

# Enhanced salinity stress tolerance in transgenic chilli pepper (*Capsicum annuum* L.) plants overexpressing the wheat antiporter (*TaNHX2*) gene

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**Abstract** Transgenic chilli pepper (*Capsicum annuum* L.) plants tolerant to salinity stress were produced by introducing the wheat  $\text{Na}^+/\text{H}^+$  antiporter gene (*TaNHX2*) via *Agrobacterium*-mediated transformation. Cotyledonary explants were infected with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pBin438 that contains a wheat antiporter (*TaNHX2*) gene driven by the double CaMV 35S promoter and *NPT II* gene as a selectable marker. PCR and semiquantitative RT-PCR analysis confirmed that the *TaNHX2* gene had been integrated and expressed in the  $T_1$  generation of transgenic pepper plants as compared to the non-transformed plants. Southern blot analysis further verified

the integration and presence of *TaNHX2* gene in the genome of chilli pepper plants. Biochemical assays of these transgenic plants revealed enhanced levels of proline, chlorophyll, superoxide dismutase, ascorbate peroxidase, relative water content, and reduced levels of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), malondialdehyde compared to wild-type plants under salt stress conditions. The present investigation clearly showed that overexpression of the *TaNHX2* gene enhanced salt stress tolerance in transgenic chilli pepper plants.

**Keywords** *TaNHX2* · RT-PCR · Southern blot · Transgenic pepper

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## Introduction

Soil salinity is the most prevalent abiotic stress that severely reduces the growth and yield of many important food crops worldwide to a great extent. About 20 % of world irrigated agricultural land was adversely salt affected in more than 100 countries (Flowers and Yeo 1995; Rengasamy 2006). High-salinity stress disturbs ion homeostasis in plant cells. Ion-specific stresses resulting from altered  $\text{K}^+/\text{Na}^+$  ratio and  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations are detrimental to plants. This ion flux causes a loss of homeostasis and plants respond to this condition by removing the sodium from the cytosol, through the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter. A vacuole  $\text{Na}^+/\text{H}^+$  antiporter, sequestering excess  $\text{Na}^+$  into the vacuole by using the

electrochemical gradient of protons generated by the vacuolar  $H^+$ -translocating enzymes ( $H^+$ -ATPase and the  $H^+$ -PPase), has been identified as an important salt tolerance determinant (Apse et al. 1999; Gaxiola et al. 1999; Quintero et al. 2000; Blumwald 2000; Zhang and Blumwald 2001; Zhang et al. 2001; Zhu 2003; Yamaguchi and Blumwald 2005; Leidi et al. 2010). Different  $Na^+/H^+$  antiporters have been identified and intensively studied in Arabidopsis, rice, beetroot, cotton, maize, salsa, clover, and tomato (Gaxiola et al. 1999; Fukuda et al. 1999; Xia et al. 2002; Wu et al. 2004; Chen et al. 2007; Li et al. 2007; Tang et al. 2010; Bhaskaran and Savithamma 2011). Among the several  $Na^+/H^+$  antiporter genes, the wheat *TaNHX2* gene is one of the salt stress-induced gene. The over expression of the *TaNHX2* gene in transgenic soybean, alfalfa, tomato, and rice showed improved tolerance to salinity (Cao et al. 2011; Zhang et al. 2012; Yarra et al. 2012; Wu et al. 2012). Bojórquez-Quintal et al. (2014) hypothesized that the expression of HKT1 transporter could induce accumulation of proline there by increases salt tolerance in *Capsicum chinense* Jacq. Salt tolerance is a complex mechanism controlled by many genes (Bojórquez-Quintal et al. 2014). Therefore, manipulating the expression level of certain salt stress-induced genes is necessary to improve an agronomically important crop plants.

Chilli pepper (*Capsicum annum* L.) is an important vegetable and indispensable spice crop of the Solanaceae family and grown for supplying its pungent fruits, which are used both ripe and green to impart pungency to the food. Green chillies are rich in vitamin A and C, minerals, and proteins while dry chillies are rich in vitamin A and D which offers hotness and pungency to the food. The demand for chillies has increased recently because of their high nutraceutical compound capsaicin. Compared to the other vegetable crops, economic yield of chilli pepper plants are more adversely affected by biotic (Egea et al. 2002; Venkataiah et al. 2003; Suzuki and Mori 2003,) and abiotic stress (Ochoa-Alejo and Ramírez-Malagón 2001; Subramanyam et al. 2011). Transgenic approaches provide a powerful tool for gene function analysis in plants. However, chilli pepper plants are still recalcitrant to current transformation technologies, limiting the genetic improvement against biotic and abiotic stress (Kothari et al. 2010). To our knowledge only one report has been published on

the metabolic engineering of salt tolerance in chilli pepper using an osmotin gene (Subramanyam et al. 2011). Hence, developing salt-tolerant chilli pepper plants is essential for sustaining food production.

In the present study, transgenic chilli pepper plants were generated by overexpressing the wheat  $Na^+/H^+$  antiporter gene *TaNHX2* via *Agrobacterium*-mediated transformation, and further, possible mechanisms of salt stress tolerance for chilli pepper plants were addressed by biochemical assays.

