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ORIGINAL ARTICLE

Expressing class I wheat *NHX* (*TaNHX2*) gene in eggplant (*Solanum melongena* L.) improves plant performance under saline condition

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Abstract

Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous edible crop, and salt stress adversely affects its growth, development, and overall productivity. To cope with excess salinity, vacuolar Na^+/H^+ antiporters provide the best mechanism for ionic homeostasis in plants under salt stress. We generated transgenic eggplants by introducing wheat *TaNHX2* gene that encodes a vacuolar Na^+/H^+ antiporter in to the eggplant genome via *Agrobacterium*-mediated transformation using pBin438 vector that harbors double35S:*TaNHX2* to confer salinity tolerance. Polymerase chain reaction and southern hybridization confirmed the presence and integration of *TaNHX2* gene in T_1 transgenic plants. Southern positive transgenic eggplants showed varied levels of *TaNHX2* transcripts as evident by RT-PCR and qRT-PCR. Stress-inducible expression of *TaNHX2* significantly improved growth performance and Na^+ and K^+ contents from leaf and roots tissues of T_2 transgenic eggplants under salt stress, compared to non-transformed plants. Furthermore, T_2 transgenic eggplants displayed the stable leaf relative water content and chlorophyll content, proline accumulation, improved photosynthetic efficiency, transpiration rate, and stomatal conductivity than the non-transformed plants under salinity stress (200 mM NaCl). Data showed that the T_2 transgenic lines revealed that reduction in MDA content, hydrogen peroxide, and oxygen radical production associated with the significant increase of antioxidant enzyme activity in transgenic eggplants than non-transformed plants under salt stress (200 mM NaCl). This study suggested that the *TaNHX2* gene plays an important regulatory role in conferring salinity tolerance of transgenic eggplant and thus may serve as a useful candidate gene for improving salinity tolerance in other vegetable crops.

Keywords *TaNHX2* · *Solanum melongena* · Salt stress · Vegetables

Abbreviations

RT-PCR	Reverse transcription PCR
qRT-PCR	Quantitative real-time PCR
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
GPX	Guaiacol peroxidase
GR	Glutathione reductase
MDA	Malondialdehyde

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Introduction

Global agricultural productivity is subject to increasing environmental constraints, particularly to salinity due to their high magnitude of impact and wide distribution. Traditional breeding programs trying to improve abiotic stress tolerance have had some success but are limited by the multigenic nature of the trait. The yield and productivity of many crops including vegetables are hampered by the enormous amounts of soluble salts in soil in many parts of the world (FAO 2002; AVRDC 2006). Salt stress affects each phase of vegetable crop development including morphology, physiological function, yield, and nutritional value (Zhuang et al. 2014; Shahbaz et al. 2012; Prasad et al. 2014). To meet the food supply, it is an essential to produce salt-tolerant crops, which can be sustained on salt-affected lands. Among crops, vegetables play vital role in the human diet because of their nutritional importance in providing vitamins, carbohydrates, proteins, and mineral nutrients.

Plants have developed a specialized network of cation channels across the cellular and vacuolar membranes to regulate the movement of Na^+, K^+ and their balanced availability for cellular functions (Almeida et al. 2017; Blumwald 2000; Shi et al. 2002). The membrane and vacuolar Na^+/H^+ antiporters afford the best mechanism for ionic homeostasis in plants under salt stress. A vacuole Na^+/H^+ antiporter actively moves Na^+ into the vacuole by H^+-ATPase and H^+-PPase coupled with the vacuolar H^+-translocating enzymes, with the H^+-ATPase and the H^+-PPase producing electrochemical H^+ gradients (Liang et al. 2018; Jiang et al. 2010; Bassil and Blumwald 2014).

Physiological and biochemical data had suggested that Na^+/H^+ antiporters are involved in intracellular ion (Na^+), pH regulation, and K^+ homeostasis in plants (McCubbin et al. 2014**;** Leidi et al. 2010; Gaxiola et al. 1999; Blumwald 2000). The significant role of *NHX* genes has been highlighted with the generation of salt-tolerant transgenic plants through the overexpression of *NHX* genes in a wide variety of species (Zeng et al. 2017; Li et al. 2017; Kumar et al. 2017; Sahoo et al. 2016; Fan et al. 2015; Bhaskaran and Savithramma 2011; Tang et al. 2010; Gaxiola et al. 1999; Fukuda et al. 1999; Xia et al. 2002; Wu et al. 2004; Chen et al. 2007; Li et al. 2007). The three vacuolar Na^+/H^+ antiporter genes, namely, *TaNHX1*, *TaNHX2*, and *TaNHX3*, have been functionally characterized in wheat. All these genes complement the growth of salt sensitive yeast mutants under salt stress conditions (Brini et al. 2005; Yu et al. 2007; Lu et al. 2014). The ectopic expression of *TaNHX1* (Brini et al. 2007) and *TaNHX3* (Lu et al. 2014) in tobacco conferred salt stress tolerance. The class I *NHX* gene *TaNHX2* also played critical role to confer salt stress tolerance to salt sensitive yeast mutants (Yu et al. 2007). Significant progress has been reported to enhance salinity stress tolerance by expressing *TaNHX2* gene in higher plants such as soybean, alfalfa, and rice (Cao et al. 2011; Zhang et al. 2015; Wu et al. 2012) and including vegetables, i.e., tomato (Yarra et al. 2012) and chili pepper plants (Bulle et al. 2016).

Eggplant or brinjal or aubergine (*Solanum melongena* L.) and tomato belong to Solanaceae family (Daunay 2008) native to India and China. Eggplant is a commonly grown vegetable plant including potatoes and tomatoes (Doganlar et al. 2002). Increasing demand for vegetables globally boosted the vegetable production. The substantial rise in production has been particularly essential in key vegetable crops such as eggplant, tomato, onion, cucumber, cauliflower, pepper, lettuce, carrot, and spinach (Koike et al. 2007). Eggplant is considered to be a salt-sensitive vegetable (Bresler et al. 1982). However, tolerance varies among eggplant varieties (Unlukara et al. 2010). Shalhevet et al. (1983) observed that 50% yield loss of eggplant at irrigation water salinity, having electrical conductivity of 8.5 dS m^{-1}. Salinity stress in eggplant severely affects the growth and development at the germination and seedling stages (Akinci et al. 2004). It has been observed that salinity stress in eggplant markedly diminishes both fruit weight and number of fruits per plant (Abbas et al. 2010). Improving the salinity stress tolerance of eggplant has become a primary objective in most eggplant growing zones. Although ample improvement has been made in eggplant genetic transformation, achievement in developing transgenic eggplants with high salt tolerance has been limited. Until now, very few studies have been reported to enhance the salinity tolerance of eggplant by expressing bacterial mannitol-1-phosphodehydrogenase (*mtlD*) (Prabhavathi et al. 2002), oat arginine decarboxylase, *adc* gene (Prabhavathi and Rajam 2007), and yeast halo tolerance gene HAL1 (Kumar et al. 2014).

To date, no vacuolar Na^+/H^+ antiporter genes were introduced in eggplant genome to enhance salt stress tolerance. In our study, *TaNHX2* expression in eggplant significantly improves the plant growth under salt stress, strictly associated with the improvement of related physiological processes, including the increased antioxidant enzymatic activities and enhanced contents of photosynthetic parameters. These findings clearly demonstrated that *TaNHX2* acts as an important regulator in salinity tolerance of plants and can be used as a gene resource for molecular breeding of salt-tolerant crop cultivars.

