Ectopic expression of *Vigna radiata*'s vacuolar Na$^+$/H$^+$ antiporter gene (*VrNHX1*) in indica rice (*Oryza sativa* L.)

Md. Nazmul Hasan [a], Fahmid H Bhuiyan [b], Hammadul Hoque [a], Nurnabi Azad Jewel [a], Md Ashrafuzzaman [a], Shamsul H. Prodhan [a],[*]

[a] *Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh*
[b] *Plant Biotechnology Division, National Institute of Biotechnology, Ganakbari, Ashulia, Savar, Dhaka-1349, Bangladesh*

ARTICLE INFO

ABSTRACT

It is essential to develop high salt-tolerant rice varieties in order to cultivate the salt-affected lands. In this study, Na$^+$/H$^+$ exchanger 1 (NHX1) gene isolated from *Vigna radiata* L. Wilczek was transferred in Bangladesh Rice Research Institute (BRRI) developed two indica rice genotypes BRRI Dhan28 and BRRI Dhan29 using in-planta approach for improvement of salinity tolerance. Embryonic axes of matured dehusked rice seeds were injured and co-cultivated with *Agrobacterium* strain harboring *VrNHX1* gene and finally regenerated. GUS histochemical assay and PCR amplification of GUS-a and *VrNHX1* were performed to confirm the transformation. Expression confirmation was done by semi-quantitative RT-PCR. Under salinity stress, transgenic lines showed higher chlorophyll, relative water content and decreased electrolyte leakage, proline content, lipid peroxidation level, and catalase enzyme activity which represent the better physiology than control plants. Moreover, under salinity stress (150 mM), transgenic lines exhibited superior growth and salt tolerant than non-transgenic plants.

Abbreviations

NHX1	Na$^+$/H$^+$ exchanger 1
BRRI	Bangladesh Rice Research Institute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
VrNHX1	*Vigna radiata* L. Wilczek Na$^+$/H$^+$ exchanger 1
GUS	β-glucuronidase
WT	Wild Type
ROS	Reactive Oxygen Species
LB	Luria Broth
CTAB	Cetyltrimethylammonium Bromide
RWC	Relative Water Content
CAT	Antioxidant Enzymes Catalase

1. Introduction

Rice is the world's second biggest grain crop, trailing only maize, and provides nourishment for people all around the world. Asia produces and consumes around 90.5 percent of the world's rice (FAOSTAT, 2017). Furthermore, food is the most important supply for about 2.4 billion people in Asia, with the exception of Pakistan and certain regions of India and China, supplying two-thirds of calories for the majority of Asian people, mostly through rice-based meals [1]. The world's population is quickly increasing, and important challenges for global agriculture include producing 87 percent more food by 2050 for an additional 2.3 billion people [2]. Abiotic stresses, which comprise salt, drought, submersion, heat, and cold, are the major threat to agricultural production and cause significant yield losses across vast areas [3]. The increased demand for food crops is limited by saltiness. Salinity stress affects more than 20% of agricultural lands globally (about 900×10^6 hectares), and the number is growing by the day [4]. Salinity stress has a deleterious impact on plant development and growth. When a plant is stressed by salinity, it goes through osmotic stress, which affects its growth [5]. Ion toxicity happens when the salt levels are so high that ion homeostasis and growth can't keep going. This causes secondary stresses like membrane damage, nutrient imbalances, problems with reactive oxygen detoxification (ROS), differences in antioxidant enzymes, and reduced stomatal aperture [6]. Salinity stress makes it difficult for seeds to germinate, roots to grow, plants to grow tall, and fruits to develop. All of these factors contribute to a significant decrease in rice yield [6–8]. This occurrence has triggered research on salt tolerance to improve rice, the world's largest crop. If there is a lot of salt in the environment, high

levels of Na$^+$ create a wide electrochemical gradient that makes it easier for Na$^+$ to move passively into the cell through K$^+$ transporters, leading to high levels of Na$^+$ in the cytosol [9]. When a plant is stressed by salinity, a lot of Na$^+$ enters the cell. This increases the concentration of Na$^+$ within the cell, impairing enzyme activation and protein synthesis [10]. Na$^+$ penetrates leaf cells and is pushed into the cell vacuole before it reaches hazardous levels of enzymatic activity. Vacuolar Na$^+$/H$^+$ antiporters regulate this pumping action [11]. During salinity stress, Na$^+$/H$^+$ activity increases even though it increases more in salt-tolerant species than in salt-sensitive ones [12]. Recent advances in genetic engineering and biotechnology have enabled researchers to develop stress-resistant plant varieties by incorporating external genes into sensitive plants. Scientists have overexpressed the vacuolar transporter (NHX1) and increased salinity tolerance in *Arabidopsis* [13], tobacco [14, 15], rice [16] brassica [17], tomato [18–20] and wheat [21]. Overexpression of the *AtNHX1* (vacuolar Na$^+$/H$^+$ antiport from *Arabidopsis thaliana*) gene in *Arabidopsis* plants increases growth and development in soil with up to 200 mM NaCl. This salt tolerance was connected to higher-than-normal levels of *AtNHX1* gene activity, indicating that salt tolerance in plants may be engineered [13]. Overexpression of the *AtNHX1* gene in tobacco plants resulted in better seed germination rates and good seedlings in the presence of hazardous NaCl concentrations (200 mM NaCl) [14]. Rice transformed with the *OsNHX1* gene (vacuolar Na$^+$/H$^+$ antiport from *Oryza sativa*) was capable of withstanding mild NaCl stress and greatly reduced stress-induced injury by evoking advances in root growth, water uptake, and relative water content, and it had no negative impact on plant growth and development, demonstrating that *OsNHX1* has immense potential for developing salinity tolerance in rice [16]. Also, overexpressing the AtNHX1 gene in brassica and wheat and the TaNHX2 gene from wheat in tomato led to better growth and tolerance to salinity [17–21].

Mungbean (*Vigna radiata* L. Wilczek) is a commonly cultivated legume in South and Southeast Asia, China, Canada, and Australia, and has already been described as a moderately stress-resistant plant [22]. It was observed that most mungbean cultivars can withstand salt concentrations ranging from 9 to 18 dS/m. 42 mungbean and black gram cultivars were examined in 1/5 Hoagland nutrient solution at five salinity levels (3–18 dS/m) in an experiment where some cultivars, such as S 72, H 45, No. 525, Madira, and RS-4, were discovered to be extremely salinity tolerant. Mungbean callus grown in sand culture with Hoagland's nutrient solution and 0–350 mol/m^3 NaCl added showed the same salt tolerance as the whole plant. This suggests that mungbean can handle salinity at cellular level [23]. It was reported that the *Vigna radiata* L. Wilczek Na$^+$/H$^+$ antiporter (*VrNHX1*) participates in the homeostasis of cell ions [24]. Constitutive expression of the mungbean vacuolar Na$^+$/H$^+$ antiporter gene (*VrNHX1*) in *Arabidopsis thaliana* led to improved salt tolerance. Ectopic expression of the *VrNHX1* gene in cowpea increased salt resistance and growth and development under 200 mM NaCl stress [24]. These findings indicate that the *VrNHX1* gene has a high potential for enhancing plant salinity tolerance. BRRI Dhan28 and BRRI Dhan29 are the most popular high-yielding varieties in Bangladesh. However, these two varieties are susceptible to salinity stress, and productivity suffers greatly due to salinity stress. Even though salinity-affected areas have salinity levels higher than 4 dS/m, these varieties can handle salinity stress below 4 dS/m [25]. Overexpression of the *Vigna radiata* L. Wilczek Na$^+$/H$^+$ antiporter (*VrNHX1*) in BRRI Dhan28 and BRRI Dhan29 can improve salt tolerance, which may aid in the development of salinity-tolerant rice varieties for salinity-affected areas. *Agrobacterium*-mediated genetic transformation in plants is the most common method because it is easy to use, transfers DNA fragments with clear ends and little rearrangement, is effective, cheap, can transfer large DNA fragments, and only adds small numbers of copies of genes to plant chromosomes. Because of this, *Agrobacterium*-mediated DNA transformation has become a popular way to change many crop species [26]. In this study, *Agrobacterium*-mediated transformation of the Na$^+$/H$^+$ exchanger 1 (NHX1) gene isolated from *Vigna radiata* L. Wilczek

was transferred to two rice varieties, BRRI Dhan28 and BRRI Dhan29, using an *in-planta* approach (a technique for transforming complete plants or plant tissues without the use of callus culture) to assess tolerance to salinity.