## Materials and methods

### Plant material

Seeds of *C. annum* (Cv G4) were obtained from regional agricultural research station (RARS), Lam, Guntur, Andhra Pradesh, India. The seeds were surface-sterilized under aseptic conditions in a laminar flow chamber by immersion in an aqueous solution of 0.1 % (w/v) mercuric chloride ( $HgCl_2$ ) for 3–4 min followed by 4–5 rinses with autoclaved deionized water, and then blot dried. Surface-sterilized seeds were placed on Murashige and Skoog's basal salt solution (Murashige and Skoog 1962) supplemented with 2 % sucrose and 0.8 % (w/v) agar (Himedia, India). The medium was adjusted to pH  $5.7 \pm 0.1$ , autoclaved at 120 °C and 1.1 kPa for 20 min, and allowed to cool before the addition of surface-sterilized seeds. The seeds were germinated in a growth room environment at  $25 \pm 2$  °C with a light regime of 16 h and irradiance of  $65 \mu mol m^{-2} s^{-1}$  provided by two fluorescent lamps (Philips, India, 110 W). Cotyledons of germinated seedlings (8–10 days old) without petiole and apical parts were cut into desirable size each of 1.0 cm<sup>2</sup> and used as explants.

### Transformation vector and bacterial strain

The plasmid pBin438 containing the *TaNHX2* gene driven by a double Cauliflower Mosaic Virus (CaMV) 35S promoter (Yu et al. 2007) was kindly provided by Professor Shouyi Chen, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pBin438-*TaNHX2* was used for stable transformation of chilli pepper.

## Plant transformation

Cotyledons from 10-day-old plants were used as explants for transformation. Explants were placed on pre-culture medium [PCM = MS Salts + 0.5 mg l<sup>-1</sup> thidiazuron (TDZ) + 0.2 mg l<sup>-1</sup> indole-3-acetic acid (IAA)] for 2 days and then infected with *A. tumefaciens* for 15 min at 28 °C with gentle shaking. Explants were blotted on sterile filter paper to remove excess bacterial solution before transfer to co-cultivation medium [CCM = MS salts + 0.5 mg l<sup>-1</sup> TDZ + 0.2 mg l<sup>-1</sup> IAA + 100 µM acetosyringone (AS)] and incubated in the dark at 25 ± 1 °C for 2 days. After co-cultivation, cotyledon explants were transferred to selective shoot induction medium (SSIM = MS Salts + 0.5 mg l<sup>-1</sup> TDZ + 0.2 mg l<sup>-1</sup> IAA + 80 mg l<sup>-1</sup> kanamycin + 200 mg l<sup>-1</sup> cefotaxime). For shoot proliferation and an initial selection, cotyledon explants were cultured for 3 weeks on regeneration medium (MS Salts + 0.5 mg l<sup>-1</sup> TDZ + 0.2 mg l<sup>-1</sup> IAA) containing 80 mg l<sup>-1</sup> kanamycin and 250 mg l<sup>-1</sup> cefotaxime. After 3 weeks of shoot proliferation, shoots (2–4 mm length) that had emerged from wounded ends of co-cultivated cotyledon explants were separated and transferred to selective shoot elongation medium (SSEM = MS Salts + 0.5 mg l<sup>-1</sup> TDZ + 0.2 mg l<sup>-1</sup> IAA + 10 mg l<sup>-1</sup> AgNO<sub>3</sub> + 80 mg l<sup>-1</sup> kanamycin + 200 mg l<sup>-1</sup> cefotaxime). After 3–4 weeks, the elongated shoots were transferred onto shoot rooting media (SRM = ½ MS Salts + 40 mg l<sup>-1</sup> kanamycin + 200 mg l<sup>-1</sup> cefotaxime). The regenerated plantlets with well-developed shoots and roots were finally transferred to plastic pots filled with compost and then transferred to the greenhouse for seed production. The experiments were repeated at least three times and three replicates kept per experiment with approximately 225 explants for transformation studies. The data were statistically analyzed by analysis of variance (ANOVA, *P* < 0.05) followed by Duncan's (Duncan 1955) multiple range test for mean comparison.

## PCR confirmation of transgenic plants

For an initial selection of transformants, total genomic DNA was isolated from young leaves of putative transgenic and wild-type plants using a standard cetyl trimethylammonium bromide (CTAB) protocol, as described by Murray and Thompson (1980). PCR was

performed by using a thermocycler (Biorad-C1000, Hercules, CA, USA). A primer set (forward 5'-ATGG GGTACCAAGTGGTGGC-3' and reverse 5'-ATGA GAGGTAGGCCATGAGC-3') was used to amplify a specific 800 bp of DNA sequence in transgenic plants corresponding to the *TaNHX2* gene. For the detection of *TaNHX2* gene, PCR conditions were set as initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 30 s, extension at 72 °C for 50 s, and final extension at 72 °C for 10 min. Plasmid DNA served as a positive control, and DNA from a non-transformed control plant was used as negative controls. Amplified PCR products were visualized and photographed using gel documentation system (Bio-rad; Gel docXR+) after electrophoresis on a 1 % (w/v) agarose (Seakem LE, Kolen, Germany) gel and detected by ethidium bromide staining.