Materials and methods

Plant material

Seeds of elite cultivars of eggplant (*Solanum melongena* L.) PPL variety were procured from National Seeds Corporation Ltd., Secunderabad, India. Seeds were imbibed for 6 h in distilled water and then surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 3–5 min, washed three times with sterile distilled water, and germinated on the surface of MS (Murashige and Skoog 1962) basal medium with 15 g l^{-1} sucrose and 4 g l^{-1} agar. The pH was adjusted to 5.8 before autoclaving. The cultures were maintained under 16 h photoperiod, 25 °C, relative humidity of 60–65%, with fluorescence light (60 μmol m^{-2} s^{-1}). Young and fully expanded leaves of 3–4 cm in length and 2–3 cm in width were excised from all parts of the shoot grown for a month on MS medium and used as explants for transformation experiments.

Binary plasmid and *Agrobacterium* strain

Agrobacterium tumefaciens strain LBA4404 harboring a binary vector pBin438-*TaNHX2* was used for transformation of eggplant. The binary vector pBin438 containing wheat Na^+/H^+ antiporter (*TaNHX2*) gene driven by a double Cauliflower Mosaic Virus (CaMV) 35S promoter (Supplementary Fig. S1) was generously provided by Professor Shouyi Chen and

Jinsong Zhang, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, P. R. China.

Generation of transgenic eggplants

Leaf segments derived from in vitro-regenerated 1-month-old plants were used as explants for transformation and were pre-cultured for 2 days on MS medium augmented with 11.10 μM 6-Benzylaminopurine (BAP) and 2.85 μM Indole-3-acetic acid(IAA). Pre-cultured leaf explants were used to infect with *Agrobacterium* suspension harboring pBin438-*TaNHX2* plasmid for 10 min. The infected explants were blotted on a sterile filter paper and transferred onto co-cultivation medium for 2 days. During co-cultivation, explants were placed on solid MS medium with 30 g l^{-1} sucrose, 4 g l^{-1} agar, 11.10 μM BAP + 2.85 μM IAA, and 100 μM acetosyringone for 2 days maintained in the dark at 25 ± 2 °C. After co-cultivation, leaf explants were transferred onto selection medium containing MS medium with 11.10 μM BAP + 2.85 μM IAA, 100 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime and cultured under 16 h photoperiod at 25 ± 2 °C for 4 weeks. Explants were transferred to a fresh medium once every 2 weeks until kanamycin-resistant buds differentiated and shoots developed. The cultures were transferred onto fresh shoot elongation medium supplemented with 2.22 μM BAP, 100 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime for two sequences of subculture until the shoots achieved a height of 2–4 mm. After 3–4 weeks, the elongated shoots (15–20 mm) were transferred onto half-strength MS medium containing 7.35 μM IBA, 50 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime for rooting. The putative transformed plantlets with well-developed shoots and roots were shifted to pots containing organic substrate and vermiculite (3:1) mixtures, maintained in a greenhouse at 25 ± 2 °C under a 16/8-h light/dark photoperiod and a light intensity of 60 μmol m^{-2} s^{-1}.

Molecular analysis of transgenic eggplants

PCR confirmation of transgenic plants

Genomic DNA was isolated from kanamycin-resistant plants (T_0) as well as from non-transformed plants by CTAB method (Dellaporta et al. 1983) to test for the presence of *TaNHX2* gene in the putative eggplant transgenics using gene-specific primers (*TaNHX2*-1-F and *TaNHX2*-1-R; Supplementary Table S1), which was expected to produce 800 bp, corresponding to the *TaNHX2* gene. PCR amplifications were conducted with initial denaturation at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s, extension at 72 °C for 50 s, and final extension at 72 °C for 10 min. Similar PCR conditions were also used for detection of *TaNHX2* in the T_1 and T_2 generations. The amplified PCR products were separated on a 1.0% (*w*/*v*) agarose gel and visualized using a gel documentation system.

Southern hybridization analysis

Southern blot analysis was performed to verify the *TaNHX2* gene integration and copy number. Genomic DNA (~ 15 μg) isolated from PCR positive plants and non-transformed plants was digested with restriction enzyme, *Hind*III. The digested DNA was separated by electrophoresis on a 0.8% agarose gel and then blotted onto Hybond N^+ nylon membrane (GE Bioscieces, Hong Kong) according to the manufacturer's instructions. A 0.80-kb PCR product of the *TaNHX2* gene fragment was used as a probe, and its radiolabelling was carried out using BioPrime DNA Labeling System (Fischer Scientific, India). After transfer to nylon membrane and hybridizing with probe, the insertion copy number of the transgene was observed on autoradiography film.

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted using RNAiso Plus reagent (Takara, India) from the T_2 southern positive transgenic and non-transformed plants for *TaNHX2* expression analysis. DNase-treated total RNA samples (2 μg) were used for the synthesis of first strand cDNA using MaximaÆ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, India). Semi-quantitative RT-PCR was performed using primers (*TaNHX2*-2-F and *TaNHX2*-2-R; Supplementary Table S1) for amplifying a 376-bp fragment of *TaNHX2*. Eggplant adenine phosphoribosyl transferase (*APRT*, accession JX448345) used as reference gene (Gantasala et al. 2013) to check the expression levels of transgenes using primer pair (*APRT*-1-F and *APRT*-1-R; Supplementary Table S1), which give a 163-bp product with cDNA. The PCR products were analyzed on 1% agarose gel and stained with ethidium bromide.

Real-time PCR

To investigate the *TaNHX2* expression level of transgenic eggplants, quantitative real-time PCR (qRT-PCR) assay was conducted using *TaNHX2* gene-specific primers (*TaNHX2*-3-F and *TaNHX2*-3-R; Supplementary Table S1) and eggplant adenine phosphoribosyl transferase gene (*APRT*, accession JX448345) (*APRT*-2-F and *APRT*-2-R; Supplementary Table S1) as an internal control. Leaves were harvested from T_2 transgenic plants (B1, B2, B4, B5, B8, and B11) and the non-transformed plants when subjected to 200 mM salt stress. Three biological replicates and three technical replications were performed. All experiments were done in triplicate for cDNA synthesis from three batches of plants.

Evaluation of transgenic eggplants under salt stress

Salt stress of transgenic egg plants was assessed by growing T_1 transgenic and control plants in hydroponics and 1-month-old T_2-independent transgenic lines and control plants in pots and exposing them to salt stress under greenhouse conditions. T_1 transgenic lines and control plants were exposed to NaCl stress (150 mM) in hydroponic method for 2 days followed by recovery in water without NaCl for 5 days. The fresh weight and dry weight were recorded after 2 days of NaCl stress. T_2 transgenic eggplant and control plants grown in pots were irrigated with water once in 2 days with gradually increasing concentrations of NaCl (50, 100, 150, and 200 mM), and the 200 mM NaCl treatment continued till 3 weeks. The growth and phenotype were observed after salt stress.

Physiological and biochemical analysis of transgenic and WT eggplants exposed to salt stress

