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RESEARCH ARTICLE

Screening of T_3 generation *NHX1* transgenic rice lines for salt tolerance

■ **Rajashree Biradar, M. Chandra Naik and V. R. Sashidhar**

SUMMARY

Rice is the most salt sensitive crop among cereals. Salinity is one of the major a biotic stress threatening the agricultural productivity worldwide. Salinity reduces 80 per cent of rice growth. To cope up with salinity stress, plants have adapted certain mechanisms to maintain growth and productivity. If salt tolerance is enhanced in rice, that reduction in growth and yield is only 30-40 per cent instead of 80 per cent, it would greatly benefit rice productivity in saline areas. Such salt tolerant rice transgenic plants were developed by transferring *NHX1* gene (Sodiun proton antiporter). In the present study screening of T_3 transgenic rice lines was done. In plants *pgNHX1* gene catalyzes compartmentation of Na^+ into vacuoles for maintenance of a low Na^+ concentration in cytosol. Screening was done by stringent salt screening test at seed level and leaf senescence bioassay at plant level. At seed level, root and shoot growth was used as selection criterion. In transgenic plants shown root and shoot length of 24.1±3.3 and 19.7±32, respectively and control shown 4.0±1.8 and 6.0±0.8, respectively. At plant level, extent of chlorosis was used as selection criteria. Some of the transgenics showed significantly lesser chlorotic symptoms compared to wild type.

Key Words : Leaf senescence bioasay, *NHX1*, SSST

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Saline soil is one of the major problem in the world because it decreases agricultural productivity. This saline area increasing day by day. Rice is an important cereal crop of India. However, its productivity is limited by salinity because it is most salt sensitive crop among cereals. At the concentration of 200 mM NaCl treatment to the rice inhibits growth by 80 per cent. Therefore, if salt tolerance is enhanced in rice through genetic engineering, so that the reduction in growth and yield is only 30-40 per cent instead of 80 per cent, it would greatly benefit rice productivity in saline areas.

The adverse effect of NaCl salinity and its amelioration by $CaCl_2$ during early seedlings growth of two groundnut cultivars (cv. TPT-2 and cv. TCGS-29).

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NaCl treatment caused decrease in the levels of lipoxygenase (LOX) activity when compared to the seedlings treated with either $CaCl_2$ or its combination with NaCl. LOX showed a single peak with an UV absorption maximum at 235 nm on HPLC. Three major polypeptides (66 kD, 47 kD and 18.4 kD) appeared predominantly in control and treated seedlings of cotyledons in both varieties but they were absent in the embryonic axis. Cv. TPT-2 is stress tolerant than cv. TCG-29.30 mM and 15mM Ca^{2+} treatment alleviated the stress effect in cv, TPT-2 and TCGS-29, respectively (Basha *et al*., 2010).

Selection for salinity tolerance genotypes of rice based on phenotypic performance alone is less reliable and will delay in progress in breeding. Recent advent of molecular markers, microsatellites or simple sequence repeats (SSRs) were used to find out salt tolerant rice genotypes. the mapping and marker-assisted selection for salt tolerance genes in rice have been conducted. Evaluation of genetic diversity among 19 rice genotypes, representing highly tolerant as well as susceptible rice cultivars using SSR markers. Among 39 SSR markers used, 26 SSR marker loci generated polymorphic patterns and a total of 185 alleles were detected. From these 26 SSR markers, 16 SSR markers are located on the Saltol region on chromosome 1 of rice. The number of alleles per locus ranged from 3-11 with a mean of 7.1 alleles per locus. The PIC values for 26 SSR markers varied from 0.50 (RM6737) to 0.89 (RM3412) with an average PIC of 6.7. Hence, from the present study, it can be proved that SSR markers can detect high polymorphism and are very useful in studying variation among different genotypes (Davla *et al*., 2013).

Mechanisms of salt tolerance and genes responsible for that mechanism:) Exclusion: Restricting Na^+ entry into the root cells Ex: *HKT1* (High affinity K^+ transporter) and *HAT1*) Extrusion: Pumping of Na^+ from root cells into soils Ex: *SOS1* (Salt overly sensitive) Compartmentation: The vacuolar sodium sequestration is mediated by the Na^+/H^+ antiporter at the tonoplast using the proton motive force (Xue *et al*., 2004; Hasegawa *et al*., 2000 and Yamaguchi and Blumwald, 2005) Ex: *AtNHX1*(Sodium proton antiporter).