## Southern blot hybridization

Southern blot analysis was performed to verify the integration and copy number of the transgene. Genomic DNA (10 µg) from PCR positive and non-transformed plants was digested with *HindIII*. The digested DNA was separated by electrophoresis on a 0.8 % agarose gel and then blotted onto a nylon membrane (Hybond-N+, Amersham biosciences, UK). The membranes were pre-hybridized for 3 h using the membrane supplier's protocol and hybridized at 65 °C. Following hybridization for at least 16 h, the filters were washed with a solution containing 2 × SSC, 0.1 % SDS and then washed in 1 × SSC, 0.1 % SDS at 65 °C. A 0.80-kb PCR product of the *TaNHX2* gene labeled with digoxigenin (DIG) was used as a probe for DNA blot hybridization. After transfer to nylon membrane and hybridizing with DIG-labeled probe, the insertion copy number of the transgene was observed on autoradiography film.

## Gene expression analysis by semiquantitative reverse transcription PCR

To detect the expression of transgenes by reverse transcription PCR (RT-PCR), total RNA was isolated from young leaves of T<sub>1</sub> transgenic plants using the protocol described by Camacho-Villasana et al. (2002) and treated with DNase I (Takara, Dalian, China) to remove DNA traces. Total RNA (2 µg) was used as a

template for the synthesis first-strand cDNA with M-MLV reverse transcriptase, RNase H Minus kit (Promega, Madison, WI, USA). PCR of the *TaNHX2* gene was carried out according to the conditions described above. The house-keeping gene actin was used as an internal control to check the expression levels of transgenes. The primer sequences of actin were forward: 5'-CCACCACAGCCGAACGGG-3' and reverse: 5'-ACCCGGGAACATGGTGGAAACC-3', which gives a 325-bp product with cDNA. The amplified products were electrophoresed on 2.0 % agarose gel.

#### Biochemical analysis of transgenic chilli pepper plants under salt stress

To examine the salt tolerance *in vivo*, we used a method described previously (Bhaskaran and Savithramma 2011) with slight modifications. Different transgenic lines and the wild type were planted in plastic pots (with holes at the bottom) containing Agra-Vermiculite (PULL Rhenen, 3911 TX Rhenen, the Netherlands), irrigated with 50 ml of Hoagland solution for 2 weeks in the greenhouse. The concentrations of NaCl supplementation were increased incrementally by 50 mM every 4 days for each group, to the final concentration of 50, 100, 150, and 200 mM, respectively. The stress treatment comprised of three plastic pots in two replicates. After salt treatment for 2 weeks, the plants were used for biochemical analysis.

#### Estimation of free proline content and H<sub>2</sub>O<sub>2</sub> concentration

Proline content was estimated from the leaves of T<sub>1</sub> plants after different concentrations of salt treatment independently along with the non-transformed plants by using the method of Lei et al. (2007). Hydrogen peroxide levels were determined according to the method described by Velikova et al. (2000). Five hundred milligrams of leaf tissues were homogenized in an ice bath with 5 ml 0.1 % (w/v) TCA. The homogenate was centrifuged at 12,000 × g for 15 min. Fifty microliters of the extract was treated with 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The absorbency of supernatant was read at 390 nm.

#### Determination of ascorbate peroxidase (APX), superoxide dismutase (SOD) activities, and malondialdehyde (MDA) concentration

After treatment with different concentrations of NaCl, APX activity was measured according to the protocol described by Chen and Asada, 1989. Leaf tissues (500 mg) were homogenized in an ice bath with 1.5 ml 10 mM sodium phosphate buffer (1 mM EDTA, 5 mM ascorbate). The homogenate (0.5 ml) was treated with the reaction mixture (50 mM phosphate buffer, pH 7.0, 0.5 mM ascorbate, 0.2 mM H<sub>2</sub>O<sub>2</sub>). The enzyme activity was calculated at absorbency of 260 nm. Superoxide dismutase (SOD, EC1.15.1.1) activity (Wang et al. 2012) and malondialdehyde (MDA) concentration were determined in transgenic and non-transformed chilli pepper plants, using the method described previously by Subramanyam et al. (2011).

#### Leaf disk assay

The leaf disk assay was performed using a method described previously (Bhaskaran and Savithramma 2011; Jha et al. 2013) with slight modifications to analyze transgenic plants for their salt tolerance conferred by the expression of the *TaNHX2*. Healthy and fully expanded chilli pepper leaves from WT and transgenic *C. annuum* plants (T<sub>1</sub> generation) of similar age were detached. Leaf disks 5 mm in diameter were punched out and floated in 6 ml of half-strength Hoagland media with different concentrations of sodium chloride (0, 100, 150, and 200 mM) for 72 h. The leaf disks were kept under 16 h light/8 h dark at 25 ± 2 °C. The effects of this treatment on leaf disks were assessed by observing phenotypic changes. Leaf disks kept in half-strength Hoagland solution were taken as control.