2. Materials and methods

2.1. Plant materials and explant preparation

Most cultivated high-yielding rice varieties (BRRI Dhan28 and BRRI Dhan29) were collected from the Bangladesh Rice Research Institute (BRRI). Healthy mature rice seeds were initially washed 3/4 times with distilled water and dehusked. The dehusked rice seed was sterilized in 0.5 percent (w/v) NaOCl (VWR, USA) for 1 minute before being washed three times with distilled water. The seeds were then sterilized for three minutes and washed 3/4 times with distilled water using 0.1 percent mercury chloride (W/V). The seeds were then rinsed in distilled water with 2/3 drops of TWEEN 20 (JHD, Vietnam) for 2 min and finally, washed 4/5 times with sterilized distilled water, and dried on sterilized filter paper [27].

2.2. Vector and Agrobacterium strain preparation

The binary vector pCAMBIA2301 harboring the *VrNHX1* gene (Vacuolar Na$^+$/H$^+$ exchanger 1 gene isolated from the important legume *Vigna radiata* L.) and *Agrobacterium* strain EHA105 were collected from the Prof. Lingaraj Sahoo, Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India. The T-DNA of pCAMBIA2301 includes the CaMV 35S promoter-driven neomycin phosphotransferase gene (NPTII) and β-glucuronidase gene (GUS-a), respectively. The strain of *Agrobacterium* was transformed using the heat shock technique with the binary vector [28]. The transformed *Agrobacterium* strains were selected in solid LB medium supplemented with 20 mg/L of rifampicin (Sigma-Aldrich, Germany) and 50 mg/L of kanamycin (Sigma-Aldrich, Germany).

2.3. Explant infection, co-cultivation, and regeneration

Sterilized explants were immersed overnight at 35±1°C temperature in distilled water and, afterward, seed embryos were injured by utilizing the sharp tip of the needle. A single bacteria colony was inoculated in 100 ml of liquid LB medium supplemented with 20 mg/L rifampin and 50 mg/L kanamycin, which was cultured overnight at 28°C. The bacterial strains were recovered by centrifugation at 5000 rpm for five minutes and pellets were resuspended in liquid N6 medium (Duchefa, Netherlands) [29] fortified with 100 μM acetosyringone (Sigma-Aldrich, Germany). Injured explants were kept on sterilized filter paper fixed in Petri dishes and moistened with bacterial suspension culture. The infected explants were finally co-cultivated for 3 days at 35±1°C. The explants were washed with 500 mg/L cefotaxime after three days of co-cultivation and transferred into a hydroponics system containing Yoshida solution [30] supplemented with 150 mg/L kanamycin for regeneration. The regenerated putative transgenic rice seedlings were moved to soil-containing pots (supplemented with 50% compost) and grown in the greenhouse.

2.4. β-glucuronidase (GUS) assay

The reporter gene expression was constructed through the GUS histochemical assay [31]. Leaf samples from 1-month old kanamycin-resistant T$_0$ plants grown in regeneration media were employed for the GUS histochemical assay. The 0.5 cm leaf cut samples were inoculated in a 5-bromo-4-chloro-indoyl-D glucuronide (X-glucuronide) GUS substrate solution and kept in the dark at 37°C for 24 hours. After treatment, the leaf tissues were washed with 70% ethanol several times and observed under the microscope.

2.5. PCR analysis

The genomic DNA was extracted from control and putative transgenic rice seedlings following the CTAB (Cetyltrimethylammonium Bromide) method [32]. The PCR identification of the GUS-a and *VrNHX1* genes was used to confirm the transformation. Gene-specific primers for *GUS-a* (F: 5´-CCCGTGAAATCAAAAAACTC-3´ and R: 5´-TTTTCACC-GAAGTTCATGC-3´) and *VrNHX1* (F: 5´-CTCCGTTGTCTCCAT-GAACTTA-3´ and R: 5´-CAGTGAGGATGCCACTTAGATAG-3´) were used to detect a 1762bp fragment of the *GUS-a* gene and a 773bp fragment of the *VrNHX1* gene. For the GUS-a gene, PCR conditions were initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95° for 30 seconds, annealing at 56°C for 1 minute, extension at 72°C for 1.50 minutes, and final extension at 72°C for 10 minutes [24]. For the *VrNHX1* gene, PCR conditions were initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95° for 30 seconds, annealing at 62°C for 45 seconds, elongation at 72°C for 1 minute, and final extension at 72°C for 10 minutes. Using a 1.5% agarose gel, electrophoresis was conducted to detect the PCR products.

2.6. RNA extraction, cDNA synthesis, and semiquantitative RT-PCR

Following the manufacturer's instructions, total RNA was extracted from the T_1 and T_2 transgenic rice lines using TRIzol™ reagent (Invitrogen, USA). Before cDNA synthesis, extracted RNA samples were processed with RNase-free DNase I (Invitrogen) to eliminate genomic DNA. GoScript™ Reverse Transcription System (Promega, USA) was used for cDNA synthesis following the supplier's guidelines. Primer3-Plus tool was used to design specific primers for the *VrNHX1* (forward primer 5´-CTTTGACCTCGACCACATAGAC-3´ and reverse primer 5´-GTAGACCTGTCAACACTCCAAG-3´) and housekeeping rice *eEF-1α* (forward primer 5'-TTTCACTCTTGGTGTGAAGCAGAT-3' and reverse primer 5'-GACTTCCTTCACGATTTCATCGTAA-3') genes based on their corresponding CDS. The SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, USA) and the Applied Biosystems™ StepOne™ Real-Time PCR System were employed to perform a semi-quantitative RT-PCR test to assess the transcript levels of the *VrNHX1* gene in transgenic rice plants. For PCR amplification, 12.5 μL of 2 × SYBR Premix, 6.5 μL of ddH₂O, 4 μL of the template, and 1 μL (10μM) of each specific primer were used for each reaction. 300 nM ROX dye was used in each reaction as a reference dye. Thermal cycling was performed in the following order: initial denaturation at 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, 61°C for 30 seconds, and 72°C for 45 seconds.

2.7. Physiological evaluation of transgenic rice plants under salinity stress

The 21-day-old control and transgenic plants (T_2 lines) grown in a greenhouse were subjected to salinity stress. Initially, the selected plants were irrigated with distilled water enriched with 50 mM NaCl for one week, and then the NaCl concentration was raised to 150 mM progres-

$$\mu \text{ moles proline per g of fresh tissue} = \frac{[(\mu g \text{ proline/mL} \times \text{ mL toluene}) / 115.5 \, \mu g/\, \mu \text{ mole}]}{[\text{g sample } / \, 2.5]}$$

sively at 5-day intervals. From NaCl-treated control and transgenic T_2 plants, leaf samples were taken to measure the relative water content (RWC), electrolyte leakage, proline, chlorophyll, lipid peroxidation level, and antioxidant enzymes-catalase (CAT) activity.

2.8. Leaf senescence assay

The leaf senescence experiment was carried out on 30-day old control (WT) and transgenic rice plants (T_2). Healthy and completely grown leaves were collected and immediately washed in deionized water, and 1-cm diameter leaf discs were prepared and rinsed in NaCl solutions of 0-, 50-, 100- and 150-mM concentrations [33]. The phenotypic changes caused by salinity stress were observed over 7 days, and overall chlorophyll content was calculated using Arnon's method [34].