Characteristics of vacuolar transporter gene *NHX1*:

Plant *NHX1* exchangers are proteins of about 550 residues in length and have the typical transporter topology of 10-12 transmembrane domains with a hydrophilic C-terminal tail that is thought to be cytosolic. Class-I isoforms are vacuolar antiporters and class-II isoforms are endosomal antiporters. Class-I and class-II isoforms have 21-23 per cent similarity.

In a previous study conducted in Dept. of Crop Physiology UAS, Bangaluru the *PgNHX1* was over-expressed in rice by inplanta transformation analysis. 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 by using root and shoot length as selection criteria at seedling level and chlorosis symtom as a selection criteria at plant level. Gene integration was confirmed by PCR and RT-PCR. These results clearly demonstrate that *pgNHX1* transgenic rice plants over expressing *PgNHX1* gene, 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 (Sushma *et al.*, 2012).

With this background, the present investigation is necessary to screen the T_3 generation transgenic lines whether the tolerance capacity of transgenic plants continued to next generation or not. To screen the transformed transgenic the research was carried out with the following objectives: Screening of T_3 *NHX1* over expressed transgenic lines using screening of tolerant seeds by stringent salt screening test (SSST) and leaf senescence bioassay.

This paper deals with advancement study of T_3 transgenic rice plants to carry forward from previous study (Sushma *et al*., 2012) and select salt tolerant lines.

MATERIAL AND METHODS

Screening of tolerant seeds by stringent salt screening test (SSST):

SSST was a salt screening test at seed level standardized by Sushma *et al*. (2012). Bold and healthy seeds were selected and treated with Bavistine (0.1 %) for 10 min and washed with distilled water 3 to 4 times. They were then soaked in distilled water overnight and kept in Petri plates for germination. After 3 days these seedlings were transferred to Petri plates containing 350 mM NaCl. After 12 day, seedlings were transferved to

Petri plates containing filter paper rinsed with water to recover (4 days). Screening was done by selection of seedlings based on root and shoot length (at least 10 % more than wild type). Selected seedling were transferred to green house.

Screening of tolerant plants by leaf senescence assay:

The leaf senescence assay is a useful method to test the salt tolerance of plants and is a reflection of the ability of the plants to retain chlorophyll under salt stress.

Leaf samples are collected in deionized water then subjected to salt treatment. The healthy and fully expanded youngest leaves from wild type and transgenic plants (45 days old) were washed in deionized water and 1 cm diameter leaf discs were finely cut and kept on 0.6 % agar + $^1/_4$ strength Hoagland's solution + 350 mM NaCl for 4 days. Based on the retention of greenness, transgenc lines were selected.