Physiological and biochemical experiments were performed when plants exposed to 200 mM NaCl stress. The leaves and roots of untreated controls (UC, wild-type plants without salt stress), salt-treated control (SC, wild-type plants exposed to 200 mM NaCl), and salt treated (200 mM NaCl) T_2 transgenic lines (B1, B2, B4, B5, B8, and B11) were collected and used for determination of cellular Na^+ and K^+ concentrations, relative water content (RWC), total chlorophyll, proline, ascorbate and malondialdehyde (MDA) content. Quantification of H_2O_2 and O_2^- and antioxidant enzymes assay were carried out in the leaves of materials (UC, SC, T_2 lines). For Na^+ and K^+ concentration measurements, the harvested roots and leaves described above were dried before digesting with concentrated HNO_3 at 90 °C for 30 min and centrifuged at 12,000 rpm for 10 min. The digested samples were diluted with sterile Milli-Q water and analyzed for Na^+ and K^+ content in flame photometer. All measurements were conducted in triplicate. To investigate effect of salt stress on chlorophyll fluorescence of transgenic and control plants, chlorophyll fluorescence (Fv/Fm) was measured with MINI-PAM-II, photosynthesis yield analyzer (Heinz Walz GmbH, Germany). Apart from that, stomatal conductance and transpiration rate of above described plants were also measured by leaf porometer (DECAGON Devices, USA). The photosynthetic and chlorophyll fluorescence estimations were taken in the vicinity of 10:00 and 12:00 h when the encompassing light force was 1000–1200 μmol m^{-2} s^{-1}. After growth in greenhouse for 4 weeks, fully expanded leaves were harvested for estimation of RWC according to Yarra et al. (2012). The total chlorophyll content of the leaves was estimated according to the method described by Hiscox and Israelstam (1979). Antioxidant enzyme assays, ascorbate peroxidase, APX (Chen and Asada 1989); superoxide dismutase, SOD (Wang et al. 2012); guaiacol peroxidase, GPX (Chance and Maehly 1955); glutathione reductase, GR (Smith et al. 1988); free proline (Bates et al. 1973); malondialdehyde, MDA (Heath and Packer 1968); hydrogen peroxide, H_2O_2 (Sagisaka 1976); and super oxide, O_2^- (Elstner and Heupel 1976) were carried out by collecting the leaf materials from the salt stressed (200 mM NaCl) plants as well as from control plants.

Statistical analysis

All experiments were comprised of three samples and performed three times. All data were presented as mean ± SD. Statistical significance was determined by Student's *t* test. Significance was defined as $P \leq 0.05$ and indicated by asterisks.

Results

Eggplant transformation and selection of transformants

The binary vector pBin438-*TaNHX2* was introduced in to eggplant genome via *A. tumefaciens* (LBA4404)-mediated genetic transformation approach. About 70% pre-cultured leaf explants, which had been co-cultivated with *Agrobacterium*, formed shoot buds on MS medium augmented with 11.10 μM BAP + 2.85 μM IAA, 100 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime. Shoots developed from the leaf explants on media of similar composition after four successive weeks of culture (Supplementary Fig. S2b), whereas no shoots were observed from untransformed leaf explants on MS medium containing 100 mg l^{-1} kanamycin (Supplementary Fig. S2a). The elongation of shoots was achieved on MS media containing 2.22 μM BAP, 100 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime after 3 weeks of culture (Supplementary Fig. S2c). The elongated shoots were multiplied subsequently on the same media to recover putative transgenic plants (Supplementary Fig. S2d). The recovered putative transgenic lines were rooted on half-strength MS medium containing 7.35 μM IBA, 50 mg l^{-1} kanamycin, and 250 mg l^{-1} cefotaxime (Supplementary Fig. S2e). Subsequently, they were transferred to the greenhouse for acclimatization (Supplementary Fig. S2f). The T_0 transgenic plants were self-pollinated to produce T_1 seeds. These transgenic eggplants from each line were phenotypically identical and indistinguishable from control plants.

Transgene integration and expression

PCR analysis for genomic integration of the *TaNHX2* gene was carried out on T_0 plants randomly with one non-transgenic line as a negative control. The amplification reaction was carried out using *TaNHX2* gene-specific primers revealed that all the kanamycin-resistant plants became acclimatized and exhibited positive amplification. An 800-bp fragment was observed with the *TaNHX2* gene-specific primers (Fig. 1a), whereas no corresponding band was detected in the untransformed plant (Fig. 1a). The integration of the *TaNHX2* gene into the eggplant genome was further analyzed by southern hybridization for revealing the copy number of transgene. The genomic DNA of *TaNHX2* eggplant transgenics (T_2) and non-transformed plants was digested with *Hind*III and probed with *TaNHX2* gene. The transformed plants demonstrated single to three copy integration events, whereas no hybridization signal was noticed in non-transformed plants (Fig. 1b). The expression of double CaMV35S promoter–driven *TaNHX2* gene in T_2 plants was confirmed by RT-PCR using *TaNHX2* primers specific to 376-bp fragment. As expected, 376-bp band was observed and confirmed the expression of *TaNHX2* in transgenic T_2 plants, whereas no expression was observed in control plants (Fig. 2a). Further verification by real-time PCR analysis also showed the significant expression of *TaNHX2* transcript in all tested T_2 transgenic eggplants under 200 mM NaCl stress (Fig. 2b). As expected, no expression was observed in non-transformed plants (Fig. 2b).

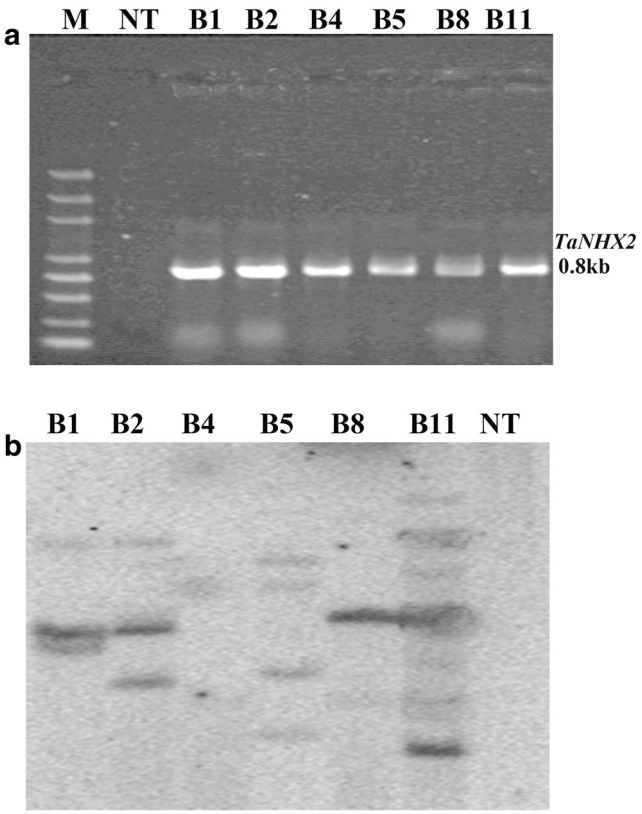

Fig. 1 Molecular analysis of transgenic lines overexpressing *TaNHX2*. **a** PCR confirmation of 0.8 kb product of *TaNHX2* in transgenic lines (B1, B2, B4, B5, B8, and B11). M marker, NT non-transformed. **b** Southern blot hybridization of *HindIII* digested genomic DNA of the six independent T_2 transgenic and NT plants using *TaNHX2* probe

Enhanced tolerance of transgenic plants to high salinity stress (150 mM and 200 mM NaCl)

To further understand the expression of *TaNHX2* improves salinity tolerance of transgenic egg plants, the performance of transgenic plants against NaCl-induced salinity stress was verified. All plants (NT and T_1 transgenic (B2, B4, B5) were watered with 150 mM NaCl in hydroponic method for 2 days in a greenhouse and allowed recovery for up to 5 days. Remarkably, transgenic plants exhibited an increased growth with more leaves, compared with non-transformed plants (Fig. 3). In addition, the transgenic plants exhibited higher fresh weights and biomass than control plants under salt treatment (Fig. 3b, c). The seedlings of three T_1 transgenic lines (B2, B4, and B5) showed 2.07, 2.2, and 1.99 times more fresh weight (Fig. 3b), and 1.52, 1.58, and 1.43 times more dry weight (Fig. 3c) respectively, as compared to seedlings of non-transformed plants.

One-month-old non-transformed plants and southern positive T_2 lines (B1, B2, B4, B5, B8, and B11) were assessed for salinity stress test grown in pots in greenhouse. These plants were irrigated with increasing concentrations of NaCl (0, 50, 100, and 150 mM) for 7 days followed by 200 mM NaCl for a period of 3 weeks (Fig. 4). The transgenic and non-transformed plants were phenotypically indistinguishable under normal conditions (0 mM NaCl) (data not shown). However, after treatment with increased salinity stress conditions, the transgenic plants appeared phenotypically close to normal and increased growth even at 200 mM NaCl (Fig. 4), whereas non-transformed plants were chlorotic with retarded growth and ultimate death (Fig. 4).