2.9. Electrolyte leakage analysis

The leaves were taken from the control (WT) and transgenic (T_2) lines and washed with deionized water to eliminate electrolytes that stick to the surface. The leaf samples were placed in closed flasks containing 10 mL of deionized water and incubated on a shaking incubator at 25°C for 24 hours. After incubation, initial electric conductivity (L_0) was measured using a conductivity meter (Hanna Instruments, HI98309). The sample-containing solutions were then autoclaved at 120°C for 20 minutes and cooled to 25°C. Final electric conductivity (L_t) was measured and recorded according to the previous report [35]. Finally, the electric leakage was calculated using the following formula:

$$\text{Electrolyte leakage (\%)} = \frac{L_t}{L_o} \times 100\% \text{Electrolyte leakage (\%)}$$
$$= \frac{L_t}{L_o} \times 100\%$$

2.10. Relative water content (RWC) analysis

To measure the RWC, similar sized leaves were collected from control (WT) and transgenic (T_2) lines. The fresh weight (FW) of harvested samples was immediately measured and recorded. After immersing the samples in deionized water for 24 hours at a 25°C temperature, the turgid weight (TW) was measured. Finally, the samples were dried at 80°C for 48 hours and their dry weight (DW) was determined. The RWC was calculated using the following formula [36]:

$$\text{Relative water content(RWC)} = \frac{FW - DW}{TW - WD} \times 100 \, \%$$

2.11. Measurement of proline content

The proline content was calculated according to the guidelines given by Bates et al. (1973) [37]. 5 mL of 3 percent aqueous sulphosalicylic acid (Sigma-Aldrich, Germany) was used to homogenize 0.1 g of plant leaves, which were then centrifuged at 12000 rpm for 15 minutes. 2 mL of filtrate was heated for 1 hour at 100°C with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin. 4 mL of toluene was poured and thoroughly mixed after chilling in ice. The toluene layer (upper pink layer) was removed, and the absorbance at 520 nm was recorded. On a fresh-weight basis, the proline content was expressed as follows:

Here, 115.5 = molecular weight of proline.

2.12. Measurement of chlorophyll

To determine total chlorophyll content, 0.1g of leaf samples were chopped into tiny pieces and immersed in an acetone solution (80%) at 4°C in the dark for three days. The suspension was then filtered using

Whatman filter paper, and absorbance at 645 nm was measured [34]. The total quantity of chlorophyll was calculated using the formula:

$$\text{Total chlorophyll} \left(\frac{\text{mg}}{\text{g}} \text{ fresh weight} \right) = \frac{(20.2 \times \text{A645} + 08.02 \times \text{A665})}{\text{Weight of tissues in gram}}$$

2.13. Lipid peroxidation level

To measure the level of lipid peroxidation in the sample that had been treated, 0.1 g of leaf sample was mixed with 2 ml of 0.25 percent TBA made with 10 percent TCA. The homogenate was heated for 30 minutes at 95°C before being quickly chilled on ice. Finally, the solution was centrifuged at 10,000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 532 nm and 600 nm. The absorbance at 600 nm was subtracted from the absorbance at 532 nm to account for nonspecific turbidity. The level of lipid peroxidation is measured in millimoles of MDA (Sigma-Aldrich, Germany) produced with an extinction coefficient of 155 mM^{-1}cm^{-1} [38].

2.14. Determination of catalase (CAT)

CAT activity was calculated using the method described by Velikova *et. al.* [39]. To prepare the reaction mixture, 0.1 mL of enzyme extract was mixed with 1.9 mL of 10 mM potassium phosphate buffer (pH 7.0). To begin the reaction, 1 mL of 0.035 percent H_2O_2 was added and thoroughly mixed. The absorbance at 240 nm was then measured after 2 and 4 minutes of H_2O_2 addition. The frequency of elimination of H_2O_2 was used to determine CAT activity, which was guided by a decrease in absorbance at 240 nm measured 2 and 4 minutes after the inoculation of H_2O_2. The activity was measured using a 40 mM^{-1}cm^{-1} extinction coefficient and represented as H_2O_2 decreased min^{-1}g^{-1} fresh weight.

2.15. Field trial of transgenic lines under salinity stress

In the field, T_2 transgenic lines and control rice plants are trailed with varying doses of NaCl. T_2 transgenic line and control rice plant seedlings are transplanted in the field (confined area). 21, and 35-day-old transplanted seedlings were treated with 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM of NaCl. Height, tiller number of grains per panicle, and 100 grain weight of transgenic lines and control rice plants were measured after maturity.

2.16. Statistical analysis

All of the investigations were carried out at random and were repeated at least three times. GraphPad Prism 8.4.3 and MS Office 365 were used to analyze the data. A two-way ANOVA, followed by a Bonferroni post hoc test, was used to assess significant differences (P<0.05). To represent the significance of the differences, different means were labeled with different characters.

3. Results

3.1. Transformation and regeneration of transgenic plants

We successfully developed the transgenic BRRI Dhan28 and BRRI Dhan29 lines in this study using an *Agrobacterium*-mediated genetic transformation method and rice seed embryo axes as explants. Infected embryonic axes of explants with *Agrobacterium* strains demonstrated relatively high regeneration rates. After three days of co-cultivation with *Agrobacterium*, the infected explants were given time to regenerate before being moved to Yoshida Solutions' hydroponic system, where the new plantlets grew well (Fig. 1 a and b). The GUS histochemical assay revealed blue staining in regenerated platelets, which are being transformed. Plantlets that appeared to have been transformed were then
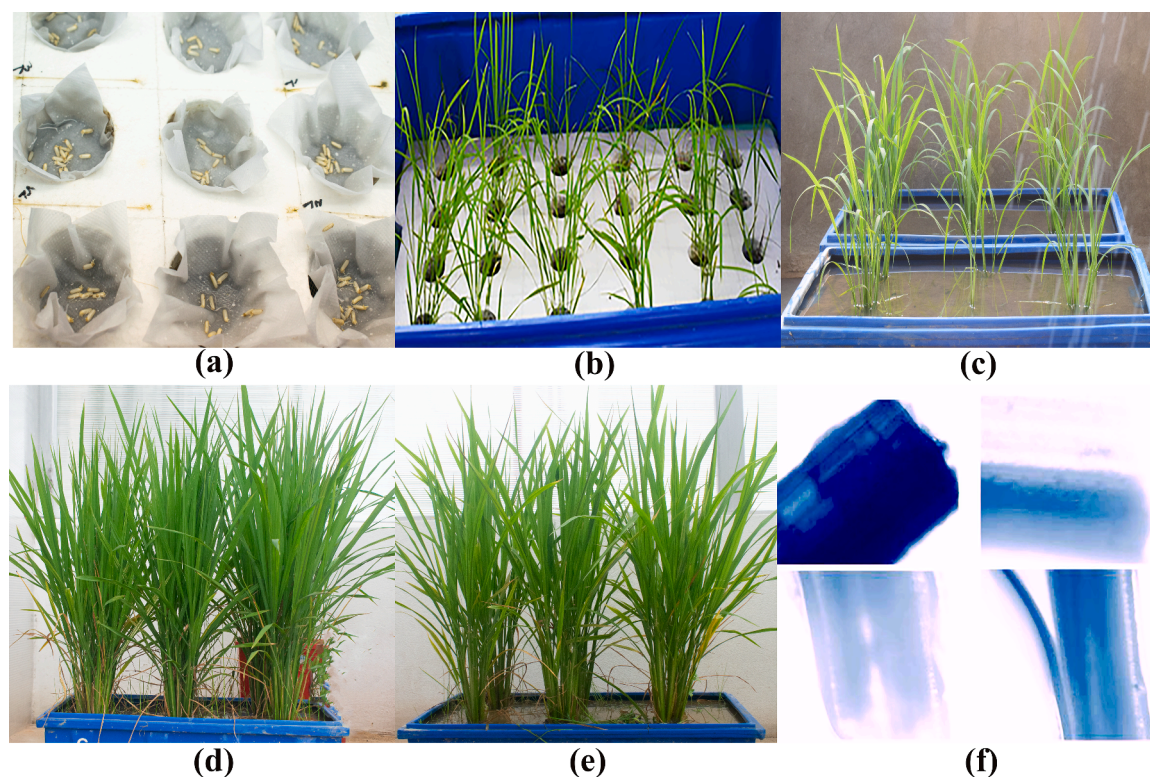

Fig. 1. Transformation, selection, and regeneration of transgenic BRRI Dhan28 and BRRI Dhan29. (a & b) Regeneration of co-cultivated BRRI Dhan28 and BRRI Dhan29 explants in Yoshida solution. (c, d, & e) Raring of selected (on the basis of GUS staining and PCR amplification) transgenic BRRI Dhan28 and BRRI Dhan29 lines in soil-containing plastic tray. (f) GUS expression in transgenic T0 BRRI Dhan28 (T0-1) and BRRI Dhan29 (T0-2) lines (bar 2mm).

placed in soil-filled plastic trays. The transgenic plantlets were chosen for further evaluation using GUS staining (Fig. 1f). Out of 475 and 434 treated explants, 16 and 12 transgenic plantlets with a frequency of 3.39 ±0.28 % and 2.74±0.35% for BRRI Dhan28 and BRRI Dhan29, respectively, were found (Supp. Table 1).