#### Evaluation of relative water and chlorophyll contents

Relative water content (RWC) and chlorophyll content from non-transformed and three transgenic lines (*Cap1*, 3, 4) were measured after 2 weeks of salt treatment. RWC was measured by using the formula

$$\text{RWC (\%)} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$

where FW, fresh weight; DW, dry weight; TW, turgid weight (Yarra et al. 2012). The total chlorophyll content of the leaves was estimated according to the method described by Hiscox and Israelstam (1979). The experiment was repeated three times with three different transgenic lines ( $n = 3$ ).

#### Determination of ion leakage

Ion leakage was measured based on the method of Fan et al. (1997). Leaves of transgenic and non-transgenic plants after salinity treatment were detached and washed in deionized water. Ion leakage was determined by measurement of electrolyte leaked from transgenic and non-transgenic plant leaves. Four leaves of each treatment (0, 50, 100, 150, and 200 mM NaCl) were immersed in 5–8 ml of 0.4 M mannitol at 23 °C with gentle shaking for 3 h, and the bathing solution was measured for conductivity. The total conductivity was determined after boiling the sample for 10 min. The conductivity due to leakage was expressed as the percentage of the initial conductivity versus the total conductivity.

#### Data analysis

The significance of the effect of the transgene under salt treatment was performed in Microsoft excel 2010 and analyzed by Student's t test or ANOVA. All data were presented as mean  $\pm$  standard deviation (SD) ( $n = 3$ ).

## Results

#### Selection and molecular confirmation of transgenic chilli pepper plants

The pBin438 vector with the *TaNHX2* gene from wheat driven by a double CaMV 35S promoter (Fig. 1a) was introduced into *C. annuum* L. through *Agrobacterium*-mediated transformation (Figure S2). Ten-day-old cotyledons were pre-cultured on PCM (Table S1) for 2 days and then infected with *Agrobacterium* followed by 2 days of co-cultivation. When co-cultivated explants were cultured on selection medium (SSIM media, Table S1) for 3 weeks, multiple shoot buds (10–12) initiated (Fig. S1a, c, d) from the cut ends of cotyledon explants, whereas no shoot buds

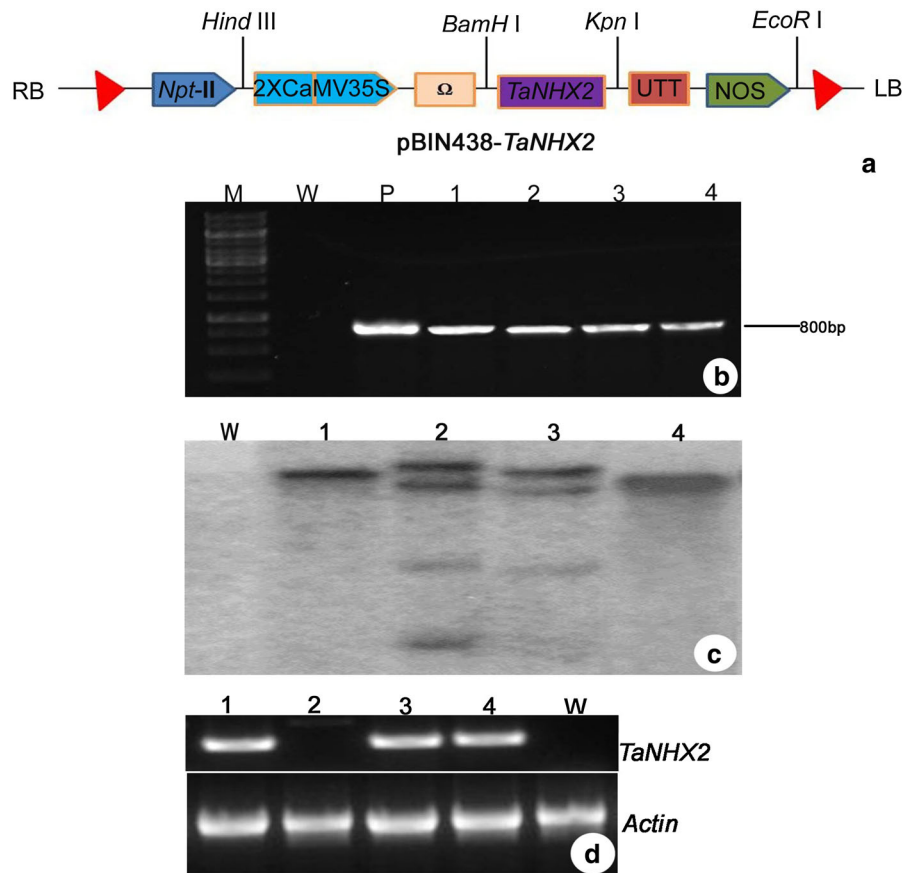
were observed from untransformed cotyledons (Fig. S1b). For initial elongation, the explants that survived on selection medium were transferred to MS media containing 0.5 mg l<sup>-1</sup> TDZ + 0.2 mg l<sup>-1</sup> IAA + 10 mg l<sup>-1</sup> AgNO<sub>3</sub> + 80 mg l<sup>-1</sup> kanamycin + 200 mg l<sup>-1</sup> cefotaxime (SSEM medium, Table S1) for 2 weeks (Fig. S1e), subsequently transferred to fresh SSEM medium, and the number of shoots elongated was recorded in the next 30 days (Fig. S1f). The explants showed elongation with 5–7 shoots on average, and the shoots longer than 3 cm (Fig. S1g) were excised and subcultured on SRM medium (Table S1; Fig. S1h) for rooting. Based on the percent of co-cultivated cotyledon explants producing shoots on kanamycin medium, the average transformation efficiency of 18.17 % was achieved (Table S2). After 10–15 days on SRM medium, putative transgenic plantlets with well-developed roots were transferred to pots (Fig. S1i) and raised to maturity to obtain T<sub>1</sub> seeds in greenhouse conditions.