Increased ion content (Na^+ and K^+) in transgenic plants

To determine whether overexpression of *TaNHX2* enhances the concentration of ions in transgenic plants, Na^+ and K^+ in leaf and root tissues from transgenic and non-transformed were measured before and after salt treatment. Na^+ content of leaves and roots increased in transgenic plants than non-transformed plants at 200 mM NaCl (Fig. 5a, d). Similarly, treatment with 200 mM NaCl led to significantly increase of K^+ contents in both leaf and root tissues of transgenic plants compared to non-transformed plants (Fig. 5b, e). The similar results were observed that the K^+/Na^+ ratio of leaves and roots of transgenic plants was also higher than that of non-transformed plants (Fig. 5c, f). The transgenic plants

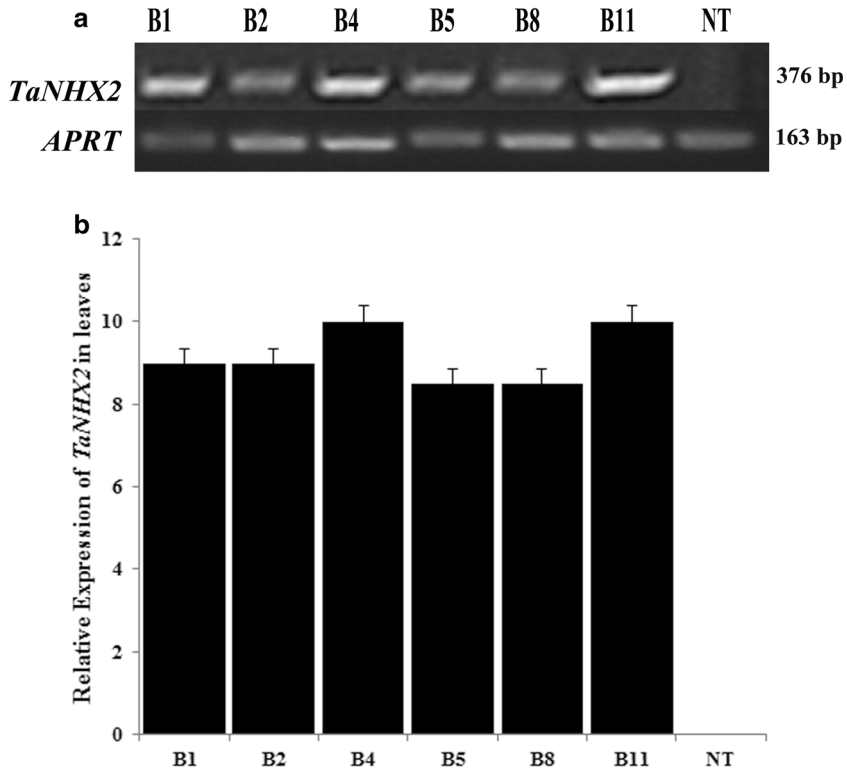

Fig. 2 Expression analysis of *TaNHX2* gene in T_2 transgenic eggplants. **a** *TaNHX2* expression analysis by semi-quantitative RT-PCR in southern positive T_2 transgenic lines (B1, B2, B4, B5, B8, and B11) using *TaNHX2* gene-specific primers. Eggplant adenine phosphoribosyl transferase gene (*APRT*) as internal control. **b** Real-time RT-PCR analysis used to further precisely quantified the expression levels of *TaNHX2* in transgenic eggplants (B1, B2, B4, B5, B8, and B11). *APRT* gene was used as an internal control

accumulated more Na^+ and K^+ in both leaves and roots than non-transformed plants which were treated with NaCl, indicating that the overexpression of *TaNHX2* induced the accumulation of Na^+ and K^+ under salt stress conditions.

Physiological responses in transgenic eggplants under salt stress

To explore the consequence of *TaNHX2* overexpression on salinity tolerance of eggplants, we examined the following physiological parameters when transgenic and non-transformed plants subjected to 200 mM NaCl stress: relative water content, chlorophyll content, proline, ascorbate, and MDA contents. In the absence of salinity stress, no significant differences were found for these physiological parameters tested between non-transgenic and transgenic lines (data not shown). Relative water content and chlorophyll content of transgenic plants was higher than that of non-transformed plants under salt stress (Fig. 6a, b). The percentage of relative water content in transgenic lines was ranging from 77 to 79%, while it was only 45% in salt treated non-transformed plants (Fig. 6a). Interestingly, chlorophyll content of transgenic eggplants was significantly higher when subjected to 200 mM NaCl compared to non-transformed plants and 1.68–1.60-fold higher (Fig. 6b). *TaNHX2*-overexpressing plants had less MDA content (− 2.5- to − 2.2-fold) (Fig. 7a) than non-transformed plants under salinity stress, indicating salinity stress damage due to reactive oxygen species in non-transformed plants (Fig.7a). When subjected to salinity stress (200 mM NaCl), an obvious increase in proline content was observed in transgenic lines relative to the non-transformed plants (Fig.7b) and found to be 2.0- to 2.25-fold higher accumulation in transgenic plants (Fig. 7b). Quantification of H_2O_2 and O_2^- in both transgenic and non-transformed plants suggested that the transgenic lines displayed a significant inhibition in H_2O_2 and O_2^- accumulation compared to non-transformed plants under salinity stress (Fig. 7c, d).

Examining the consequence of salt stress on chlorophyll fluorescence of transgenic and non-transformed eggplants subjected to salinity stress (200 mM NaCl), it showed that the maximum efficiency of photosystem II (Fv/Fm) was found be increased significantly (1.60–2.00-fold) in transgenic compared to non-transformed plants (Fig. 8a). Similarly, the transpiration rate and stomatal conductance were higher in transgenic eggplants compared to non-transformed plants (Fig. 8b, c). Transgenic plants maintained 1.45–1.75-fold higher transpiration rate (Fig. 8b) and 1.35–1.75-fold higher stomatal conductance (Fig. 8c) than non-transformed plants.

Expression of *TaNHX2* improves ROS scavenging capacity in transgenic eggplants

Antioxidant enzymes are responsible for scavenging of ROS in plants. Subsequent assays were carried out to investigate superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR)