3.2. Molecular analysis of transgenic plants

DNA from control (WT) and transformed plantlets (T_0 and T_1) were extracted and confirmed in gel electrophoresis to assess transgene insertion. The extracted DNA was then subjected to PCR amplification utilizing GUS-a and *VrNHX1* gene-specific primers. There was no amplification of these genes in control rice plantlets, but the existence of *VrNHX1* and GUS-a genes with predicted band sizes of 1.726 kb and 773 bp in transgenic BRRI Dhan28 and BRRI Dhan29 rice plantlets confirmed gene integration. (Fig. 2 a and b). Expression of the transgene was confirmed using semi-quantitative RT-PCR. Total RNA was isolated from the control (WT) and transformed (T_1 and T_2) plantlets and cDNA was synthesized from extracted RNA. The expression of the *VrNHX1* gene in transgenic BRRI Dhan28 and BRRI Dhan29 rice plantlets was confirmed by semi-quantitative RT-PCR with bands of an expected size of 104 bp. In control (WT) plantlets, no band was observed (Fig. 2c). A housekeeping gene, eEF-1, was also studied in both transgenic and non-transgenic rice plants (Fig. 2d).

3.3. Physio-biochemical analysis of transgenic plants

3.3.1. Leaf disc assay

To evaluate the salinity stress resistance capacity of transgenic rice plants, leaf disk assays of WT (BRRI Dhan28: WT-1 and BRRI Dhan29: WT-2) and transgenic T_2 BRRI Dhan28 (T_2-1) and BRRI Dhan29 (T_2-2) were conducted (Fig. 3a). Data analysis revealed that 50 mM NaCl stress did not significantly affect transgenic lines or WT plants. However, when the NaCl concentration increased, both the transgenic lines and the WT plants were impacted. With 100 mM and 150 mM NaCl stresses, the leaf discs of WT plants displayed a substantial amount of bleaching and

turned pale quickly due to chlorosis. However, the transgenic lines exhibited less bleaching and stayed greener longer than the WT plants. To measure the extent of chlorosis, the total chlorophyll content of the evaluated leaf discs of WT and transgenic lines was computed. Analysis of the data showed that transgenic lines had more chlorophyll than WT plants. This meant that transgenic lines that expressed the *VrNHX1* gene had less chlorosis. There were no significant changes in chlorophyll content between WT and transgenic lines in the untreated condition. After the rice plants were treated with 100 mM and 150 mM NaCl, the transgenic T_2-1 and T_2-2 lines with the *VrNHX1* gene had more chlorophyll than the WT-1 and WT-2 plants (Fig. 3b).

3.3.2. Salinity stress assay

Transgenic T_2 BRRI Dhan28, BRRI Dhan29, and WT rice plants were evaluated for salinity tolerance in soil conditions containing 150 mM NaCl solutions (at a 2-day interval). In terms of tolerance to 150 mM NaCl, transgenic rice plants gave better physiology than WT plants (Fig. 4). The findings showed that the Na^+/H^+ antiporter has a beneficial effect on salt tolerance.

3.3.3. Relative water content (RWC) and electrolyte leakage analysis

There were no significant changes in relative water content and electrolyte leakage in WT (WT-1 and WT-2) and transgenic T_2 BRRI Dhan28 and BRRI Dhan29 lines under unstressed conditions (0 mM NaCl). WT plants lost more electrolytes under salinity stress (150 mM NaCl) than transgenic BRRI Dhan28 and BRRI Dhan29 lines (Fig. 5d). Transgenic lines, on the other hand, have greater relative water content than WT plants (Fig. 5b).

3.3.4. Proline content

There were almost no significant changes in proline content in controls (WT-1 and WT-2) and transgenic T_2 BRRI Dhan28 and BRRI Dhan29 lines under normal conditions (0 mM NaCl). In the absence of stress, both the WT and transgenic lines accumulated comparable proline content. In contrast, in stressful conditions (150 mM NaCl), the WT rice plants showed higher proline content compared to the transgenic T_2
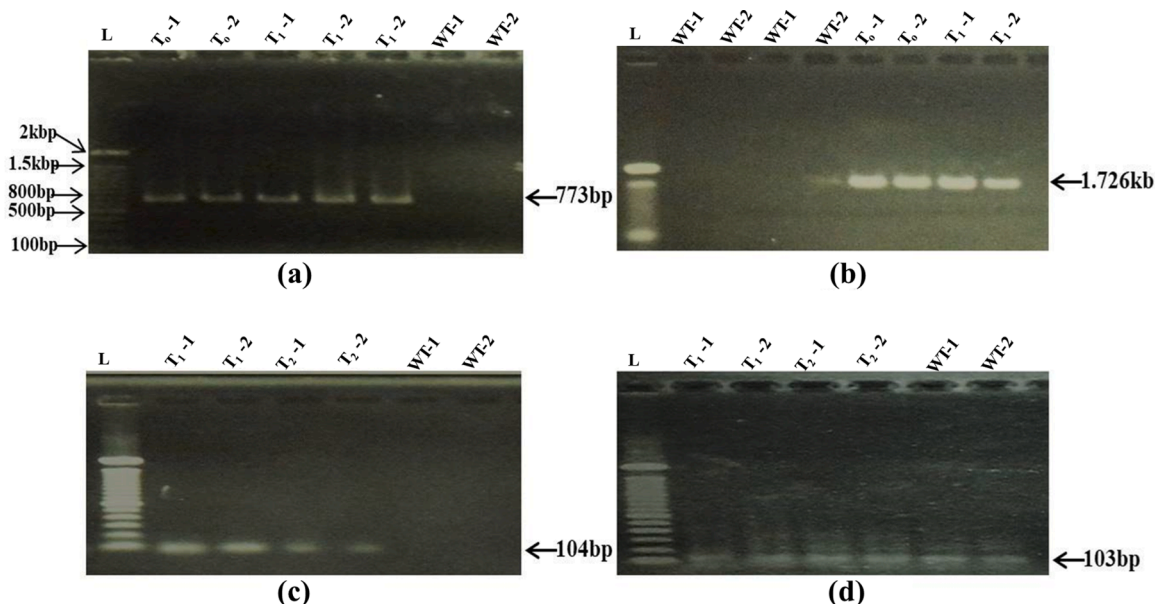