Molecular analysis for the detection of genomic integration of the transgene (*TaNHX2*) was carried out on T<sub>1</sub> transgenic plants. The PCR analysis of fragment of 800 bp with *TaNHX2* gene primers was amplified from genomic DNA of four (*Cap1*–4) kanamycin-resistant transgenic lines, whereas corresponding bands were not detected in the non-transformed control plants with both the primers (Fig. 1b). Stable integration of the *TaNHX2* gene into the genomic DNA was further confirmed by Southern blot analysis of transformed *C. annuum* plants. Among the four transgenic lines tested, two lines (Fig. 1c) showed single integration, and the other lines showed multiple integration. No probe hybridization was observed in the control plants (Fig. 1c). The expression of *TaNHX2* in T<sub>1</sub> transgenic plants was confirmed by RT-PCR analysis; a 800-bp amplified fragment, specifically amplified by the gene specific primers, confirmed the expression of *TaNHX2* in three (*Cap1*, *Cap3*, and *Cap4*) of the four Southern-positive T<sub>1</sub> plants (Fig. 1d), whereas no expression was found in transgenic (*Cap2*) and wild-type plants (Fig. 1d).

#### Overexpression of *TaNHX2* in chilli pepper enhanced tolerance to the salt stress

To investigate the growth of *TaNHX2*-overexpressing lines under salt stress in soil, chilli pepper plants were irrigated with different concentration of Hoagland





**Fig. 1** Molecular confirmation of transgenic Chilli pepper (*Capsicum annuum* L.) plants. **a** Schematic map of plant expression gene construct pBIN438-*TaNHX2*. Expression of the *TaNHX2* is driven by the double cauliflower mosaic virus 35S promoter. **b** PCR amplification of the *TaNHX2* gene (800 bp) from the genomic DNA of T<sub>1</sub> transgenic Lanes 1–4 (*Cap-1*–4) and Lane M is 1 Kb Molecular weight marker ladder; Lane W is wild-type plant (negative control), Lane P is pBIN438-*TaNHX2*

(positive control). **c** Analysis of transgenic chilli pepper plants (Lanes 1–4) by southern blot hybridization after digesting genomic DNA with *Hind*III and probing with digoxigenin labeled *TaNHX2*. **d** Reverse transcriptase PCR of T<sub>1</sub> transgenic lines. Amplification was observed in three transgenic lines (*Cap-1*, *Cap-3*, and *Cap-4*) while there was no amplification from line 2 (*Cap-2*) and wild-type (W) plants; *Actin* gene was used as an internal control

solution containing 0, 50, 100, 150, and 200 mM NaCl for 15 days. As shown in Fig. 2, under normal conditions, there was no obvious morphological differences between the wild-type and transgenic plants. However, during the treatment with increasing concentration of NaCl, the growth of wild-type plants was inhibited, and most of the plants were severely stunted at above 100 mM of NaCl (Fig. 2d), whereas the transgenic chilli pepper plants grew well and showed a normal phenotype at different concentrations of NaCl (Fig. 2a–c). These results indicate that the growth of chilli pepper plants overexpressing *TaNHX2* under high salt concentrations showed increased tolerance than that of the wild-type plants.

#### Overexpressing *TaNHX2* induces antioxidant systems and proline accumulation

To evaluate the oxidative stress induced by high salt concentrations, We performed physiological and biochemical assessments to determine the chlorophyll content, leaf relative water content, proline, H<sub>2</sub>O<sub>2</sub> accumulation, and the activities of SOD, APX, and MDA contents in three transgenic (*Cap-1*, *Cap-3*, *Cap-4*) and wild-type plants (WT) under different salt stress conditions (0, 50, 100, 150, and 200 mM NaCl) (Fig. 3). We also performed leaf disk incubation assay in transgenic (*Cap-3*) and WT lines. The damage caused by stress was reflected in the degree of bleaching

**Fig. 2** Phenotypic differences of 2 months-old greenhouse grown wild-type and T<sub>1</sub> transgenic plants expressing the *TaNHX2* antiporter under different concentrations of salt stress. **a–c** T<sub>1</sub> transgenic chilli pepper plants (*Cap-1*, *Cap-3* and *Cap-4*) irrigated with different concentrations of NaCl. **d** Wild-type Chilli pepper plants irrigated with different concentrations of NaCl