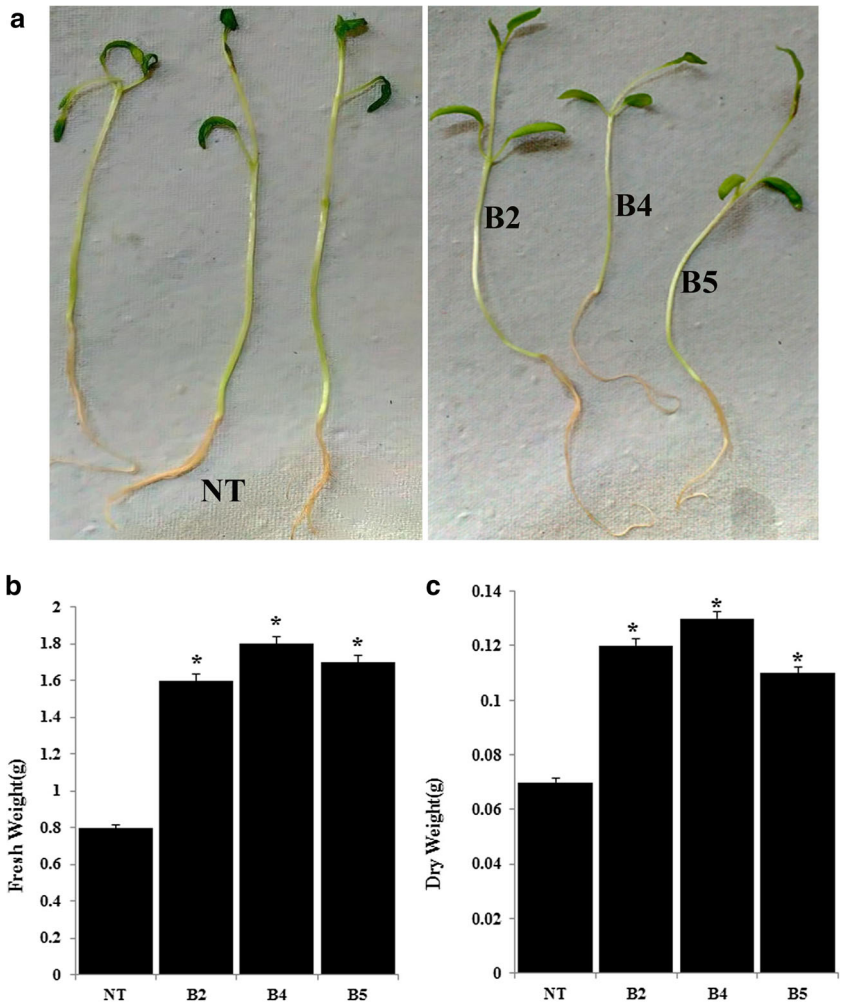

Fig. 3 **a** Phenotypic performance of non-transformed (NT) and transgenic T_1 seedlings (B2, B4, B5) after salt stress (150 mM) for 2 days. **b** Fresh weight. **c** Dry weight in *TaNHX2* transgenic and NT seedlings after salt stress (150 mM). Values are mean ± SD ($n = 3$). Asterisks indicate significant difference compared with NT at $P \leq 0.05$

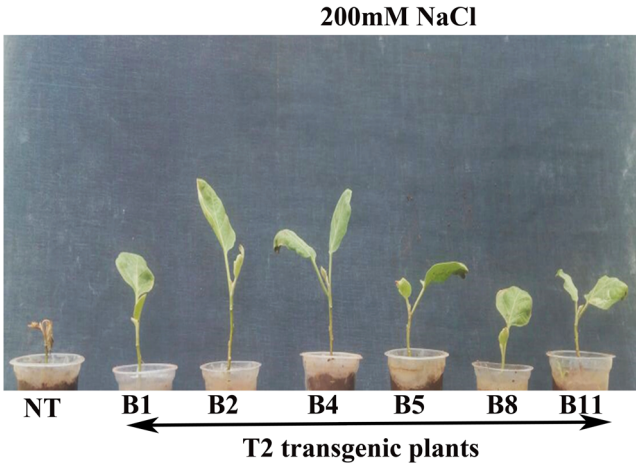

Fig. 4 Enhanced salt tolerance (200 mM NaCl) of *TaNHX2* overexpressing transgenic eggplants. One-month-old non-transformed plants and southern positive T_2 lines (B1, B2, B4, B5, B8, and B11) were subjected salinity stress test grown in pots in greenhouse. These plants were irrigated with increasing concentrations of NaCl (0, 50, 100, and 150 mM) for 7 days followed by 200 mM NaCl for a period of 3 weeks. NT plants show severe chlorosis caused by Na^+ toxicity, whereas the transgenic eggplants show normal phenotype

activities in transgenic and non-transformed plants subjected to 200 mM NaCl stress. In non-transformed plants, the SOD, APX, GPX, and GR activities were lower under salinity stress. However, in transgenic lines, the salinity stress effects on SOD, APX, GPX, and GR activities were significantly alleviated than the non-transformed plants (Fig. 9a–d). The T_2 transgenic eggplants exhibited 2.42–2.43-fold SOD (Fig. 9a), 1.61–1.75-fold APX (Fig. 9b), 1.35–1.42-fold GPX activity (Fig. 9c), and 1.46–1.73-fold GR activity (Fig. 9d) higher than non-transformed plants.

Discussion

Salinity is one of the main threats to sustainable agriculture worldwide (Yadav et al. 2011; Hasegawa 2013). Excessive salinity in soil and irrigation water significantly disturbs the growth, development, and productivity of vegetable crops (FAO 2002; AVRDC 2006). The *TaNHX2* gene discussed in this study belongs to class I *NHX* family localized in vacuolar membrane which is the unique feature of this class.

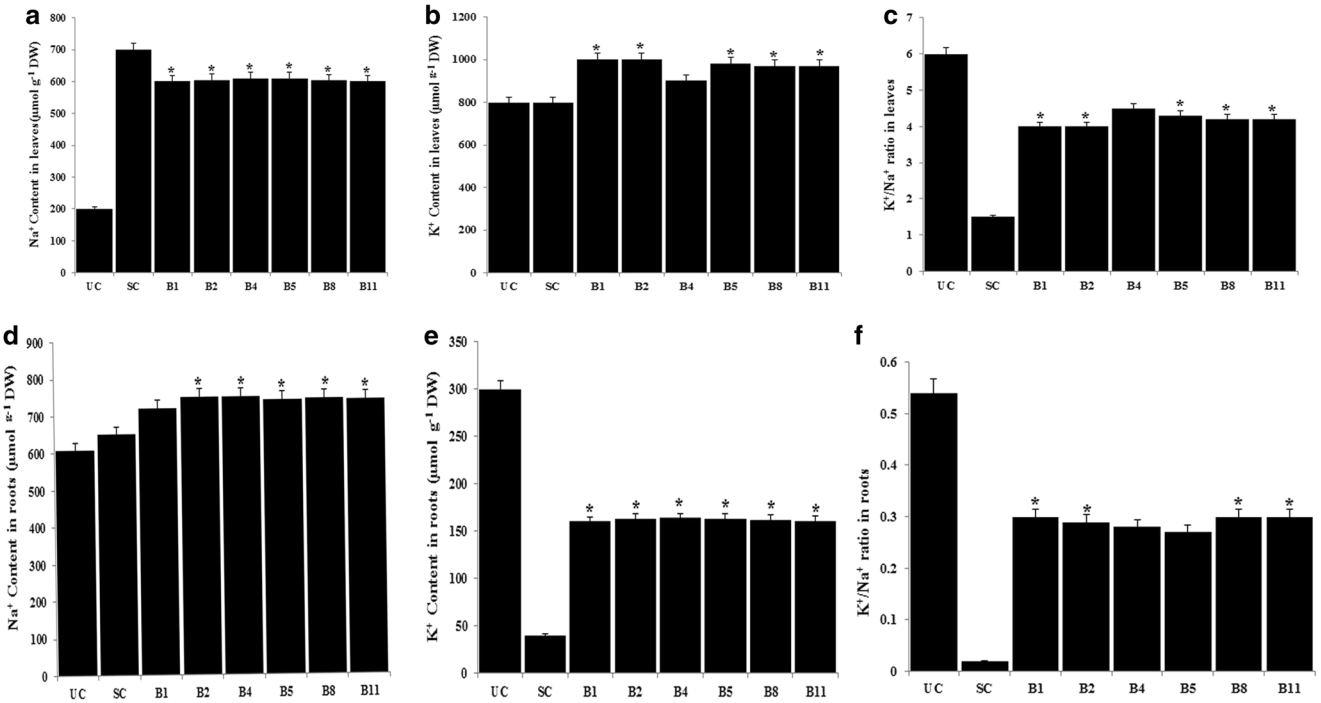

Fig. 5 Sodium (Na^+), potassium (K^+) ion contents, and K^+/Na^+ ratios of leaves and roots from T_2 transgenic eggplants and non-transformed plants (UC, SC) under salt stress (200 mM NaCl). **a** Na^+ content in leaves. **b** K^+ content in leaves. **c** K^+/Na^+ in leaves. **d** Na^+ content in roots. **e** K^+ content in roots. **f** K^+/Na^+ in roots. Ion content measurements are expressed in "μmol g^{-1} DW." UC untreated control, SC salt-treated control. B1, B2, B4, B5, B8, and B11: salt-treated transgenic plants. Values are mean ± SD ($n = 3$). Asterisks indicate significant difference compared with SC at $P \leq 0.05$

Previous studies have shown that *TaNHX2* gene can enhance the salinity tolerance in few species (Cao et al. 2011; Zhang et al. 2015; Wu et al. 2012; Yarra et al. 2012; Bulle et al. 2016). Therefore, to improve salinity tolerance of plants through overexpression of *TaNHX2* gene by transgenic technology has attracted ample attention of plant researchers. Eggplant is an essential vegetable for diet consumption worldwide, and the growth and development of eggplants have been affected by salinity stress, leading to the substantial drop of eggplant productivity. In this work, we intended to improve salinity stress tolerance of eggplants by overexpression of *TaNHX2* gene through *Agrobacterium* mediated transformation.