Fig. 2. Confirmation of transgenic BRRI Dhan28 and BRRI Dhan29 via PCR detection of GUS-a end VrNHX1 gene and expression analysis of VrNHX1 gene through semiquantitative RT-PCR. (a) VrNHX1 gene (773 bp) amplification in transgenic BRRI Dhan28 (T0-1 and T1-1) and BRRI Dhan29 (T0-2 and T1-2) lines; no amplification found in control (WT) BRRI Dhan28 (WT-1) and BRRI Dhan29 (WT-2) plants. (b) GUS-a gene amplification (1.726 kb) in transgenic BRRI Dhan28 (T0-1 and T1-1) and BRRI Dhan29 (T0-2 and T1-2) lines; no amplification detected in control (WT) BRRI Dhan28 (WT-1) and BRRI Dhan29 (WT-2) plants. (c) VrNHX1 gene (104 bp) expression in transgenic BRRI Dhan28 (T1-1 and T2-1) and BRRI Dhan29 (T1-2 and T2-2) lines; no expression in control (WT) BRRI Dhan28 (WT-1) and BRRI Dhan29 (WT-2) plants. (d) Rice eEF-1 gene expression; constitutive expression was observed in all analyzed samples. L: 100bp DNA ladder (Nippon gene, cat. no. 316-06951).
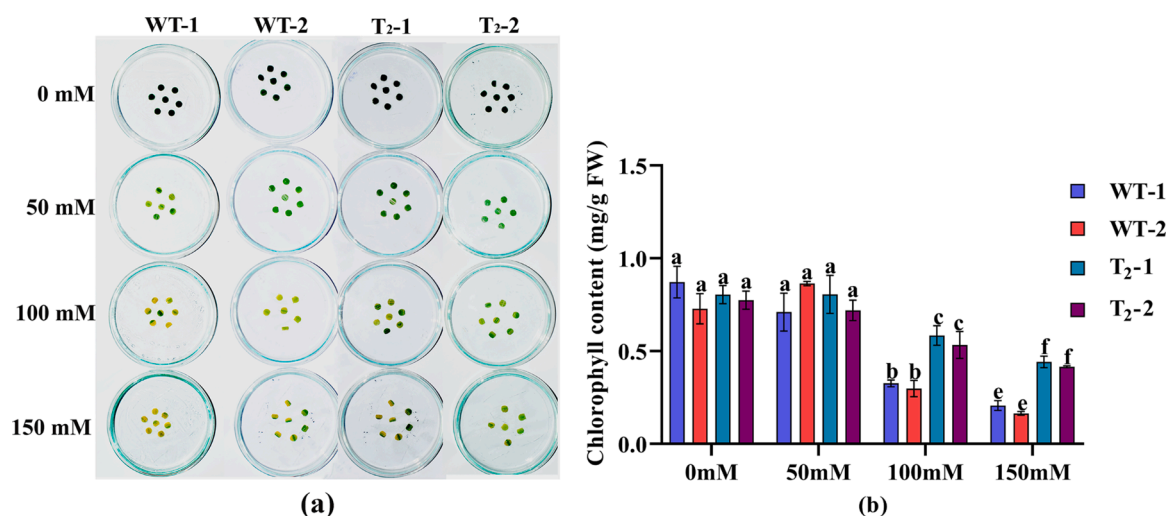
Fig. 3. (a) Leaf disk assay of transgenic BRRI Dhan28 (T2-1) and BRRI Dhan29 (T2-2) lines and control (WT) BRRI Dhan28 (WT-1) and BRRI Dhan29 (WT-2) plants under different levels of salinity stress. When WT plants were compared to transgenic lines, there was a significant quantity of chlorophyll leaching. (b) Transgenic plants have higher chlorophyll content than WT plants. The data represent the mean SEM (Standard Error of the Mean), and significant values were determined using Bonferroni analysis at P 0.05 and are represented by different letters.
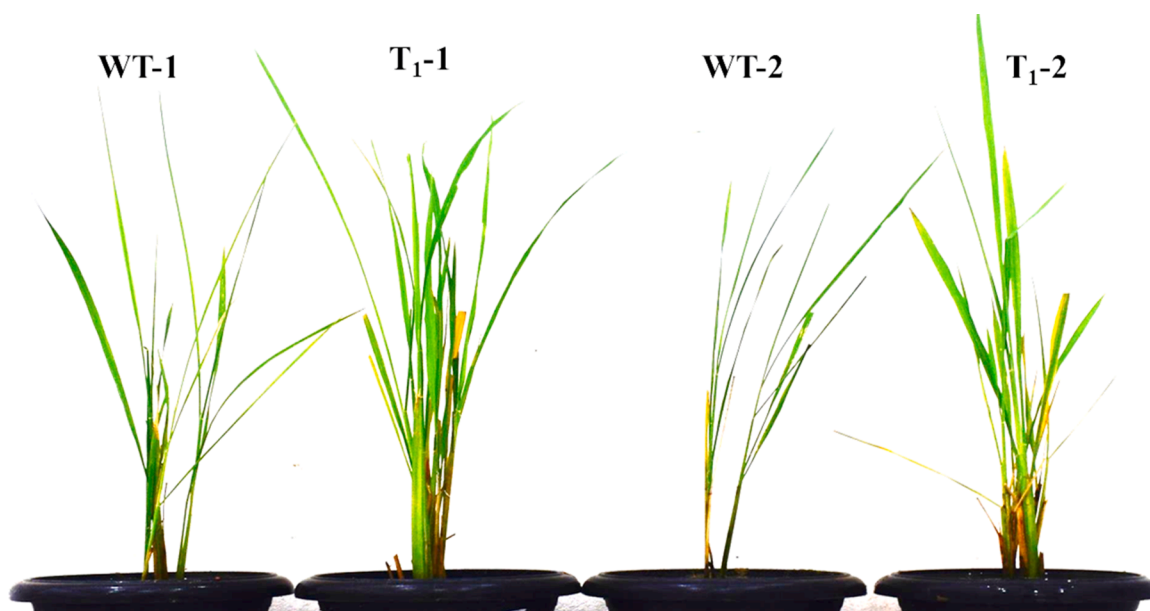

Fig. 4. Effect of salinity stress on T2 transgenic BRRI Dhan28 (T2-1) and BRRI Dhan29 (T2-2) lines and WT BRRI Dhan28 (W-1) and BRRI Dhan29 (WT-2) plants. Transgenic lines and WT plants were irrigated with 150 mM NaCl solutions in plastic pots for 7 days before being examined. Transgenic lines outgrew WT plants in terms of growth.

BRRI Dhan28 and BRRI Dhan29 lines (Fig. 5c).

3.3.5. Measurement of chlorophyll

In physiological and 150 mM NaCl stressed conditions, the chlorophyll and anthocyanin content of WT (WT-1 and WT-2) and transgenic T$_2$ BRRI Dhan28 and BRRI Dhan29 lines were determined. Data analysis revealed that there were no significant differences in chlorophyll content between WT and transgenic lines expressing the *VrNHX1* gene under normal physiological conditions. However, in response to salinity stress (150 mM NaCl), the WT plants produced less chlorophyll than the transgenic lines. Transgenic lines, on the other hand, have much greater chlorophyll content than WT plants (Fig. 5a).

3.3.6. Catalase (CAT) enzyme activity

Catalase and peroxide enzyme activity were calculated in WT (WT-1

and WT-2) and transgenic T$_2$ BRRI Dhan28 and BRRI Dhan29 lines under stressed and unstressed conditions. Under normal physiological conditions, there was no significant change in catalase and peroxide enzyme activity between WT and transgenic BRRI Dhan28 and BRRI Dhan29 lines. But under stressful conditions (150 mM NaCl), catalase enzyme activity was much higher in transgenic plants expressing the *VrNHX1* gene than in WT plants (Fig. 6a).

3.3.7. Lipid peroxidation level

In normal growth conditions, the amounts of hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) in (WT-1 and WT-2) and transgenic T$_2$ BRRI Dhan28 and BRRI Dhan29 lines were found to be identical, with no significant changes. With an increase in salinity stress (150 mM NaCl), substantial amounts of NBT and DAB staining were increased in WT rice plants compared to the transgenic BRRI Dhan28 and BRRI
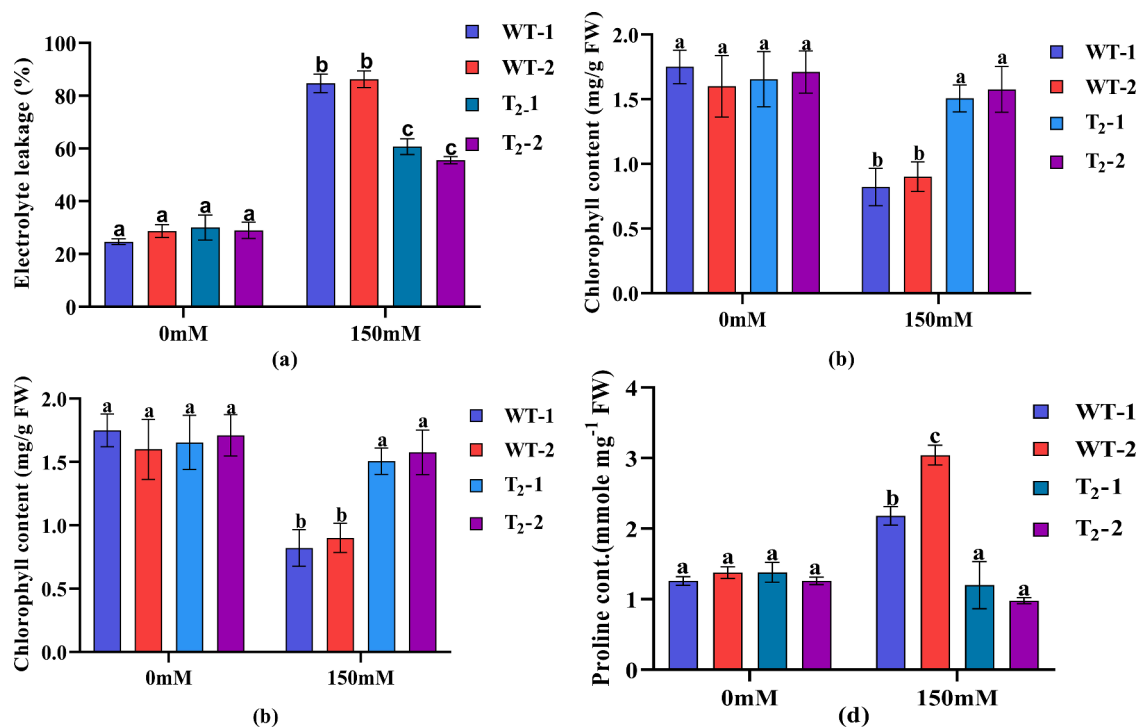
Fig. 5. Physiological parameters were examined under salt salinity (150 mM), and comparisons were made between WT BRRI Dhan28 (W-1) and transgenic BRRI Dhan28 (T2-1) as well as WT BRRI Dhan29 (W-2) and BRRI Dhan29 (T2-2) lines, as well as WT BRRI Dhan28 (W-1): (a) Chlorophyll content; (b) Relative water content; (c) Proline content; and (d) Electrolyte leakage. The data represent the mean SEM (Standard Error of the Mean), and significant values were determined using Bonferroni analysis at P 0.05 and are represented by different letters.
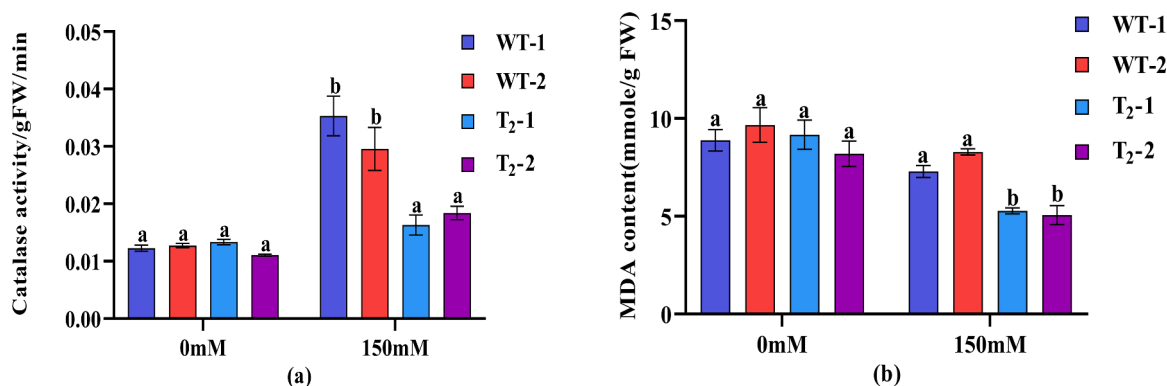