RESULTS AND DISCUSSION

The results are presented in Fig. 1a and 1b, around

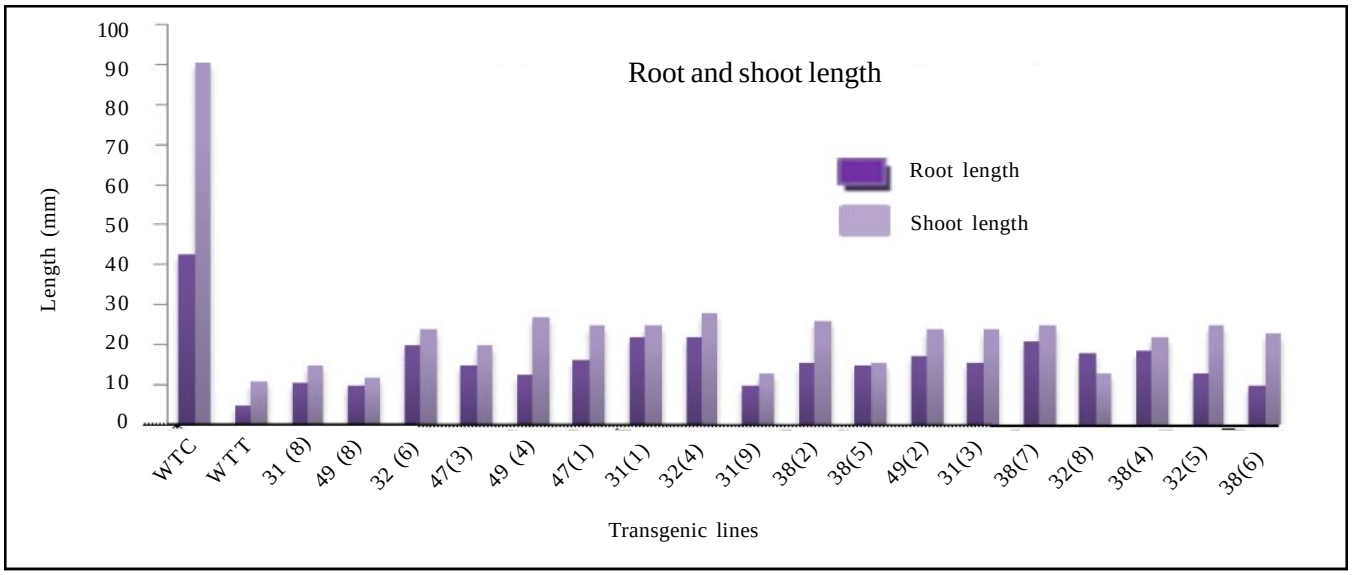

Fig. 1a : Growth difference between wild type control, transgenic lines and wild type treated

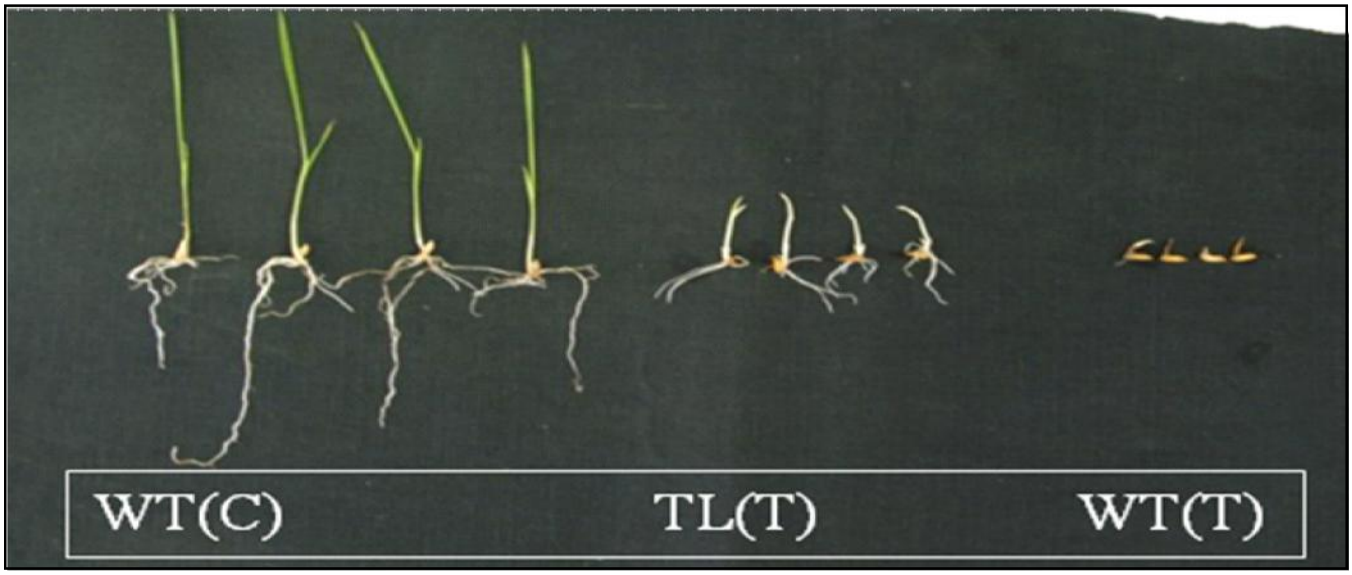

Fig. 1b : Root and shoot length of the seedlings. WT(C): Wild type seedling without treatment, TL(T): Transgenic line treated, WT(T) : Wild type seedling with 350mM NaCl