Salinity-induced fresh weight reduction is a common phenomenon for most of the crop plants and studied (Mozafariyan et al. 2013). Plant dry matter content is a worthwhile consideration to evaluate the plant strategy for the use and procurement of resource. Dadkhah and Grrifiths (2006) reported that plant dry matter is significantly reduced under salinity stress conditions. The effect of salt stress (150 mM NaCl) on biomass of transgenic eggplants overexpressing *TaNHX2* was evaluated at seedling stage, and the T_1 lines

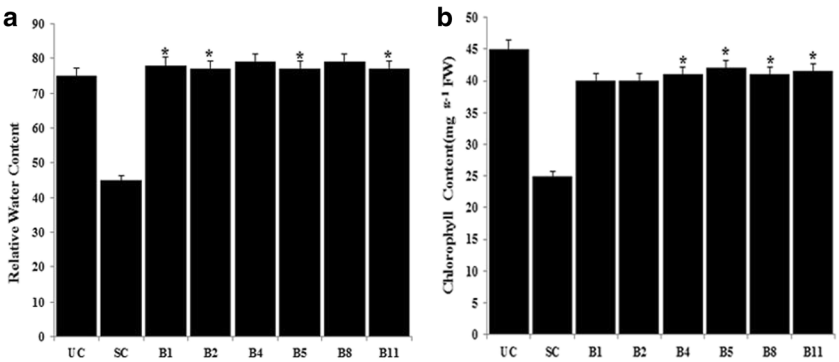

Fig. 6 Stabilized relative water content (RWC) and chlorophyll content in *TaNHX2* overexpressed T_2 transgenic eggplants under salt stress (200 mM NaCl). **a** Relative water content (%). **b** Chlorophyll content (mg g^{-1} FW). UC untreated control, SC salt-treated control. B1, B2, B4, B5, B8, and B11: salt-treated transgenic plants. Values are mean ± SD ($n = 3$). Asterisks indicate significant difference compared with SC at $P \leq 0.05$

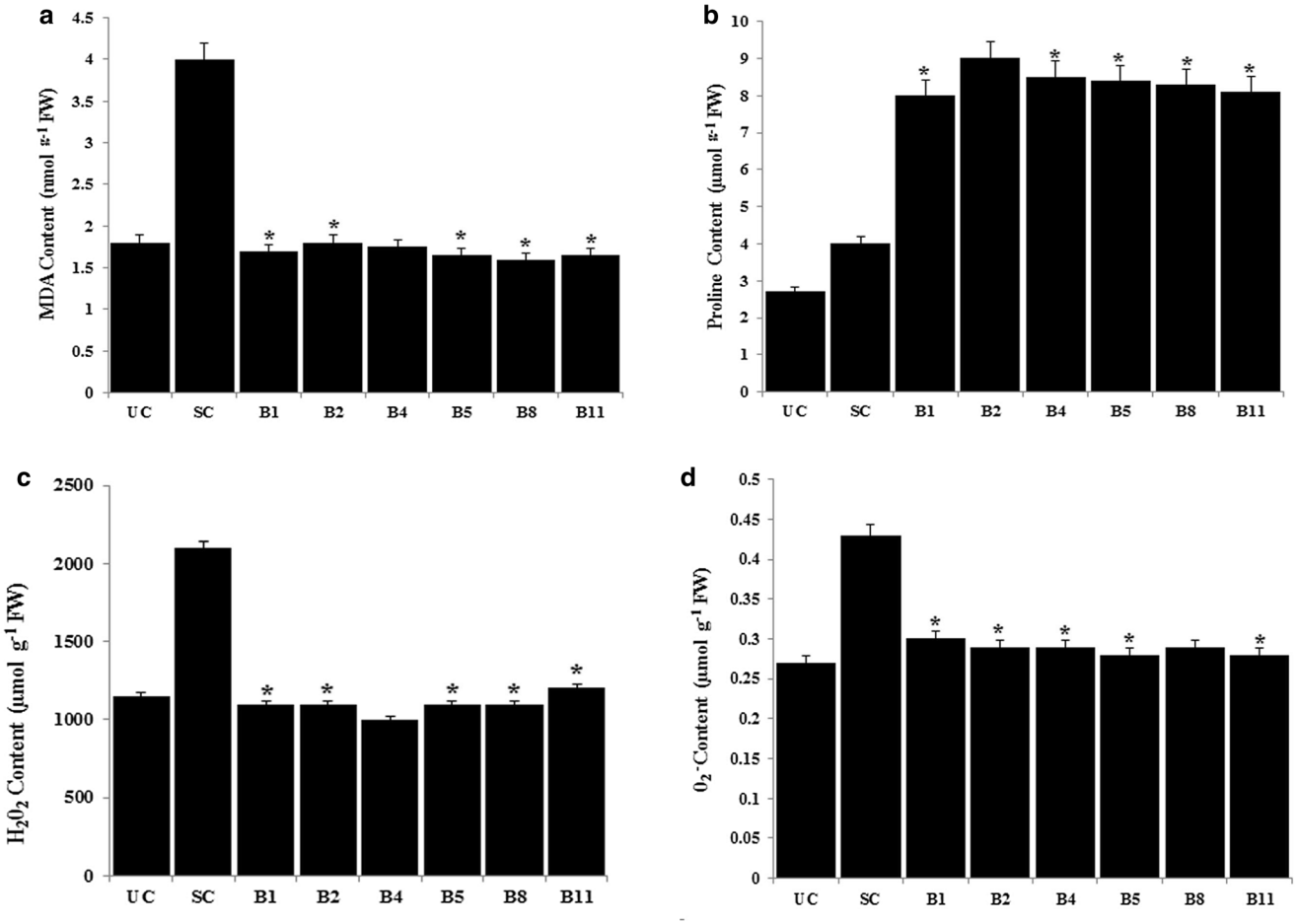

Fig. 7 Analysis of lipid peroxidation, proline accumulation, and ROS scavenging capacity in T_2 transgenic eggplants and non-transformed plants (UC, SC) under salt stress (200 mM NaCl). **a** Levels of lipid peroxidation expressed in terms of MDA content (nmol g^{-1} FW). **b** Changes in the level of proline accumulation (µmol g^{-1} FW). **c** H_2O_2 content (µmol g^{-1} FW). **d** O_2^- content (µmol g^{-1} FW). UC untreated control, SC salt-treated control. B1, B2, B4, B5, B8, and B11: salt-treated transgenic plants. Values are mean ± SD ($n = 3$). Asterisks indicate significant difference compared with SC at $P \leq 0.05$

exhibited significantly higher fresh and dry weight compared with non-transformed plants. This significant increase in biomass under salinity stress clearly indicated that the transgenic eggplants withstand to salinity stress conditions.