Fig. 6. Analysis of catalase enzyme activities and lipid peroxidation levels under salinity stress (150 mM) and comparison between transgenic BRRI Dhan28 (T2-1) and BRRI Dhan29 (T2-2) lines and WT BRRI Dhan28 (W-1) and BRRI Dhan29 (WT-2) plants: (a) catalase activity and (b) lipid peroxidation level. The data represents the mean SEM (Standard Error of the Mean), and significant values were determined using Bonferroni analysis at P 0.05 and are represented by different letters.

Dhan29 lines, indicating that WT plants produced more hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-). The lipid peroxidation level was calculated using the MDA content. In an unstressed condition, there was no significant change in MDA content between WT and transgenic lines. However, when stressed (NaCl), WT plants produced more MDA than transgenic BRRI Dhan28 and BRRI Dhan29 lines. This demonstrated that transgenic rice plants had lower levels of lipid peroxidation (Fig. 6b).

3.3.8. Transgenic lines under salinity stress

Transgenic rice plants (transgenic BRRI Dhan28 and BRRI Dhan29) performed better than control rice plants (WT-1 and WT-2) after 15 days of salt treatment (50 mM, 100 mM, 150 mM, 200 mM, and 250 mM). The control rice plants were almost died under 100 mM salt treatment. Data study of agronomical features of transgenic lines revealed that they

outperformed control rice plants. The majority of the control rice plants perished relatively immediately after being exposed to 100 mM NaCl, and all of the control rice plants died after being exposed to above 100 mM NaCl dosages (Supp. Fig. 1). Agronomical characteristics analysis reveals that transgenic rice plants treated with 50 mM, 100 mM, 150 mM, and 200 mM NaCl performed similarly to control rice plants (no NaCl treatment) with no significant changes (Supp. Table 2). Transgenic rice plants survived 250 mM NaCl treatment but performed poorly when compared to transgenic lines treated with lower doses of NaCl.

4. Discussion

Plants rely on the vacuolar Na^+/H^+ antiporter NHX1 to transport Na^+ ions from the cell cytoplasm to the vacuoles, as well as to maintain cellular homeostasis and pH [40]. By compartmentalizing deleterious

cytosolic Na$^+$ ions, Na$^+$/H$^+$ antiporters reduce the detrimental effect and influence on development and growth in plants under salinity stress, improving salinity stress resistance [41]. *Vigna radiata* L. Wilczek is a common plant in Southeast Asia that tolerates moderate salinity stress. The Na$^+$/H$^+$ antiporter from *Vigna radiata* L. Wilczek (*VrNHX1*) has been shown to be important for maintaining the balance of ions in cells [42].

In this study, the *VrNHX1* gene isolated from *Vigna radiata* L. Wilczek was transferred into two high-yielding salinity-stress sensitive varieties, BRRI Dhan28 and BRRI Dhan29, to improve salinity-tolerance through *Agrobacterium*-mediated transformation. The *Agrobacterium*-mediated transformation method is a successful method for transferring genes into plants that makes use of a naturally occurring crown gall disease mechanism. It is used in fundamental research and commercial applications because it is more suited than other methods such as particle gun bombardment or microinjection [43, 44]. The *Agrobacterium*-mediated transformation process allows the transferred DNA to enter the nucleus, where it may integrate into the genome and pass on to the next generation. Again, the transferred DNA may remain in the nucleus without integrating and continue to be transported to the targeted products [45]. The selection of a suitable explant is critical for effective transformation, and mature rice seed-derived embryos were used as explants in this experiment because they are acquiescent, reproducible, and have a high frequency of transformation efficiency, as previously described in several reports [46–48]. The embryos of rice seeds were carefully wounded with a precise and sharp needle to avoid over-damaging the embryos and causing them to fail to germinate, as the success rate of transformation is greatly dependent on this stage [47]. The density of *Agrobacterium* and co-cultured time are the important factors for successful transformation since too much bacteria can promote overgrowth, which damages the explants and reduces transformation efficiency [49–52]. Three days of co-culture with 0.5 (OD600) *Agrobacterium* suspensions at density of 0.5 (OD600) yielded transformation frequencies of 3.39±0.28 % and 2.74±0.35%, respectively, in this research.

The appropriate acetosyringone concentration, which ranges between 100 and 400 µM depending on the genotype, is another critical component for successful transformation. Previous research has shown that adding 150 µM acetosyringone boosts transformation efficiency in rice and other plants [53, 54] and we found transformants in this investigation by adding this quantity of acetosyringone. With the advancement of plant biotechnology, *an Agrobacterium*-mediated genetic transformation is now a popular approach for generating stress and disease-resistant plants [55, 56]. Salinity stress is a severe limitation on rice production, particularly in coastal areas, and it is worsening in climate-vulnerable nations such as Bangladesh, where rice is the primary source of food [54]. To restore rice farming to these saline-affected areas, salt-tolerant rice varieties must be developed. Increased external Na$^+$ levels provide a significant electrochemical gradient that favors passive Na$^+$ transfer into the cell via K$^+$ transporters, leading to a high cytosolic Na+ concentration that has a detrimental impact on the plant [9]. Overexpression of the vacuolar Na$^+$/H$^+$ antiporter (NHX1) is important for salinity tolerance because it compartmentalizes cytosolic Na$^+$ in the vacuole [57, 58]. Here, we successfully developed transgenic BRRI Dhan28 and BRRI Dhan29 lines, which were confirmed using GUS histochemical assay, PCR amplification of GUS and target gene, and finally, expression of transgene was confirmed using semi-quantitative RT-PCR (Fig. 2), seeing as GUS histochemical assay, PCR, and RT-PCR are the most commonly used procedures to validate transgene incorporation and expression [24, 35, 59, 60].

