \leq 0.05$.

CaMV35S is a universally popular constitutive promoter which is used in many cases for gene expression (Jones et al. 2008). In previous studies it was reported that *Oryza sativa* vacuolar Na^+/H^+ antiporter can play an important role in salt tolerance of rice under CaMV35S (Fukuda et al. 2004) and Actin1D (Islam et al. 2009) promoters. However, in those studies no comparable analysis between the two promoters was done. This study shows that the performance of Actin1D and CaMV35S promoter are similar in rice.

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