Compared with the non-transformed plants, the T_2 eggplants exhibited improved salinity tolerance. After exposure to high salinity stress (200 mM NaCl), the non-transformed plants displayed growth inhibition, chlorosis, and even death, whereas the T_2 plants maintained their normal growth and survival. These results indicate that overexpression of *TaNHX2* gene under salt stress is likely responsible for the increased compartmentalization of Na^+ into vacuoles confers the improved salt tolerance (Gaxiola et al. 2001, 2007; Yamaguchi et al. 2013; Yarra et al. 2012, Bulle et al. 2016, Sahoo et al. 2016).

Ion homeostasis with low Na^+ and high K^+ concentrations in the cytoplasm is vital for maintaining normal metabolic and physiological processes, e.g., the activity of many cytosolic enzymes (Zhu 2003). A commensurate increase was found in the Na^+/K^+ contents in leaves and roots of *TaNHX2* transgenic compared with non-transformed plants under salt stress conditions, indicating that Na^+/H^+ antiporter gene *TaNHX2* improved the salt tolerance by increasing Na^+ accumulation and retained K^+/Na^+ equilibrium. However, accumulation of Na^+ content in roots is significantly higher than the leaves in transgenic plants. This is possibly advantageous for the normal growth and development of transgenic plants under slat stress conditions. The occurrence of higher Na^+ and K^+ contents in leaves and roots of transgenic plants overexpressing *NHX* genes was also reported in mungbean (Sahoo et al. 2016), sweet potato (Fan et al. 2015), and alfalfa (Li et al. 2011). Our results strongly supported that *NHX* proteins largely functioned as $Na^+/K^+(H^+)$ antiporter and played significant role in Na^+/K^+ homeostasis by regulating their uptake, transport, and compartmentalization.

Measurement of relative water content (RWC) is necessary to know the plant water status under salinity stress to ascertain that up to what extent cellular water content is retained, as all

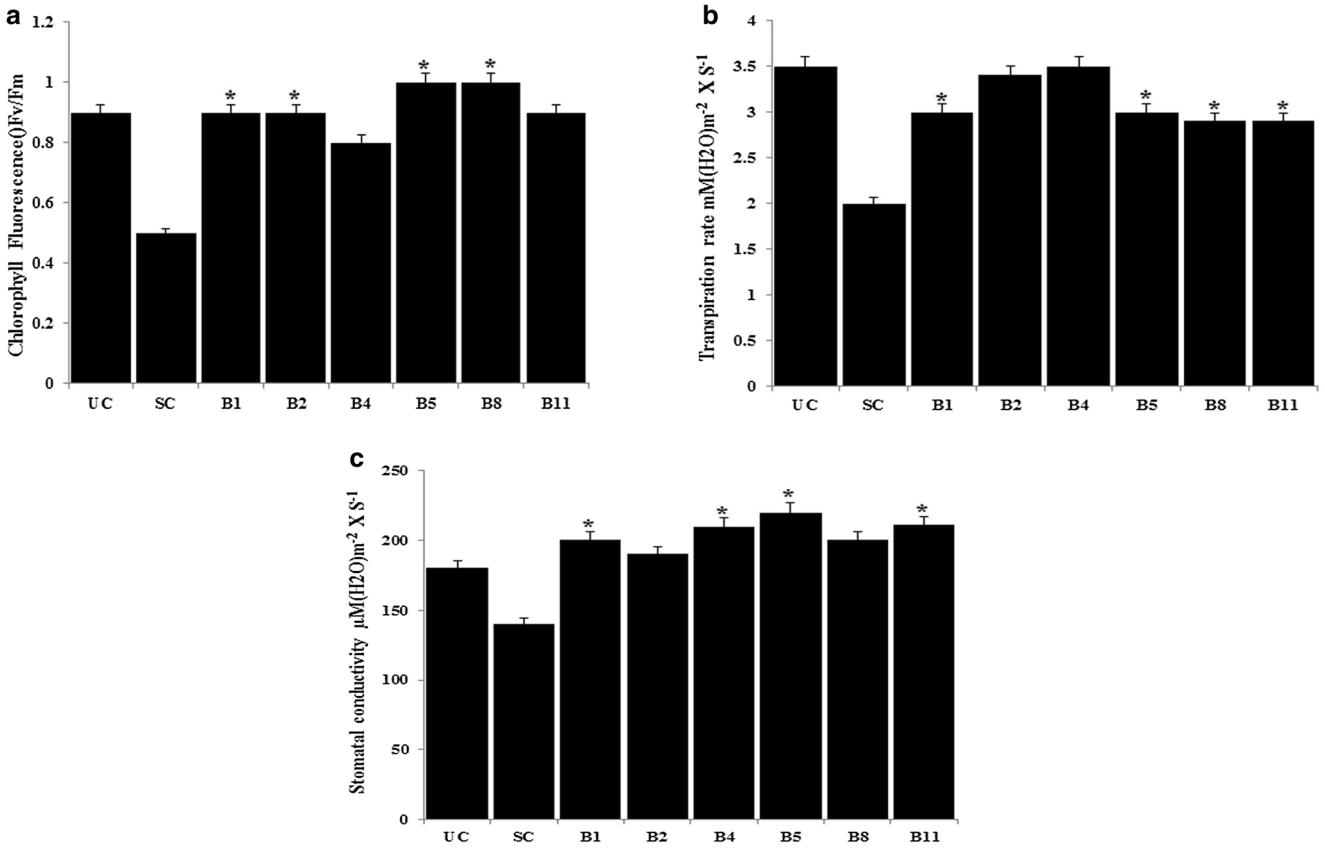

Fig. 8 Photosynthetic performance in the leaves of T_2 transgenic eggplants and non-transformed plants (UC, SC) under salt stress (200 mM NaCl). **a** PSII photosynthetic efficiency (Fv/Fm). **b** Transpiration rate (mM (H_2O) $m^{-2}x\ s^{-1}$). **c** Stomatal conductance ($\mu M(H_2O)\ m^{-2}x\ s^{-1}$). UC untreated control, SC salt-treated control. B1, B2, B4, B5, B8, and B11: salt-treated transgenic plants. Values are mean ± SD ($n = 3$). Asterisks indicate significant difference compared with SC at $P \leq 0.05$

metabolic activities within the cell are dependent on the availability of adequate amount of water (Haripriya et al. 2010; Ashraf et al. 2011). Shaheen et al. (2013) reported that there is a significant decrease in relative water content of eggplants under salinity stress. In contrast, *TaNHX2* overexpressing transgenic eggplants displayed significant increase in relative water content compared to non-transformed plants under high salinity conditions (200 mM NaCl). Our results are in agreement with the previous reports, where relative water content of the *TaNHX2* expressing transgenic plants is significantly higher compared to non-transformed plants under salt stress conditions (Bulle et al. 2016; Yarra et al. 2012).

At 200 mM NaCl salt stress, the decrease in chlorophyll contents in the transgenic plants was lesser than the non-transformed plants, indicating a positive association between the expression of *TaNHX2* and salinity stress tolerance in leaf tissues. These finding are consistent with the previous reports of *TaNHX2* overexpression in tomato (Yarra et al. 2012) and chili pepper plants (Bulle et al. 2016). Photosynthetic apparatus is sensitive and easily damaged under salt stress conditions (Sixto et al. 2006), which leads to reduction of the complete plant growth and development. The transgenic eggplants expressing *TaNHX2* gene were found able to retain higher chlorophyll fluorescence ratio (Fv/Fm), higher transpiration rate, and higher stomatal conductivity under 200 mM NaCl stress compared to non-transformed plants. These observations indicated that the overexpression of *TaNHX2* in eggplant alleviated the inhibition of photosynthesis and PSII photoinhibition under salt stress conditions. This is consistent with previous reports, which suggest that *NHX* gene expression in transgenic plants protects the damage of photosynthetic apparatus under salt stress conditions (Kumar et al. 2017).