Salinity stress, in general, inhibits plant growth, reduces photosynthesis, and induces protein synthesis. Plants' physiological response to salinity stress is a complicated process that requires the collective action of many genes, and the overexpression of one gene can impact the expression of others [5, 61]. By compartmentalizing the Na$^+$ in the vacuoles, transgenic plants that overexpressed the NHX1 antiporter were easily able to keep the cytoplasmic enzymes in a good physiological state [61]. Some indicators (Supp. Table 3), such as proline content, chlorophyll content, lipid peroxidation level, catalase enzyme activity (CAT), relative water content, and electrolyte leakage, provide information about the plant's physiological status [62–64]. Under salinity stress, *VrNHX1* expressing T$_2$ BRRI Dhan28 and BRRI Dhan29 lines had increased chlorophyll content, indicating reduced damage from salinity stress, which might be related to higher Na$^+$ compartmentalization. When plants are exposed to salinity stress, RWC values tend to decrease and are considered an essential measure of water retention capacity [65]. We observed that RWC was considerably greater in transgenic rice lines than in WT rice lines. When *VrNHX1* was overexpressed in transgenic rice lines, less electrolyte was released from the leaves than from wild-type rice plants. Transgenic rice lines had also lower levels of proline, lipid peroxidation, and catalase enzyme activity, indicating that the plant had more osmoprotectants [35]. Under salinity stress, data analysis of all parameters reveals that transgenic rice lines have better physiology than wild-type plants (Figs. 5 and 6). The leaf disc assay also confirms T$_2$ BRRI Dhan28 and BRRI Dhan29 rice lines having higher physiological state than WT plants. The T$_2$ BRRI Dhan28 and BRRI Dhan29 rice lines and WT plants were also evaluated under 150 mM saline water. T$_2$ BRRI Dhan28 and BRRI Dhan29 rice lines grew faster than WT plants after 7 days of salinity stress, confirming that the transgenic lines had stronger salt tolerance than WT plants. Furthermore, field observations of transgenic T$_2$ lines under salinity stress revealed better agronomical features compared to control plants, demonstrating enhanced salinity tolerance in transgenic lines. Overexpression of vacuolar Na$^+$/H$^+$ antiporter (NHX1) in other plants such as *Vigna unguiculata* [24] *Ricinus communis* L. [35], *Arabidopsis thaliana* [24, 66], and *Miscanthus sinensis* Anderss [67] yielded comparable findings and imparted salt resistance.

5. Conclusion

The production of salt-tolerant rice cultivars is essential for cultivating salt-affected agricultural regions and fulfilling future food demands for growing populations. Here, we developed transgenic BRRI Dhan28 and BRRI Dhan29 rice expressing the *VrNHX1* gene through *Agrobacterium*-mediated transformation with a frequency of 3.39±0.28 % and 2.74±0.35% respectively. Transgenic lines with more chlorophyll, higher relative water content, and lower electrolyte leakage, proline content, lipid peroxidation level, and catalase enzyme activity had superior physiology than WT plants. Under salinity (150 mM) stress, transgenic lines outgrew WT plants in terms of growth and salt resistance. For sustainable agriculture, these transgenic rice varieties that can withstand more salinity could be grown in salty coastal areas.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon request.

Supplementary information

Supp. Fig. 1: Field trial of transgenic lines (T$_2$ BRRI Dhan28 and BRRI Dhan29) under salinity stress: a) 21-day old transgenic lines subjected to salinity stress, b) 35-day old transgenic lines subjected to salinity stress, c) mature rice of transgenic rice lines subjected to salinity stress, and d) control plants subjected to salinity stress.

CRediT authorship contribution statement

Md. Nazmul Hasan: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft. **Fahmid H Bhuiyan:** Methodology. **Hammadul Hoque:** Investigation, Writing – review & editing. **Nurnabi Azad Jewel:** Investigation. **Md Ashrafuzzaman:** Supervision, Writing – review & editing. **Shamsul H. Prodhan:** Conceptualization, Validation, Supervision, Writing – review & editing.

Declaration of Competing Interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00740.

References

[1] S.S. Gnanamanickam, Rice and its importance to human life, in Biological control of rice diseases, Springer, 2009, pp. 1–11.

[2] G.S. Gupta, Land degradation and challenges of food security, Rev. Eur. Stud. 11 (2019) 63.

[3] I.N.B.L. Reddy, et al., Salt tolerance in rice: focus on mechanisms and approaches, Rice Science 24 (3) (2017) 123–144.

[4] Y. Bu, et al., Adverse effect of urease on salt stress during seed germination in Arabidopsis thaliana, Febs Letters 589 (12) (2015) 1308–1313.

[5] B. Gupta, B. Huang, Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization, International journal of genomics 2014 (2014).

[6] W. Liang, et al., Plant salt-tolerance mechanism: A review, Biochemical and biophysical research communications 495 (1) (2018) 286–291.

[7] A. Kroes, et al., Plant-mediated interactions between two herbivores differentially affect a subsequently arriving third herbivore in populations of wild cabbage, Plant Biology 18 (6) (2016) 981–991.

[8] P. Rengasamy, Soil processes affecting crop production in salt-affected soils, Functional Plant Biology 37 (7) (2010) 613–620.

[9] T.M.L. Hoang, et al., Improvement of salinity stress tolerance in rice: challenges and opportunities, Agronomy 6 (4) (2016) 54.

[10] T.M.D.R. Na, tolerance and Na+ transport in higher plants, Ann Bot (Lond) 91 (2003) 503–527.

[11] T. Yamaguchi, et al., Vacuolar Na+/H+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca2+-and pH-dependent manner, Proceedings of the National Academy of Sciences 102 (44) (2005) 16107–16112.

[12] F. Hauser, T. Horie, A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K+/Na+ ratio in leaves during salinity stress, Plant, cell & environment 33 (4) (2010) 552–565.

[13] M.P. Apse, et al., Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis, Science 285 (5431) (1999) 1256–1258.

[14] S. Zhou, et al., Enhanced V-ATPase activity contributes to the improved salt tolerance of transgenic tobacco plants overexpressing vacuolar Na+/H+ antiporter AtNHX1, Biotechnology letters 33 (2) (2011) 375–380.

[15] V. Jegadeeson, et al., Expression of wild rice Porteresia coarctata PcNHX1 antiporter gene (PcNHX1) in tobacco controlled by PcNHX1 promoter (PcNHX1p) confers Na+-specific hypocotyl elongation and stem-specific Na+ accumulation in transgenic tobacco, Plant Physiology and Biochemistry 139 (2019) 161–170.

[16] S. Liu, et al., Overexpression of OsVP1 and OsNHX1 increases tolerance to drought and salinity in rice, Journal of Plant Biology 53 (6) (2010) 444–452.

[17] H.-X. Zhang, et al., Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium

[18] accumulation, Proceedings of the National Academy of Sciences 98 (22) (2001) 12832–12836.

[18] P.H. Kumari, et al., Overexpression of a plasma membrane bound Na+/H+ antiporter-like protein (SbNHXLP) confers salt tolerance and improves fruit yield in tomato by maintaining ion homeostasis, Frontiers in plant science 7 (2017) 2027.

[19] M. Maach, et al., Overexpression of LeNHX4 improved yield, fruit quality and salt tolerance in tomato plants (Solanum lycopersicum L.), Molecular biology reports 47 (6) (2020) 4145–4153.

[20] R. Yarra, et al., Overexpression of a wheat Na+/H+ antiporter gene (TaNHX2) enhances tolerance to salt stress in transgenic tomato plants (Solanum lycopersicum L.), Plant Cell, Tissue and Organ Culture (PCTOC) 111 (1) (2012) 49–57.

[21] Z.-Y. Xue, et al., Enhanced salt tolerance of transgenic wheat (Tritivum aestivum L.) expressing a vacuolar Na+/H+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na+, Plant science 167 (4) (2004) 849–859.

[22] D.E.P. Sumalapao, et al., Tolerance of mung bean (Vigna radiata (L.) wilczek) to lactic acid, a potential herbicide: growth and morphology, J. Anim. Plant Sci 28 (1) (2018) 138–145.

[23] B. HanumanthaRao, R.M. Nair, H. Nayyar, Salinity and high temperature tolerance in mungbean [Vigna radiata (L.) Wilczek] from a physiological perspective, Frontiers in Plant Science 7 (2016) 957.

[24] S. Mishra, et al., Cloning and functional characterization of a vacuolar Na+/H+ antiporter gene from mungbean (VrNHX1) and its ectopic expression enhanced salt tolerance in Arabidopsis thaliana, PloS one 9 (10) (2014), e106678.