500 transgenic T_3 seeds were pre-germinated and treated at 350 mM NaCl concentrations in 0.6 per cent agar media for 12 days along with wild type seeds in each plate. Untreated wild type seeds were also maintained for comparison of growth reduction (Control). The growth of both wild type as well as transgenics was inhibited in agar media contained 350 mM NaCl. However, the inhibition of growth was more in wild type seedlings compared to T_3 transgenics. After salt treatment, seedlings were put for recovery in water. Four days after recovery, around 120 seedlings of the T_3 transgenic lines had recovered (24 %), whereas only 1.71 per cent of wild type seedlings were recovered. Besides some of the transgenics showed significantly higher root and shoot growth compared to wild type (Fig 1a and b). Around 120 T_3 seedlings showed at least 10 per cent higher root and shoot length than wild type (Table 1). Therefore, out of 500 T_3 seedlings, 120 seedlings were selected from stringent salt screening test (seed level/primary screening) and put into cups filled with soilrite for further analysis (Table 2).

Seedlings of T_3 which had passed the stringent salt tolerance test were put for recovery in the green house (Fig. 2). These plants that were selected by SSST were subjected to a second level of screening at plant level.

The leaf senescence assay is a useful method to test the salt tolerance of plants and is a reflection of the ability of the plants to retain chlorophyll under salt stress. In this bioassay the leaf bits were kept on 0.6 per cent agar media along with $^1/_4$ strength Hoagland's solution and 350 mM NaCl for 4 days. After four days based on the retention of greenness, lines were selected. In plate 3 WTC showed more greenness compared to treated plates. In treated plates transgenic lines showed more greenness compared to WTT.

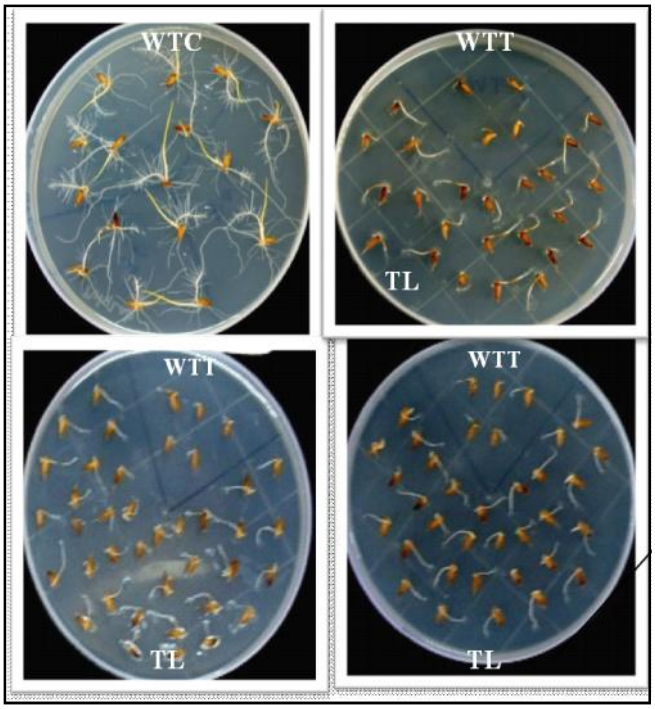

Fig. 2 : Imposition of stringent salt screening test (SSST) on T_3 seeds

Table 1: Root and Shoot length measurements (SSST) and severity of leaf symptoms (Extent of chlorosis) caused by NaCl treatment (Leaf Senescence assay)

Transgenic line	Root length	Shoot length	LSB
ABC	43	90	T
Wild type treated	4.0±1.8	6.0±0.8	S
Transgenic	24.1±3.3**	19.7±3.2**	T to MT

** indicate significance of value at P=0.01

Table 2: Recovery and selection percentage of T_3 seeds after stringent salt screening test

	Total No. of seeds used for salt screening	No. of seedlings recovered	% of recovery	No. of selected seedlings with higher root and shoot length	% of selection
Transgenic line	500	120	24%	95	4.5
Wild type	350	6	1.71	-	-