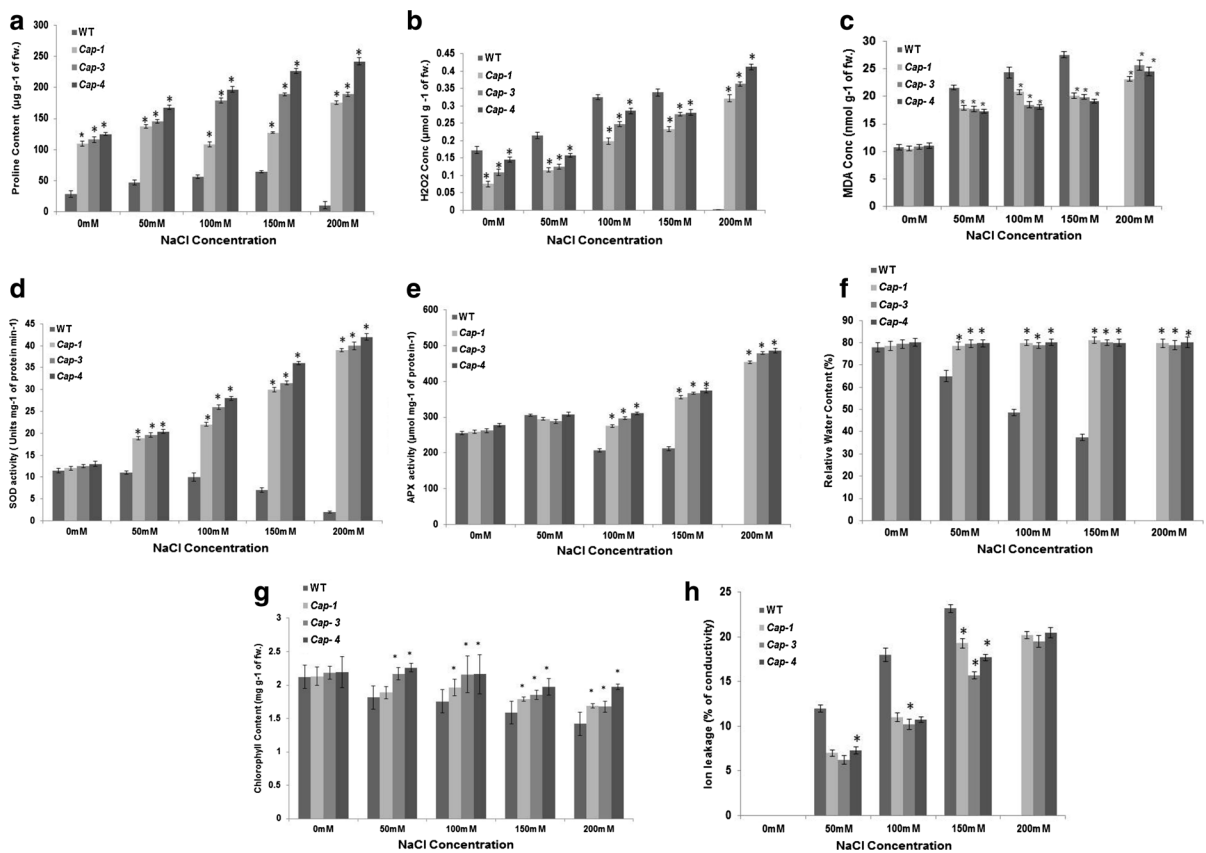


observed in the leaf disks after 72 h. Deterioration of the WT plant leaf disks was visually observed and correlated with a substantial loss of chlorophyll in comparison with transgenic line (*Cap-3*) (data not shown).

Proline is an important osmoprotectant that protects cells from damage under salt stress. As shown in Fig. 3a, the proline concentration was increased in

transgenic lines when compared to wild-type chilli pepper plants. In presence of 150 and 200 mM salt concentration, proline content was significantly higher in *TaNHX2*-overexpressing lines (*Cap-3* and *Cap-4*) than wild-type control plants (Fig. 3a).

Free radical formation and membrane damage levels were analyzed by observing the H<sub>2</sub>O<sub>2</sub> and



**Fig. 3** Biochemical analysis of wild-type and  $T_1$  transgenic plants grown under normal conditions and different salt treatments. **a** Proline content ( $\mu\text{g g}^{-1}$  of fw.). **b** Hydrogen peroxide concentration ( $\mu\text{mol g}^{-1}$  of fw.). **c** Malondialdehyde concentration ( $\text{nmol g}^{-1}$  of fw.). **d** Superoxide dismutase

activity (Units  $\text{mg}^{-1}$  of protein  $\text{min}^{-1}$ ). **e** APX activity ( $\mu\text{mol mg}^{-1}$  of protein  $\text{min}^{-1}$ ). **f** Relative water content. **g** Chlorophyll content. **h** Ion leakage (% of conductivity). The graph represents mean  $\pm$  SD value and asterisks indicate significant differences compared with WT values ( $*P < 0.05$ )