[25] Salam, M., et al., BRRI dhan47: a salt tolerant variety for the boro season. International Rice Research Notes, 2007.

[26] E. Nyaboga, et al., Agrobacterium-mediated genetic transformation of yam (Dioscorea rotundata): an important tool for functional study of genes and crop improvement, Frontiers in Plant Science 5 (2014) 463.

[27] H. Md. Nazmul, et al., The Effect of Plant Growth Regulators (PGRs) on Efficient Regeneration of 12 Recalcitrant Indica Rice (Oryza Sativa L.) Genotypes, American Journal of Biochemistry and Biotechnology 17 (2) (2021).

[28] A. Froger, J.E. Hall, Transformation of plasmid DNA into E. coli using the heat shock method, J Vis Exp (6) (2007) 253.

[29] S. Bakshi, et al., Improved Agrobacterium-mediated transformation of cowpea via sonication and vacuum infiltration, Plant Cell Reports 30 (12) (2011) 2281–2292.

[30] S. Yoshida, Routine procedure for growing rice plants in culture solution, Laboratory manual for physiological studies of rice (1976) 61–66.

[31] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, The EMBO journal 6 (13) (1987) 3901–3907.

[32] Doyle, J.J. and J.L. Doyle, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. 1987.

[33] B. Jha, et al., Developing transgenic Jatropha using the SbNHX1 gene from an extreme halophyte for cultivation in saline wasteland, PLoS One 8 (8) (2013) e71136.

[34] D.I. Arnon, Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris, Plant physiology 24 (1) (1949) 1.

[35] M.K. Patel, et al., Ectopic expression of SbNHX1 gene in transgenic castor (Ricinus communis L.) enhances salt stress by modulating physiological process, Plant Cell, Tissue and Organ Culture (PCTOC) 122 (2) (2015) 477–490.

[36] H. Barrs, P. Weatherley, A re-examination of the relative turgidity technique for estimating water deficits in leaves, Australian journal of biological sciences 15 (3) (1962) 413–428.

[37] L.S. Bates, R.P. Waldren, I. Teare, Rapid determination of free proline for water-stress studies, Plant and soil 39 (1) (1973) 205–207.

[38] R.L. Heath, L. Packer, Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation, Archives of biochemistry and biophysics 125 (1) (1968) 189–198.

[39] V. Velikova, I. Yordanov, A. Edreva, Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines, Plant science 151 (1) (2000) 59–66.

[40] J. Feng, et al., GhNHX3D, a Vacuolar-Localized Na+/H+ Antiporter, Positively Regulates Salt Response in Upland Cotton, International journal of molecular sciences 22 (8) (2021) 4047.

[41] P.M. Hasegawa, Sodium (Na+) homeostasis and salt tolerance of plants, Environmental and experimental botany 92 (2013) 19–31.

[42] W. Fan, et al., Elevated compartmentalization of Na+ into vacuoles improves salt and cold stress tolerance in sweet potato (Ipomoea batatas), Physiologia plantarum 154 (4) (2015) 560–571.

[43] H. Oltmanns, et al., Generation of backbone-free, low transgene copy plants by launching T-DNA from the Agrobacterium chromosome, Plant physiology 152 (3) (2010) 1158–1166.

[44] H. Mubeen, et al., Gene transformation: methods, uses and applications, Journal of Pharmaceutical and Biological Sciences 4 (2) (2016) 54.

[45] H.-H. Hwang, M. Yu, E.-M. Lai, Agrobacterium-mediated plant transformation: biology and applications, The Arabidopsis Book (2017) 15.

[46] Y. Hiei, T. Komari, Agrobacterium-mediated transformation of rice using immature embryos or calli induced from mature seed, Nature protocols 3 (5) (2008) 824–834.

[47] S. Shimizu-Sato, et al., Agrobacterium-mediated genetic transformation of wild Oryza species using immature embryos, Rice 13 (1) (2020) 1–13.

[48] G. Yu, et al., Optimization of mature embryo-based tissue culture and Agrobacterium-mediated transformation in model grass Brachypodium distachyon, International journal of molecular sciences 20 (21) (2019) 5448.

[49] P. Pérez-Piñeiro, et al., Agrobacterium-Mediated Transformation of. Transgenic Plants-Advances and Limitations, IntechOpen, 2012.

[50] P.F. Alfenas, et al., Transgenic passionfruit expressing RNA derived from Cowpea aphid-borne mosaic virus is resistant to passionfruit woodiness disease, Fitopatologia Brasileira 30 (1) (2005) 33–38.

[51] X.-f. Yang, et al., A high-efficiency Agrobacterium tumefaciens mediated transformation system using cotyledonary node as explants in soybean (Glycine max L.), Acta physiologiae plantarum 38 (3) (2016) 60.

[52] P. Pérez-Piñeiro, et al., Agrobacterium-mediated transformation of wheat: general overview and new approaches to model and identify the key factors involved. Transgenic Plants-Advances and Limitations, Intech Open Access Publisher, Rijeka, Croatia, 2012, p. 326.

[53] N. Hasan, et al., Development of partial abiotic stress tolerant Citrus reticulata Blanco and Citrus sinensis (L.) Osbeck through Agrobacterium-mediated transformation method, Journal of Genetic Engineering and Biotechnology 17 (1) (2019) 1–9.

[54] S.U. Sikdar, et al., Agrobacterium-mediated PsCBL and PsCIPK gene transformation to enhance salt tolerance in indica rice (Oryza sativa), In Vitro Cellular & Developmental Biology-Plant 51 (2) (2015) 143–151.

[55] A. Ziemienowicz, Agrobacterium-mediated plant transformation: Factors, applications and recent advances, Biocatalysis and Agricultural Biotechnology 3 (4) (2014) 95–102.

[56] S. Gantait, S. Mondal, Transgenic approaches for genetic improvement in groundnut (Arachis hypogaea L.) against major biotic and abiotic stress factors, Journal of Genetic Engineering and Biotechnology 16 (2) (2018) 537–544.

[57] M.L. Demory, et al., Epidermal growth factor receptor translocation to the mitochondria, Journal of Biological Chemistry 284 (52) (2009) 36592–36604.

[58] H. Wang, Q. Ding, H. Wang, A new Na+/H+ antiporter gene KvNHX1 isolated from the halophyte Kosteletzkya virginica improves salt tolerance in transgenic tobacco, Biotechnology & Biotechnological Equipment 32 (6) (2018) 1378–1386.

[59] M. Mehrotra, I. Sanyal, D. Amla, RETRACTED ARTICLE: High-efficiency Agrobacterium-mediated transformation of chickpea (Cicer arietinum L.) and regeneration of insect-resistant transgenic plants, Plant cell reports 30 (9) (2011) 1603–1616.

[60] A. Oropeza-Aburto, et al., Agrobacterium tumefaciens mediated transformation of the aquatic carnivorous plant Utricularia gibba, Plant methods 16 (2020) 1–11.

[61] G. Onaga, K. Wydra, Advances in plant tolerance to abiotic stresses, Plant genomics 10 (2016) 229–272.

[62] K. Maghsoudi, et al., P5CS expression level and proline accumulation in the sensitive and tolerant wheat cultivars under control and drought stress conditions in the presence/absence of silicon and salicylic acid, Journal of Plant Interactions 13 (1) (2018) 461–471.

[63] A. Oraee, A. Tehranifar, Evaluating the potential drought tolerance of pansy through its physiological and biochemical responses to drought and recovery periods, Scientia Horticulturae 265 (2020), 109225.

[64] M. Noori, et al., Evaluation of water deficiency impacts on antioxidant enzymes activity and lipid peroxidation in some tomato (Solanum lycopersicum L.) lines, Indian Journal of Agricultural Research 52 (3) (2018) 228–235.

[65] M.A.S. Polash, et al., Effect of salinity on osmolytes and relative water content of selected rice genotypes, Trop Plant Res 5 (2) (2018) 227–232.

[66] J.B. Sottosanto, Y. Saranga, E. Blumwald, Impact of AtNHX1, a vacuolar Na+/H+ antiporter, upon gene expression during short-and long-term salt stress in Arabidopsis thaliana, BMC Plant Biology 7 (1) (2007) 1–15.

[67] Q. Sun, et al., Differential Responses of NHX1 and SOS1 Gene Expressions to Salinity in two Miscanthus sinensis Anderss. Accessions with Different Salt Tolerance, Phyton 90 (3) (2021) 827.