Table 3: Salt tolerance in transgenic rice plants expressing *NHX1* involved in ion sequestration

Gene	Source	Cellular roles	Target	Reference
AgNHX1	Atriplex gmelini	Na^+ vacuolar sequestration	Rice	Ohta *et at.,* 2002
PgNHX1	Pennisitum gluaccun	Na^+ vacuolar sequestration	Rice	Verma *et al.,* 2007
OsNHX1	*Oryza sativa*	Na^+ vacuolar sequestration	Rice	Fukuda *et al.,* 2004

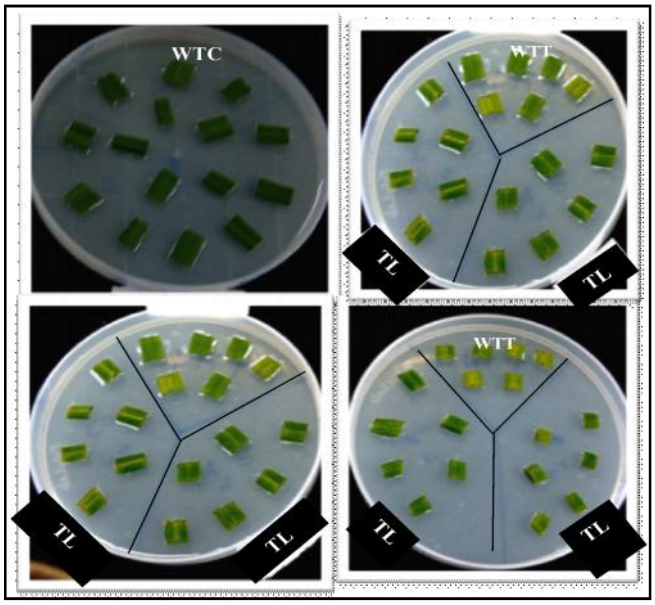

Fig. 3 : Leaf senescence bioassay

95 plants were found to be tolerant based on the leaf senescence assay *i.e.*, these plants remained green as compared to salt stress wild type which had turned yellow (Fig. 3).

The tonoplast *NHX1* Na^+/H^+ exchanger catalyzes the exchange of Na^+ for H^+ across the vacuolar membranes. It regulates internal pH, cell volume and sodium level in the cytoplasm and maintains the intracellular ion homeostasis. Transgenic Arabidopsis and tomato plants over expressing *AtNHX1* accumulated abundant quantities of the transporter in the tonoplast and exhibited substantially enhanced salt tolerance (Apse *et al*., 1999; Quintero *et al*., 2002 and Zhang and Blumwald, 2001). Similar result was also achieved in transgenic rice expressing *AgNHX1* gene (Ohta *et al*., 2002). These results implicate the pivotal function of the *NHX* family in vacuolar compartmentalization of Na^{2+}.

Genes encoding vacuole-type Na^+/H^+ antiporters have been isolated from number of plant species, including glycophytic species Arabidopsis thaliana (Apse *et al*., 1999 and 2003 and Gaxiola *et al*., 1999), *Oryza sativa* (Fukuda *et al.*, 2004), *Triticum aestivum* and *Zea mays* (Zorb *et al.*, 2005), halophytic species *Atriplex gmelini* (Hamada *et al.*, 2001), *Mesembryanthemum crystallinum*, *Atriplex dimorphostegia* and *Suaeda salsa* (Ma *et al*., 2004). The difference in salt tolerance among plant species could result from differences in regulatory circuits or from gene alleles coding for key salt tolerance genes.

In a recent review, Yamaguchi and Blumwald (2005) have emphasized the need for routine stress tolerance screens to obtain potential salt-tolerant lines among the putative T_3 transformants. This procedure has been generally followed in many studies including those dealing with overexpression of genes for ion homeostasis (Chen *et al.*, 2007).

In this study, a stringent salt screening test similar to Zhao *et al.* (2006) approach was followed and root and shoot growth of T_3 putative transformants was used as a selection criterion. Only those seedlings which had recovered and had a significantly higher root and shoot length (more than 10 % compared to wild type plants) were selected and transferred to green house.

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