MDA content in transgenic and wild-type plants under different concentrations of NaCl. Salt stress caused a significant increase in the level of  $\text{H}_2\text{O}_2$  in wild-type plants with an increase in NaCl concentration. In comparison with the wild-type plants, all transgenic lines exhibited a slow pace increase in  $\text{H}_2\text{O}_2$  levels (Fig. 3b). MDA is considered as a marker of oxidative lipid injury, and its levels were widely used as a measure of damage due to various biotic and abiotic stresses. Based on Fig. 3c, under normal conditions, *TaNHX2*-overexpressing transgenic lines did not differ significantly from the wild-type plants. After salt treatment for 2 weeks, the MDA concentrations in *TaNHX2*-overexpressing lines were significantly decreased than wild-type plants (Fig. 3c). In comparison with the transgenic plants, the MDA concentrations were increased in wild-type plants, thereby

increasing oxidative damage to membranes and reducing the resistance to salt stress. Our data suggested that *TaNHX2* overexpression in chilli pepper plants reduces oxidative lipid injury and increases the better tolerance of transgenic chilli pepper plants grown under high salt concentrations.

The activities of the antioxidant enzymes APX and SOD were similar in transgenic and non-transgenic plants under optimal growth conditions. When subjected to salt stress, all transgenic lines exhibited significantly increased SOD and APX activities compared to the wild-type plants (Fig. 3d, e). As shown in Fig. 3f, under normal conditions, the RWC of all transgenic lines did not change significantly compared to the wild-type plants. However, when subjected to saline conditions, all transgenic lines exhibited an increased RWC. The percentage of RWC in the wild-



type and transgenic plants were 37.4 and 80.2 %, respectively, at different stress conditions (Fig. 3f). We also estimated leaf chlorophyll content of both transgenic (*Cap-1*, *Cap-3*, *Cap-4*) and the wild-type plants under different salt stress conditions (50, 100, 150, 200 mM NaCl). The transgenic plants retained significant amount of chlorophyll content compared to the wild-type plants under salt stress (Fig. 3g).

#### Analysis of ion leakage

The ion content of wild-type and transgenic chilli pepper plants were analyzed. When plants were grown under normal growth conditions, the transgenic chilli pepper plants were morphologically similar to wild-type plants. However, after salt treatment the transgenic plants showed lower levels of ion leakage than that of wild-type plants (Fig. 3h). These data suggested that lesser damage has been occurred in the membrane system of transgenic chilli pepper plants expressing the *TaNHX2* gene under salt stress.

#### Discussion

Progressive changing of global climatic conditions could lead to serious losses in agricultural productivity, as well as adversely affect biodiversity and global food security (Wheeler and von Braun 2013; Gill et al. 2014). Crop plants frequently encounter a variety of abiotic stresses among which salinity stress is one of the major cause of crop failure worldwide (Gill et al. 2014). On a world scale, no toxic substance restricts plant growth more than does salt. Almost three quarters of the earth surface is covered by salt water. Plants have created complex mechanisms, which help them in adapting to adverse environmental conditions. One such mechanism in higher plants is the sequestration of  $\text{Na}^+$  in the central vacuole by making use of putative  $\text{Na}^+$  (or)  $\text{K}^+/\text{H}^+$  antiporters (Apse et al. 1999; Gaxiola et al. 1999).

Genetic engineering of pepper (*Capsicum annum* L.) for abiotic stress tolerance is crucial for improving agricultural productivity is dependent upon the development of efficient and reliable regeneration and transformation protocols. Significant progress has been reported in the chilli pepper regeneration and transformation. However, regeneration of chilli pepper is highly genotypic dependent, and efficiency of

*Agrobacterium*-mediated transformation has been limited in this system. (Wang et al. 1991; Ye et al. 1993; Christopher and Rajam 1997; Jayashankar et al. 1997; Subhash and Christopher 1997; Manoharan et al. 1998; Li et al. 2003; Lee et al. 2004). In this work, we addressed the use of a synthetic cytokinin (TDZ) which induced multiple shoots after 30 days of culture, but they did not elongate further. Previous studies reported the influence of silver nitrate ( $\text{AgNO}_3$ ) on multiple shoot production and elongation (Valera-Montero and Ochoa-Alejo 1992; Li et al. 2003). In the present study we added  $\text{AgNO}_3$  to the SSEM for multiple shoot production and elongation (Fig. S1e, f). For root induction transgenic chilli pepper plants were transferred to hormone-free media and we observed a better rooting on half-strength MS medium containing  $40 \text{ mg l}^{-1}$  kanamycin and  $200 \text{ mg l}^{-1}$  cefotaxime (Table S1; Fig. S1h). These results were consistent with the studies by Yarra et al. (2012). The highest transformation efficiency of 18.17 % was achieved by using this protocol (Table S2).

Overexpression of *TaNHX2* gene was previously described in different plant species, including soybean, alfalfa, tomato and rice (Cao et al. 2011; Zhang et al. 2012; Yarra et al. 2012; Wu et al. 2012). However, very little is known about the role of *TaNHX2* in salt stress tolerance.