It has been known that variation in the levels of free proline content is a common phenomenon in plants response to salinity stress (Liu and Zhu 1997; Armengaud et al. 2004). We also found the increased amounts of proline contents in transgenic plants than non-transformed plants under high saline conditions (200 Mm NaCl), suggesting that *TaNHX2* may induce the proline synthesis genes to confer salt tolerance in eggplants. This is consistent with the results obtained from overexpressing *TaNHX2* in chili pepper plants (Bulle et al. 2016), *RtNHX1* in Arabidopsis (Li et al. 2017), and *AtNHX1* in sweet potato (Fan et al. 2015).

Oxidative stress is an indication, evidenced by the accumulation of reactive oxygen species ROS in plants when subjected to salinity stress (Verslues et al. 2007). However, plants

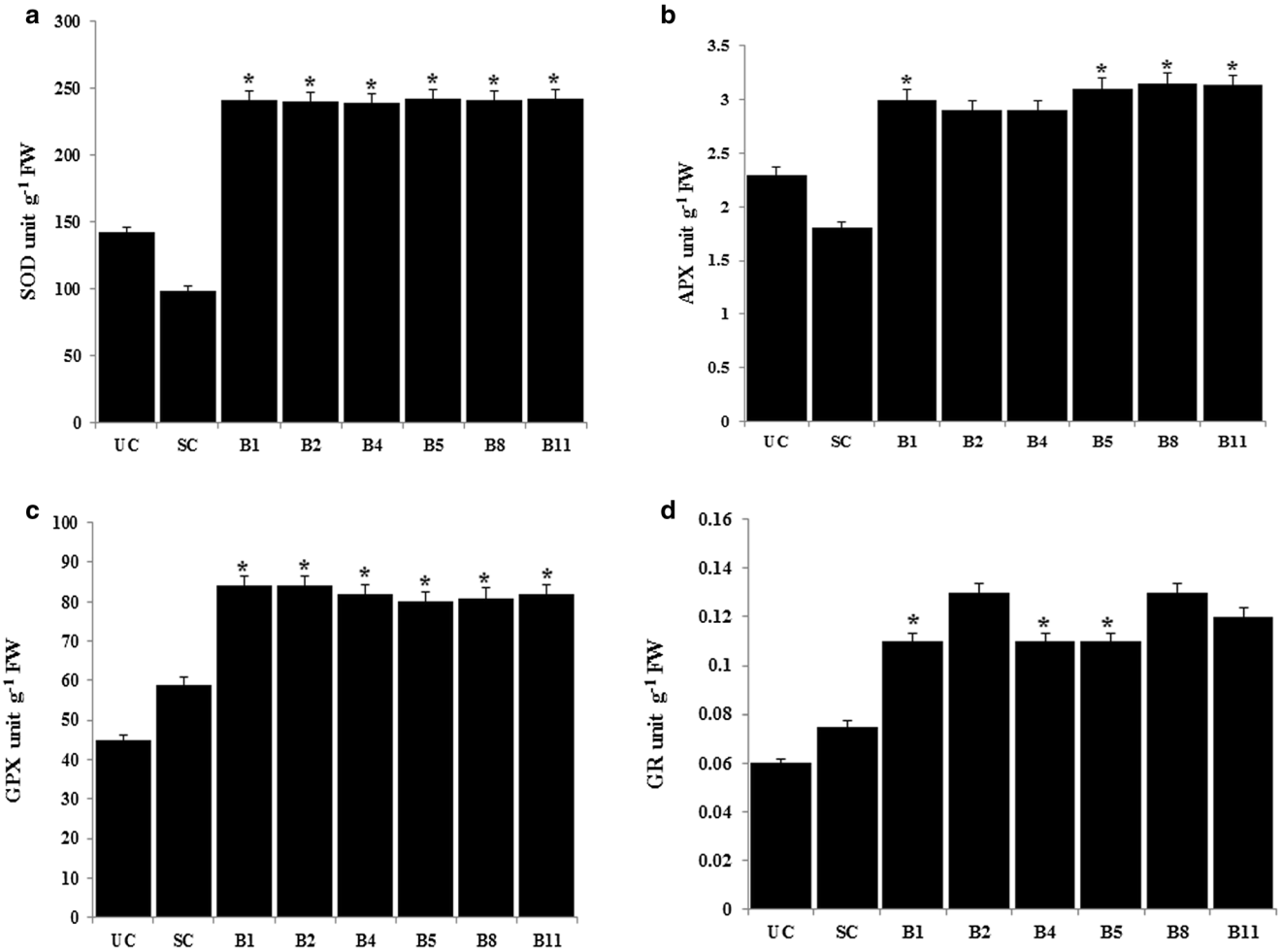

Fig. 9 Effect of salt stress (200 mM NaCl) on antioxidant responses of T_2 transgenic eggplants and non-transformed plants (UC, SC). **a** Changes in superoxide dismutase enzyme activity (SOD, unit g^{-1} FW). **b** Changes in ascorbate peroxidase enzyme activity (APX, unit g^{-1} FW). **c** Changes in guaiacol peroxidase enzyme activity (GPX, unit g^{-1} FW). **d** Changes in glutathione reductase enzyme activity (GR, unit g^{-1} FW). UC untreated control, SC salt-treated control. B1, B2, B4, B5, B8, and B11: salt-treated transgenic plants. Values are mean ± SD ($n = 3$). Asterisks indicate significant difference compared with SC at $P \leq 0.05$

have adopted a complex antioxidant system to detoxify stress-induced ROS, in which various enzymes play vital roles, in order to scavenging ROS and protecting the cells against oxidative stress (Jaleel et al. 2009; Miller et al. 2010). In salt-stressed eggplants, increased oxidative stress was observed with improved H_2O_2 and O_2^- contents, however significantly less H_2O_2 and O_2^- and increased SOD, APX, GPX, and GR activities in *TaNHX2* transgenic eggplants in comparison with non-transformed plants. Lipid peroxidation generally happens through accumulation of MDA content when plants subjected to abiotic stresses (Verslues et al. 2007). Indeed, *TaNHX2* transgenic plants displayed less accumulation of MDA content in leaves compared to non-transformed plants, indicating effectively improved cell membrane homeostasis leads to the salinity tolerance of transgenic eggplants. Previous studies indicated that transgenic plants overexpressing *NHXs* were more effective in scavenging reactive oxygen species because they had improved antioxidant enzyme activity and decreased MDA content (Li et al. 2017; Sahoo et al. 2016; Bulle et al. 2016; Gouiaa et al. 2012; Wei et al. 2011; Wang et al. 2016). Taken together, all these results indicated that *TaNHX2* gene was able to confer salt tolerance in transgenic eggplants and might facilitate the transgenic plants to make the indispensable osmotic and antioxidant adjustments.

In conclusion, our results confirmed that heterologous expression of *TaNHX2* gene explicitly improved salt tolerance and growth of transgenic eggplants, through enhanced sequestration of ions into the vacuoles, unhampered photosynthesis, altering the activation of ROS scavenging system, and the levels of protective compounds, such as MDA and proline. However, the fact that overexpression of *TaNHX2* dramatically enhances salinity tolerance of eggplants suggests that this gene will have the potential to prominently improve stress tolerance in other vegetable crops like eggplant. Further field trials of transgenic eggplants under severe environmental conditions such as salinity remain to be carried out in order to

grow them in salt-affected regions. This data laid a concrete foundation for engineering *NHX* genes like *TaNHX2* for developing salinity stress-tolerant edible crop plants without any growth defects.

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Author contributions RY and PBK conceived the experiment. RY performed the experiment. RY and PBK analyzed the data. RY and PBK wrote the manuscript. All authors approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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