In the present investigation, we generated transgenic *TaNHX2* overexpressing chilli pepper plants ( $T_1$ ) that were sustained at high salt concentration. PCR and Southern blot analysis confirmed that *TaNHX2* gene has been integrated and expressed in the  $T_1$  transgenic chilli pepper plants. RT-PCR revealed that three of the four transformed chilli pepper lines (*Cap-1*, *Cap-3*, and *Cap-4*) were expressing the *TaNHX2*, but the line 2 (*Cap-2*) did not show detectable levels of transcripts. This may be due to the low level or no expression of *TaNHX2*. Significant studies were reported with an inverse relation between transgene copy number and transgene expression level (Koprek et al. 2001; Kohli et al. 2003). In this study we used different *TaNHX2*-overexpressing lines (single and multiple insertion copies) for biochemical analyses. The transgenic lines (*Cap-4*) expressing *TaNHX2* accumulated a markedly superior level of proline, chlorophyll, SOD, ascorbate peroxidase (APX), RWC in their leaves under severe saline conditions (200 mM NaCl) as compared with the multiple gene transgenic lines.

In this study, we showed that expression of the *TaNHX2* gene in transgenic chilli pepper plants enhanced salinity stress tolerance. Biochemical assays of these transgenic plants revealed enhanced levels of proline, chlorophyll, RWC, SOD, and APX activities and reduced levels of H<sub>2</sub>O<sub>2</sub>, MDA contents, indicating that the *TaNHX2* gene is playing an important role in the salt stress tolerance mechanism in chilli pepper plants.

Accumulation of osmolytes is the most common and important responses of plants, subjected to salt stress (Zhou et al. 2014). Among the different compatible solutes, proline has been proposed to act as an important compatible osmolyte and osmoprotective compound, and its accumulation has been reported in plants exposed to high salinity (Zhang et al. 2001; Subramanyam et al. 2011; Zhou et al. 2014). In the present investigation, the transgenic chilli pepper plants expressing *TaNHX2* accumulated more proline than non-transgenic wild-type plants (Fig. 3a). Under high (150 and 200 mM) salt concentration, proline content was significantly higher in *TaNHX2*-overexpressing lines (*Cap-3* and *Cap-4*) than wild-type control plants (Fig. 3a) suggesting that the expression of *TaNHX2* might activate the key enzymes of the proline biosynthetic pathway (Bursy et al. 2007), whereas the increase in RWC and chlorophyll content in transgenic lines were higher than that of non-transgenic plants (Fig. 3f, g). Therefore, it has been explained that the *TaNHX2* gene expression in transgenic chilli pepper plants improved the osmotic adjustment of cells by maintaining RWC and chlorophyll content to restore the growth of plants in response to damage caused by salinity stress. The results of the present study are in concurrence where the *TaNHX2* gene expression in transgenic tomato (Yarra et al. 2012).

In plants, during salinity stress, the formation of reactive oxygen species (ROS) increases as by-products of various metabolic pathways (Mishra et al. 2011; Nounjan et al. 2012). To overcome salt-mediated oxidative stress, plants detoxify ROS by up-regulating antioxidative enzymes like SOD and APX. (Türkan and Demiral 2009; Dong et al. 2010; Mishra et al. 2011).

H<sub>2</sub>O<sub>2</sub> is an important ROS, which causes apoptosis in plants (Houot et al. 2001). In the present study, the non-transgenic chilli pepper plants showed more concentration of H<sub>2</sub>O<sub>2</sub> than transgenic chilli pepper plants at different NaCl concentrations (Fig. 3b). However, in comparison with the wild-type plants,

salt stress caused a slow pace increase in H<sub>2</sub>O<sub>2</sub> in transgenic chilli pepper plants (Fig. 3b). This slow increase in H<sub>2</sub>O<sub>2</sub> level probably is responsible for the much better tolerance of transgenic chilli pepper plants grown under high salt concentration. The activities of SOD, APX, and the concentration of MDA are typically used to measure oxidative damage to membranes in response to salt stress (Mittova et al. 2004). In our study at the biochemical level, under different concentrations of NaCl the activities of SOD and APX were increased significantly (Fig. 3d, e). Under normal conditions, no *TaNHX2*-overexpressing transgenic lines showed a significantly altered MDA concentration, whereas under salt stress, *TaNHX2*-overexpressing transgenic lines showed increased levels of MDA but lesser than that of wild-type plants (Fig. 3c). According to these experimental data, the decrease in the concentrations of MDA at higher salt concentrations in *TaNHX2*-overexpressing transgenic lines is due to the high activity of SOD and APX. The obtained results were in agreement with earlier reports (Subramanyam et al. 2011; Zhou et al. 2014). The results suggest that *TaNHX2* has been integrated in the genome of transgenic chilli pepper plants, thereby decreases oxidative damage to membranes and increase the resistance to salt stress.

In conclusion, the chilli pepper plants carrying the *TaNHX2* gene showed elevated levels of proline and induced antioxidant enzyme systems, resulting in tolerance of higher concentration of NaCl. The obtained results pave the way to understand the role of the *TaNHX2* gene under salt stress condition.

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